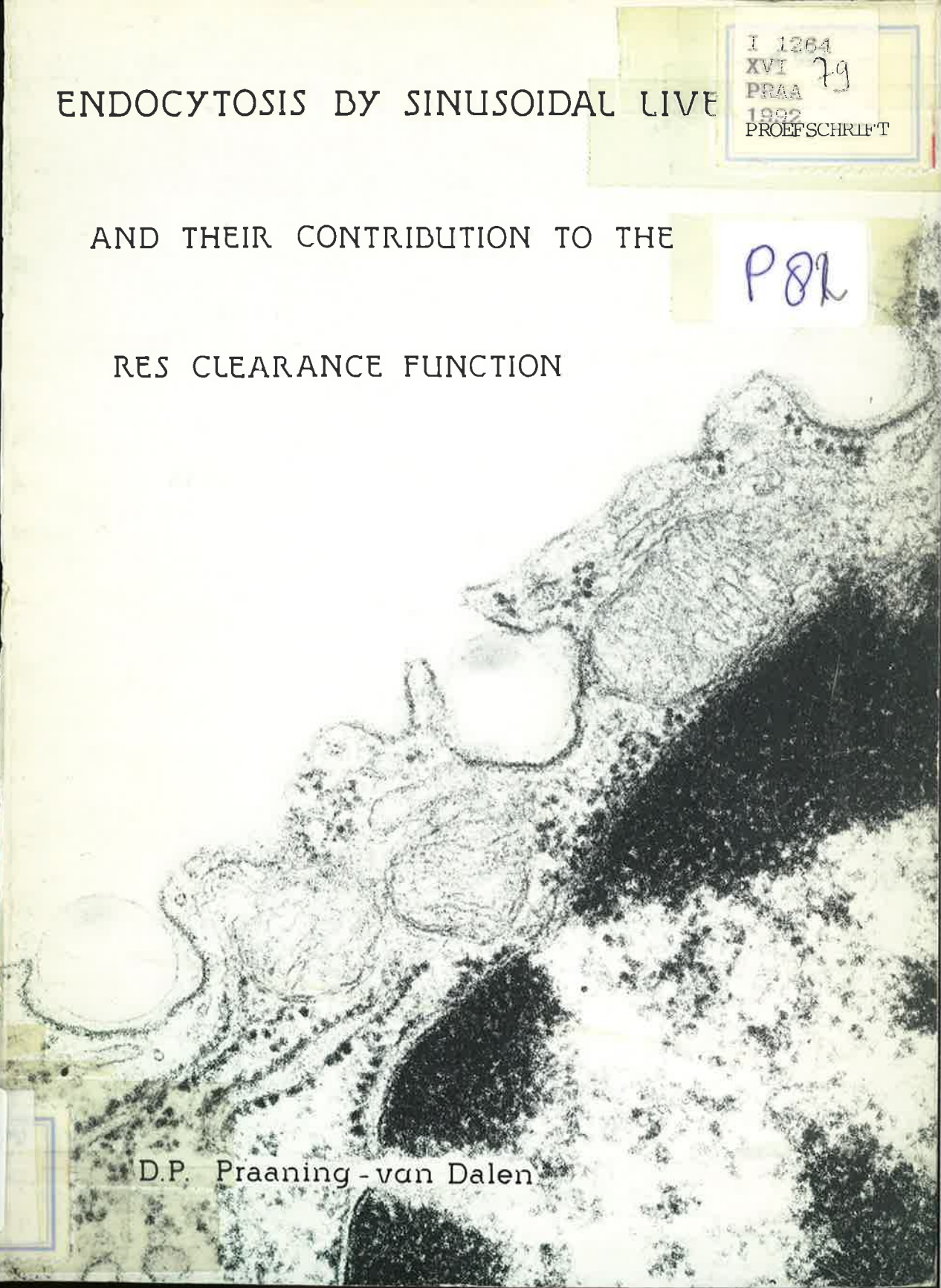
ENDOCYTOSIS BY SINUSOIDAL LIVER CELLS AND THEIR CONTRIBUTION TO THE RES CLEARANCE FUNCTION



D.P. Praaning - van Dalen

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Druk: Vadéli - 's-Gravenhage

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1983

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)

STELLINGEN

1

De bewering van Wehland et al. dat in het cytosol van gekweekte Swiss 3T3 fibroblasten geen oplosbaar clathrine aanwezig zou zijn is niet gerechtvaardigd op grond van hun experimentele resultaten.

J. Wehland, M.C. Willingham, M.G. Gallo, A.V. Rutherford, J. Rudick, R.B. Dickson & I. Pastan (1981) Cold Spring Harbor Symp. Quant. Biol. 46, 743-753.

2

Met de door Willingham et al. opgestelde theorie aangaande het endocytoseproces in fibroblasten kan een aantal stappen van dit proces niet verklaard worden.

M.C. Willingham, A.V. Rutherford, M.G. Gallo, J. Wehland, R.B. Dickson, R. Schlegel & I.H. Pastan (1981) J. Histochem. Cytochem. 29, 1003-1013.

3

In tegenstelling tot de heersende opvatting vormt "bristle-coated micropinocytosis" het enige endocytosemechanisme dat in leverendotheelcellen kan worden aangetoond.

4

De door Henshall gesuggereerde toepassing van een extract van de adelaarsvaren (Pteridium aquilinium) voor de stabilisering van ascorbinezuur in dranken verdient geen aanbeveling gezien de carcinogene eigenschappen van deze plant.

J.D. Henshall (1981) In: Vitamin C (Ascorbic Acid), (J.N. Counsell & D.H. Hornig, eds.), Applied Science Publishers, London, p. 123-137.

5

Het is betreurenswaardig dat er in de literatuur nog steeds onduidelijkheid bestaat over de exacte stereochemie van het β-lactam antibioticum ticarcilline.

6

Tegenstrijdige literatuurgegevens omtrent de capaciteit van endotheelcellen om wel of niet "antihemophilic factor" (Factor $VIII_{AHF}$) te synthetiseren zijn eerder terug te voeren op een historisch gegroeide spraakverwarring betreffende de definitie van Factor VIII dan op wezenlijk verschillende experimentele resultaten.

L.W. Hoyer, R.P. de los Santos & J.R. Hoyer (1973) J. Clin. Invest. 52, 2737-2744.
E.A. Jaffe & R.L. Nachman (1975) J. Clin. Invest. 56, 698-702.

7

Invoering van een algemene commandotaal bij het "online" literatuuronderzoek verliest veel van haar waarde omdat de door "host"-organisaties aangebrachte modificaties toch weer tot verschillende dialecten leiden.

8

Het ontbreken van een gedoogplicht van de zijde van de huurders heeft een grote achterstand veroorzaakt bij het verbeteren van de isolatie van bestaande huurwoningen ten opzichte van het peil dat bij door eigenaren bewoonde huizen is bereikt.

9

Er hadden nooit zoveel mensen voor vrede gedemonstreerd als er geen kernwapens waren geweest.

Stellingen behorende bij het proefschrift "Endocytosis by sinusoidal liver cells and their contribution to the RES clearance function"

D.P. Praaning-van Dalen

6 december 1983

1264

ENDOCYTOSIS BY SINUSOIDAL LIVER CELLS

AND THEIR CONTRIBUTION TO THE

RES CLEARANCE FUNCTION

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE
GENEESKUNDE
AAN DE RIJKSUNIVERSITEIT TE UTRECHT,
OP GEZAG VAN DE RECTOR MAGNIFICUS
PROF. DR. O.J. DE JONG,
VOLGENS BESLUIT VAN HET COLLEGE VAN DECANEN
IN HET OPENBAAR TE VERDEDIGEN OP
DINSDAG 6 DECEMBER 1983 DES NAMIDDAGS
TE 2.30 UUR

DOOR

Danielle Patricia Praaning - van Dalen

GEBOREN OP 26 JULI 1950
te 's-GRAVENHAGE

PROMOTORES: PROF.DR. D.L. KNOOK (Vrije Universiteit Brussel)
PROF.DR. C.F. HOLLANDER

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Munniksma, J., Noteborn, M., Kooistra, T., Stienstra, S., Bouma, J.M.W., Gruber, M., Brouwer, A., Praaning-van Dalen, D.P. & Knook, D.L. (1980) Fluid endocytosis by rat liver and spleen. Experiments with ^{125}I-labelled poly(vinylpyrrolidone) in vivo. Biochem. J. 192, 613-621.

Praaning-van Dalen, D.P., Brouwer, A. & Knook, D.L. (1981) Clearance capacity of rat liver Kupffer, endothelial and parenchymal cells. Gastroenterology 81, 1036-1044.

Praaning-van Dalen, D.P., Brouwer, A. & Knook, D.L. (1982) Hepatic uptake of endotoxin. Reply. Gastroenterology 83, 521-522.

Praaning-van Dalen, D.P. & Knook, D.L. (1982) Quantitative determination of in vivo endocytosis by rat liver Kupffer and endothelial cells facilitated by an improved cell isolation method. FEBS Letters 141, 229-232.

Knook, D.L., Praaning-van Dalen, D.P. & Brouwer, A. (1982) The clearance function of Kupffer and endothelial liver cells in relation with drugs and aging. In: Liver and aging (K. Kitani, ed.), Elsevier Biomedical Press, Amsterdam, 269-282.

Praaning-van Dalen, D.P., Leeuw, A.M. de, Brouwer, A., Ruiter, G.C.F. de & Knook, D.L. (1982) Ultrastructural and biochemical characterization of endocytic mechanisms in rat liver Kupffer and endothelial cells. In: Sinusoidal Liver Cells. D.L. Knook & E. Wisse (eds.), Elsevier Biomedical Press, Amsterdam, 271-278.

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CHAPTER 1

GENERAL INTRODUCTION

One of the major functions of the mammalian liver is the clearance of potentially toxic exogenous and endogenous materials from the blood. These materials include viruses, bacteria, toxins, drugs, effete cells or parts of damaged ones, denatured proteins, immune complexes as well as physiological substances which are to be eliminated or inactivated because they are present in the blood in excess, such as hormones, enzymes and clotting factors.

The efficiency by which the liver removes a substance from the blood depends on several factors, including the nature of the substance, the rate at which the substance is delivered to the liver, the size of the liver cell population involved in the clearance and the mechanisms by which the cells recognize and internalize the substance.

Certain substances, especially colloidal and particulate ones, are considered to be cleared by a specific population of liver cells which is thought to represent the major part of the reticuloendothelial system (RES) of the body. However, there is still some controversy concerning the types of liver cells that are part of the RES. Moreover, although the differences in rates at which various colloidal or particulate substances are cleared by the liver indicate that more than one mechanism of internalization exists, this possibility has generally been ignored.

The clearance capacity of the rat liver for a variety of endogenous and exogenous substances assumed to be removed by the RES will be described in this thesis. In addition, information on the cell types involved in the clearance and on the mechanisms by which the cells internalize the substances will be presented. For this purpose, experiments were performed in vivo as well as in vitro, using different types of isolated and purified liver cells.

To properly evaluate the experimental results, it is necessary to have knowledge of the main factors involved in the clearance of substances by the liver, i.e., the hepatic circulation, the architecture and cellular composition of the liver and the ultrastructural characteristics of the liver cells. These topics along with different hypotheses concerning the various cellular mecha-

nisms involved in internalization and the present concept of the RES as related to the clearance capacity of the liver will be discussed below.

1.1 THE HEPATIC CIRCULATION AND ARCHITECTURE

The blood supply of the mammalian liver is provided by two afferent vessels: the hepatic artery which delivers the oxygen rich blood and the portal vein which transports substances adsorbed from the gastrointestinal tract directly to the liver. Generally, 70-75% of the total blood supply enters the liver through the portal vein (Campra & Reynolds, 1982). The portal venous system shows no evidence of so-called autoregulation, i.e., the tendency for local blood flow to remain constant during changes in pressure, which is in contrast to what happens in the arteries (Campra & Reynolds, 1982). The blood pressure in the portal vein is rather low (6-10 mm Hg) as compared with that of the hepatic artery, where the pressure is similar to that in the aorta (Campra & Reynolds, 1982). Therefore, a slight obstruction of the portal vein can easily influence the blood supply to the liver. Since the maximum rate at which the liver can possibly clear a substance is dependent on the rate at which the substance enters it, the rate of portal blood flow can be of influence in the outcome of many clearance studies. The normal rate of blood flow through the liver has been determined to be 100-130 ml per min per 100 g liver in different species such as humans, dogs, cats and rats (Benacerraf et al., 1957a; Campra & Reynolds, 1982). The rate of portal blood flow can be altered by certain hormones and other factors (Campra & Reynolds, 1982).

Both the portal vein and the hepatic artery have numerous branches within the liver, each successive branch having a smaller diameter. The terminal branches are the sinusoids (having a diameter of about 6-7 µm in the rat (Wisse et al., 1983)), in which venous and arterial blood come together. At normal blood flow, almost all sinusoids are perfused (Goresky, 1982). The main exchanges between blood-borne substances and the liver cells occur in these sinusoids. After passage through the channels of the sinusoids, the blood is collected in branches of the hepatic or central veins, which converge in the inferior vena cava where the blood leaves the liver before entering the heart.

The smallest functional microcirculatory unit of the liver consists of one terminal portal venule and hepatic arteriole, its branching sinusoids, a bile

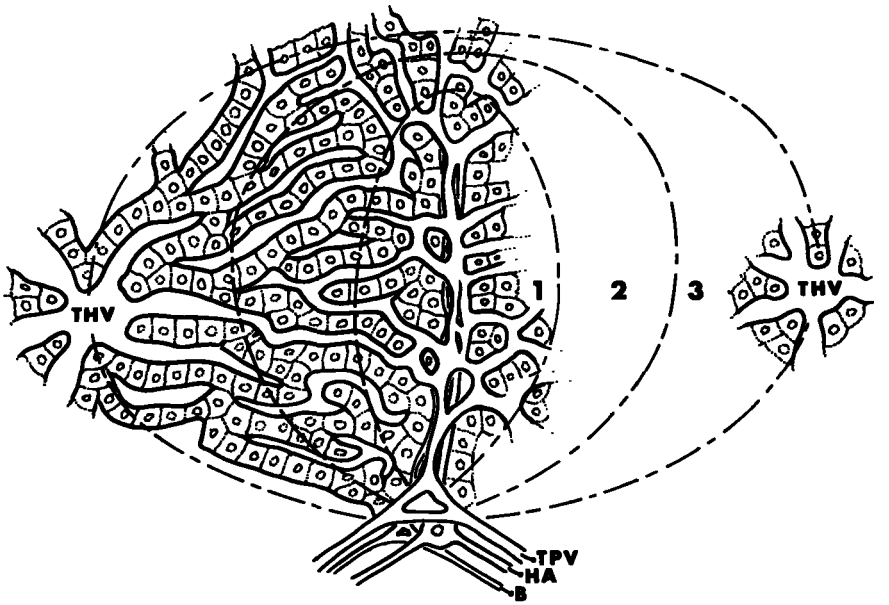

Figure 1 Smallest functional microcirculatory unit of the liver, the acinus. The terminal portal venule (TPV) and the hepatic arteriole (HA) branch into the sinusoids. The blood enters these sinusoids in zone 1 of the acinus (periportal) and subsequently flows through zone 2 (transitional) and zone 3 (perivenous), where it enters the terminal hepatic venule (THV). B: Bile ductule. According to Gumucio & Miller (1982).

ductule and the draining hepatic venule into which the sinusoids empty their contents. This unit, called the liver acinus (Rappaport, 1962), can be divided into three zones (see Fig. 1). Zone 1 is the periportal zone, zone 3 the perivenous zone and zone 2 a transitional zone. Since substances delivered by the portal and arterial blood first enter zone 1 of the acinus, it can be expected that the consuming cells in this zone will be exposed to a higher concentration of substances and oxygen than will cells situated in zone 3. Thus, although zone 1 and zone 3 cells might in principle have the same functional qualities, the clearance capacity of zone 1 cells might appear higher because there is more substance delivered to them. It must be emphasized here that in the study described in this thesis no attempt was made to discriminate between zone 1, 2 and 3 cells. The clearance capacity of cells of a specific type determined either in vivo or in vitro might therefore represent the mean capacity of these cells derived from the different zones.

1.2 CELLULAR COMPOSITION OF THE LIVER

Apart from some cells that line the arteries, veins and bile ducts, the liver is composed mainly of four morphologically different cell types: the hepatocytes or parenchymal cells, the sinusoidal endothelial cells, the Kupffer cells and the fat-storing cells. Each liver cell type is found at a specific location in the sinusoids. In the rat liver, the sinusoids run almost parallel and are separated by only one layer of parenchymal cells. The parenchymal cells, however, are not in direct contact with the sinusoidal blood but are covered by the sinusoidal endothelial cells. Between the parenchymal and the endothelial cells lies the (perisinusoidal) space of Disse.

The Kupffer cells usually lie singly on or imbedded in the endothelial lining. They are most abundant in zone 1 of the liver acinus, around the branches of the portal venules. (Wisse, 1977a; Sleyster and Knook, 1982). The fat-storing cells are situated in the space of Disse and their processes can be found under the endothelial lining.

The parenchymal cells represent the largest cell class in the liver, comprising about 60% of the total cell number of the rat liver (Knook and Sleyster, 1980). The other percentage is made up mainly by endothelial, Kupffer and fat-storing cells. The total number of endothelial cells is about 2-3 times higher than that of Kupffer or fat-storing cells. Apart from their number, the size and volume of the parenchymal cells are far more pronounced than those of the other cell types. About 80% of the total volume of the liver is represented by the parenchymal cells, whereas only 6% of it is made up by Kupffer, endothelial and fat-storing cells (Blouin, 1977). The remaining volume is formed chiefly by the lumen of the sinusoids and the space of Disse.

1.3 ULTRASTRUCTURAL CHARACTERISTICS OF KUPFFER AND ENDOTHELIAL CELLS

Since there is evidence that Kupffer and endothelial cells are the most active cells in the clearance of foreign materials from the blood (see below and also Chapter 3), the study described here was focused primarily on the characteristics and clearance capacity of these two cell types.

A detailed study of Kupffer and endothelial cells at the ultrastructural level offers three main advantages. It enables a better identification of the

cells, shows whether the ultrastructural integrity has been preserved after the cells have been isolated by enzymatic digestion of the intercellular matrix (see Chapter 2) and gives indications about their possible functions (Chapters 3,4 and 5).

At the ultrastructural level, Kupffer and endothelial cells can be easily recognized in the intact liver of a rat. Endothelial cells (Fig. 2a) possess very long flat cytoplasmic processes that surround a sinusoid, forming a tube. Neighbouring endothelial cells link up well with each other, but their cell contacts are poorly defined. Typical intercellular tight junctions and a basement membrane such as observed in other capillary endothelial cells (Palade et al., 1978) are not found in endothelial cells of the liver. In the cytoplasmic processes, sieve plates can be observed (Wisse, 1970). These are clusters of open pores each having a diameter of about 0.1 µm. Due to these sieve plates and the lack of a basement membrane, the space of Disse between the endothelial lining and the parenchymal cells is in direct contact with the blood (Fig. 2a). The cell surface area that is occupied by pores determines the so-called porosity of the cells. As a result of this porosity, no time consuming transendothelial transport as described for vascular endothelium (Palade et al. 1978) is required to provide parenchymal cells with essential substances from the bloodstream. The endothelial cells should have a high porosity to enable the entrance of substances into the space of Disse, since the pressure in the sinusoids is somewhat low (2-3 mm Hg) (Campra & Reynolds, 1982). However, due to the limited size of the pores, there is a strong mechanical selection of substances which can pass through them. Substances with a molecular weight up to 250,000 might pass through the pores without hindrance. However, larger ones such as chylomicrons with a diameter >0.1 µm will not enter the Disse space (Naito & Wisse, 1978). This selectivity of the sieving system might protect the parenchymcal cells against an excess uptake of cholesterol enriched chylomicrons as well as against injuries by, e.g., bacteria and viruses.

In contrast to other capillary endothelial cells, endothelial liver cells possess a well developed vacuolar apparatus consisting of bristle-coated micropinocytic vesicles, large uncoated ("macropinocytic") vesicles, tubular vesicles, small coated vesicles of Golgi origin and a variety of dense bodies (lysosomes) (Fig. 2a; Wisse, 1972). This considerable vacuolar apparatus indicates active uptake of extracellular fluid and the substances contained into the cells (see below). Thus, apart from the lining and filtration functions, endothelial cells might quite possibly also be involved in the clearance of

certain substances. Other organelles found in liver endothelial cells include rough and smooth endoplasmic reticulum, many free ribosomes and a Golgi apparatus. Small numbers of mitochondria are also present (Fig. 2a).

The cytoplasmic processes of the Kupffer cells (Fig. 2b) are often attached to the endothelial cells and are even able to penetrate the pores of the sieve plates. A large part of the Kupffer cell surface is exposed to the bloodstream.

The shape of Kupffer cells is variable and irregular, although they are generally more rounded than the endothelial cells. Kupffer cells do not possess sieve plates. The cell surface shows numerous microvilli, flaps and lamellipodia. In general, Kupffer cells have the appearance of macrophages. Their 70 nm thick cell coat, the "fuzzy coat", can be visualized only under specific conditions (Wisse, 1977b). However, the fuzzy coat can be easily recognized in invaginations of the cell membrane. These "worm-like structures" are typical of Kupffer cells. It has been suggested that the fuzzy coat and worm-like structures are involved in the uptake of substances from the blood (Wisse, 1977b). Inside the cytoplasm of Kupffer cells are found numerous vesicles, including bristle-coated micropinocytic vesicles, large pinocytic vacuoles and a variety of dense bodies (lysosomes) (Wisse, 1974), indicating that the cells are actively involved in the internalization of extracellular substances. Also found in Kupffer cells are rough endoplasmic reticulum, clusters of free ribosomes, a Golgi apparatus and mitochondria.

Histochemical staining for peroxidatic activity by incubation with diaminobenzidine tetrahydrochloride (DAB) (Wisse, 1974) makes possible further discrimination between Kupffer and endothelial cells. While endothelial cells

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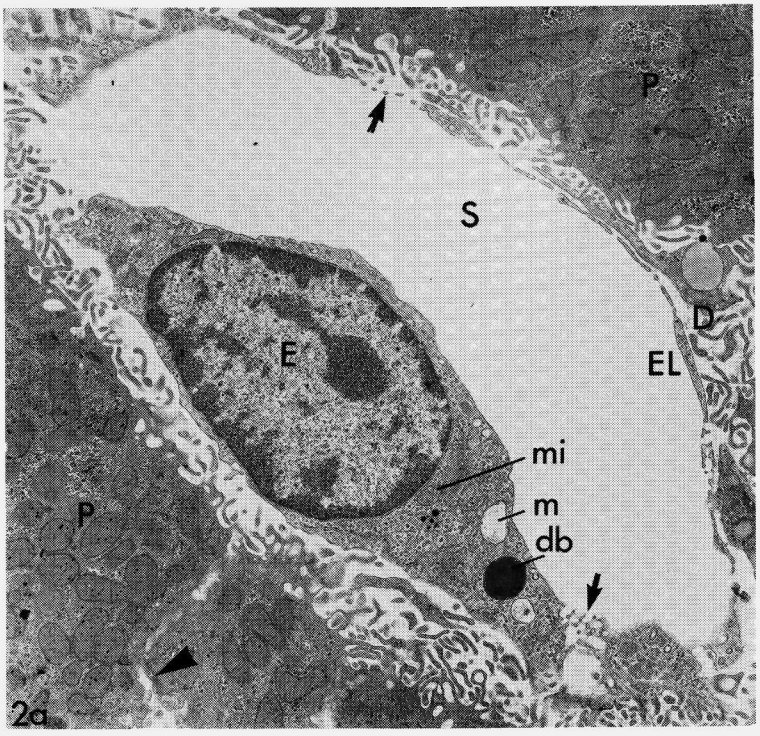
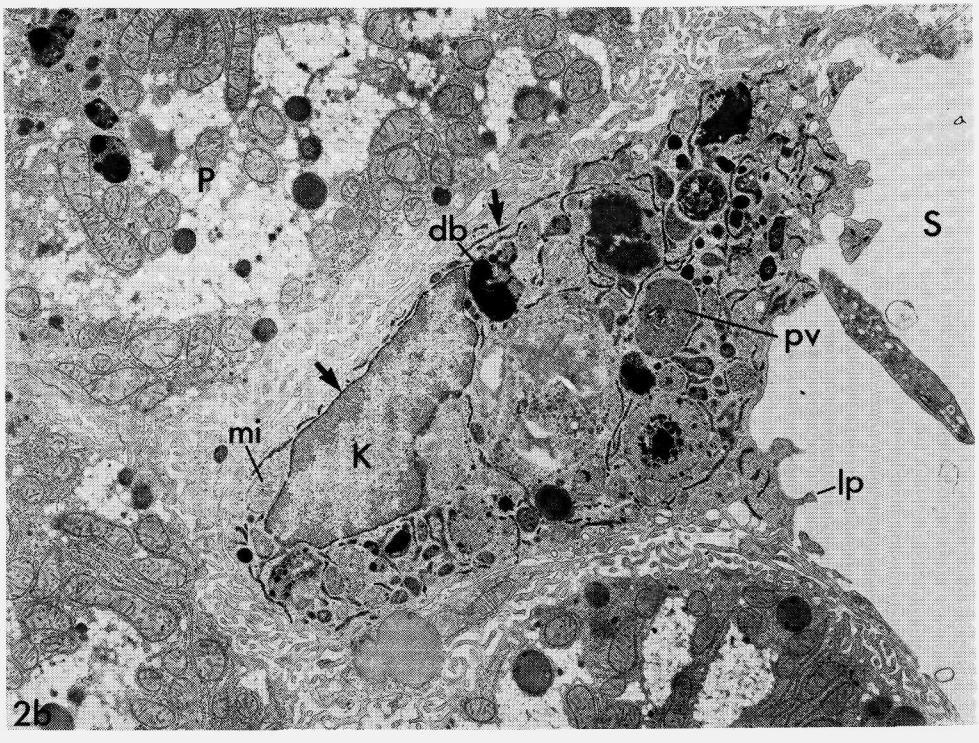
Figure 2 Ultrastructural characteristics of sinusoidal cells in situ.

a) Endothelial cell (E) possessing long flat processes that contain fenestrations (arrows). Between the endothelial lining (EL) and the parenchymal cell (P) is the space of Disse (D). A variety of organelles, including macropinocytic vesicles (m), dense bodies (db) and mitochondria (mi) can be observed in the cytoplasm of the endothelial cell. The parenchymal cells contain typical intracellular junctions (arrowhead) which are not present between the sinusoidal cells. S: sinusoid. (6,830x)

b) Kupffer cell (K), partly imbedded in the endothelial lining. The cell surface shows many lamellipodia (lp). The cytoplasm contains large pinocytic vacuoles (pv), dense bodies (db) and mitochondria (mi). Staining for peroxidatic activity is seen in the cisternae of the rough endoplasmic reticulum and the nuclear envelope (arrows). S: sinusoid. (6,830x)



2a



2b

do not stain, Kupffer cells show peroxidatic activity in the cisternae of the rough endoplasmic reticulum and the nuclear envelope (Fig. 2b). This enzymatic reaction cannot be generally used for recognition of sinusoidal cells in species other than the rat (see also Section 2.4).

TABLE I

MORPHOMETRIC DATA ON CELL ORGANELLES INVOLVED IN THE UPTAKE OF SUBSTANCES BY DIFFERENT TYPES OF LIVER CELLS

	Endothelial cells	Kupffer cells	Fat-storing cells	Parenchymal cells
Plasma membranes*	15.2	4.3	7.1	73.4
Pinocytic vesicles**	45.2	12.0	0.7	42.1
Lysosomes**	16.9	25.8	0.3	57.0
Total cell volume***	3.3	2.5	1.7	92.5

*Data from Blouin et al. (1977), expressed as % of aggregate surface area.
**Data from Blouin et al. (1977), expressed as % of aggregate organelle volume.
***Data from Blouin (1977), expressed as % of total liver cell volume.

The morphometric studies performed by Blouin et al. (1977) emphasize the possible involvement of Kupffer and endothelial cells in the clearance of substances. Table I shows the volumetric contributions of organelles involved in the process of uptake and degradation of substances (see also Section 1.4) in various liver cell types. Although the Kupffer and endothelial cells make up only 3.3 and 2.5% of the total cell volume of the liver, their contribution to the plasma membrane, the organelle which first comes into contact with a substance before internalization, is 15.2 and 4.3%, respectively. Very striking is the contribution of endothelial cells to the population of pinocytic vesicles: 45.2% of the total. This very strongly indicates that endothelial cells are active in the uptake process. Lysosomes are organelles in which most internalized substances are degraded. The contribution of the lysosomal volume of Kupffer cells to the total amount of lysosomes is very high, but also endothelial cells make a relatively high contribution to the amount of lysosomes.

1.4 ULTRASTRUCTURAL CHARACTERIZATION OF THE ENDOCYTIC PROCESS

The uptake of substances by cells can take place by three processes. Very small molecules may enter a cell simply by diffusion. For some molecules such as ions and sugars, an energy dependent transport system may be involved. Macromolecules and particles, however, which cannot enter the cell by either of these two means, have to be internalized in another way. A small part of the plasma membrane will surround some of the extracellular fluid and, after fusion of the distal parts of the membrane, an intracellular vesicle in which the fluid and the macromolecules or particles contained are trapped is formed. This process of internalization is called endocytosis. Endocytosis was first recognized in the ingestion of foreign particulate materials by macrophages. It was later discovered that endocytosis occurs in all animal cells (Goldstein et al., 1979). Since some cell types (i.e., macrophages, granulocytes) appeared to be able to endocytose larger particles (i.e., those visible by light microscopy) than others, different mechanisms of endocytosis were expected. Endocytosis was therefore roughly divided into phagocytosis (i.e., eating) and pinocytosis (i.e., drinking). Definitions based merely on the size of endocytosed particles, however, are very vague, since the point at which pinocytosis ends and phagocytosis begins is difficult to establish. As a result, the same phenomenon may be called either phagocytosis or pinocytosis by different investigators.

The first electron microscopic studies revealed that indeed morphologically different types of endocytosis exist, which seemed to be related to the size of the endocytosed particle, thus justifying the terms pinocytosis and phagocytosis. It was also recognized that some molecules or particles adsorb to the plasma membrane, whereas others do not. It is generally assumed that adsorption is a prerequisite for the phagocytic event and that the amount of fluid internalized along with a phagocytosed particle can be neglected. During pinocytosis, however, a substance can either be adsorbed to the cell membrane or be present in the fluid of the internalized vesicle. This led to a further division of pinocytosis into fluid-phase and adsorptive pinocytosis. Ultrastructural studies of the endocytic processes also revealed that there are various intracellular means for the transport of endocytosed molecules.

Thus, there seem to be three successive steps at which different endocytic mechanisms can be discriminated: 1) the events at the cell membrane, i.e., adsorption or not; 2) the mechanism of internalization, i.e., pinocytosis

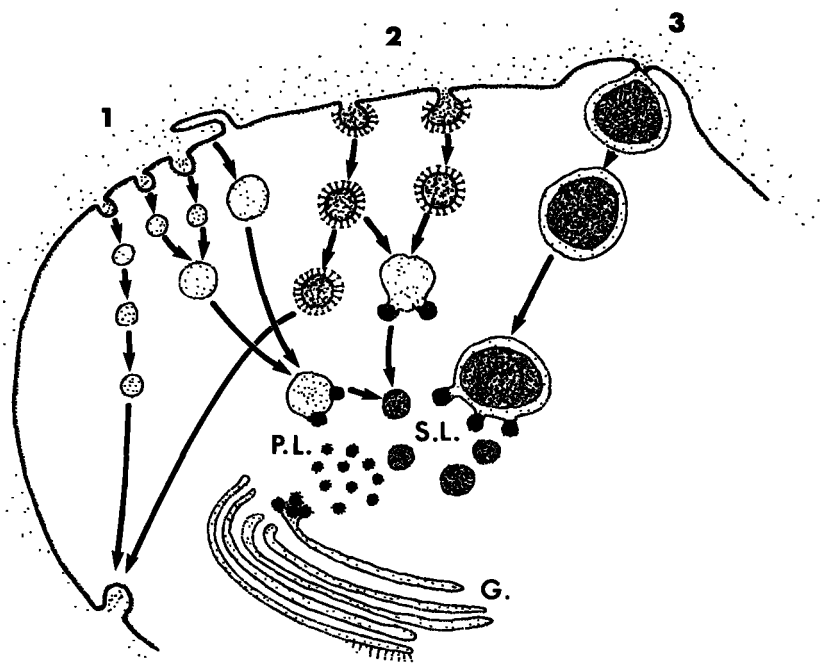


Figure 3 Mechanisms of internalization and fate of endocytic vesicles (modified from Silverstein et al., 1977). The three processes of endocytosis, i.e., fluid-phase pinocytosis, adsorptive pinocytosis and phagocytosis, are numbered 1,2 and 3, respectively. After internalization, pinocytic vesicles either traverse the cytoplasm and discharge their contents into the extracellular fluid or they fuse with primary lysosomes (PL) or secondary lysosomes (SL). The primary lysosomes are assumed to originate from an area of the endoplasmic reticulum close to the Golgi apparatus, called GERL (G): Golgi-associated region of endoplasmic reticulum, that forms lysosomes. Further explanation in text.

versus phagocytosis; 3) the intracellular route of the endocytosed material.

Using the model proposed by Silverstein et al. (1977) (Fig. 3), these three steps of endocytosis will be further explained, with special reference to observations on Kupffer and endothelial cells. During the first step of endocytosis, adsorption of a substance to the cell membrane can occur only if the membrane possesses sites to which the substance can bind (processes 2 and 3 in Fig. 3). The plasma membrane might possess binding sites for a great variety of substances. Binding of a substance to the membrane always obeys saturation kinetics but may not necessarily lead to endocytosis. If binding induces a quick cellular response in the form of endocytosis, the internalization will be a highly concentrating process as compared with fluid-

phase endocytosis (process 1). A substance which attaches to a binding site is usually called a "ligand". If a binding site shows a high specificity for a certain ligand, this site might be called a "receptor". A great variety of receptors for the endocytosis of different types of ligands has been described for various cell types (Steinman et al., 1983). Table II gives a list of some substances which have been described as binding to Kupffer and/or endothelial cells prior to endocytosis. The physiological importance for the uptake of most of the substances listed is still obscure. Ligand associated recognition groups for which Kupffer and/or endothelial cells appear to possess specific receptors include sugar groups (Hubbard et al., 1979; Kolb et al., 1979; 1980a,b), positively charged groups (Kooistra et al., 1980), Fc groups on IgG and complement C_3 (Munthe-Kaas, 1976).

Each of the three processes of endocytosis presented in Fig. 3 has its specific internalization mechanism which can be identified at the ultrastructural level.

During fluid-phase pinocytosis (process 1 in Fig. 3), internalization may occur by the invagination of a small smooth-walled part of the cell membrane. This process is called micropinocytosis. In Kupffer and endothelial cells, no smooth-walled micropinocytic invaginations are observed (Wisse, 1972). According to Silverstein, fluid-phase pinocytosis might also take place by the fusion of membrane folds which embrace the fluid to be captured, giving rise to larger smooth-walled vesicles. This process is often called macropinocytosis (Michl, 1980). In endothelial cells, the presence of large smooth-walled vesicles (diameter: 0.7 µm) which might originate from macropinocytosis has been described, but the actual internalization step of this mechanism has never been observed (Wisse, 1972). Since endothelial cells do not possess microvilli or flaps (Wisse, 1972), macropinocytosis by membrane folds seems very unlikely. No smooth-walled macropinocytic vesicles have been observed in Kupffer cells (Wisse, 1972).

Adsorptive pinocytosis (process 2 in Fig. 3) appears to occur in specialized invaginations of the plasma membrane containing a specific bristle-like coat. Therefore, these invaginations are also called bristle-coated pits and the internalization step bristle-coated micropinocytosis. Bristle-coated pits, generally having a diameter of 0.1-0.15 µm, have been described as being present on a large variety of different cell types, including Kupffer and endothelial cells (Wisse, 1977a,b). The coat of these pits consists of a special molecule, clathrin (Pearse, 1976).

TABLE II

ENDOGENOUS AND EXOGENOUS SUBSTANCES WHICH ARE INTERNALIZED BY KUPFFER AND/OR ENDOTHELIAL CELLS BY ADSORPTION

Substances	Cell type	Reference
Colloids, particles and cells:		
Colloidal gold	S	Chaudhuri et al., 1973
Colloidal carbon	K	Widmann et al., 1972; Ogawa et al., 1973
Colloidal albumin	K	Brouwer & Knook, 1977
^{99}mTc-sulphur colloid	K+E	Praaning-van Dalen et al., 1981
Galactose coated particles	K	Kolb-Bachhofen et al., 1982
Latex beads	K	Barelds et al., 1982
Liposomes	K	Wisse et al., 1976; Roerdink, et al., 1981
Cell organelles	K	Glauman et al., 1975a,b; Glauman & Trump, 1975
Zymosan	K	Ogawa et al., 1973
IgG opsonized erythrocytes	K	Munthe-Kaas, 1976; Barelds et al., 1982, Kirn et al., 1982
C_3 opsonized erythrocytes	K	Munthe-Kaas, 1976
Yeast cells	K	Barelds et al., 1982
Bacteria	K	Mills & Zucker-Franklin, 1969
Denatured proteins:		
Formaldehyde treated albumin	S	Buys et al., 1975; Nilsson & Berg, 1977; Watkins et al.,1979
Nitroguanidated albumin	S	Buys et al., 1975
Cross-linked lysozyme	S	Kooistra et al., 1980
Toxins:		
Endotoxin	K	Hirata et al., 1980; de Ruiter et al., 1981; Praaning-van Dalen et al., 1981
Ricin	S	Skilleter et al., 1981
Immune complexes and antigens:		
Immune complexes	S	Wagle et al., 1976
Carcino embryonic antigen	K	Toth et al., 1982

Hormones:		
Insulin	S	Wagle et al., 1976
Enzymes:		
Ribonuclease A	S	Kooistra et al., 1977
Ribonuclease B	S	Maynard & Baenzinger, 1981
Malate dehydrogenase	K	Bijsterbosch et al., 1982b
Lactate dehydrogenase M_4	K	de Jong et al., 1982
Lysosomal enzymes	S	Achord et al., 1978; Ullrich et al., 1979; Steer et al., 1979
	K+E	Hubbard et al., 1979
Proteins:		
Lysozyme	S	Kooistra et al., 1980
Lipoproteins:		
Very low density lipoproteins(VLDL)	S	van Berkel & Tol, 1979; van Berkel et al., 1981b
Low density lipoproteins (LDL)	S	van Berkel & Tol, 1979; Ose et al.,1980b; van Berkel et al.,1981b
High density lipoproteins (HDL)	K+E;S	van Berkel & Tol, 1979; Ose et al., 1979b; van Berkel et al., 1981b; Wandel et al., 1981
Chylomicron remnants	S	Groot et al., 1981; Lipiello et al., 1981
Acetylated LDL	S	van Berkel et al., 1982a
Acetoacetylated LDL	S	Mahley et al., 1979
Acetylated HDL	S	Wandel et al., 1981
Glycoproteins:		
Mannose terminated oligosaccharide chains	K+E;S	Hubbard et al., 1979; Maynard & Baenziger, 1981; Steer & Clarenburg, 1979; Straus, 1981
N-acetylglucosamine terminated oligosaccharide chains	K+E;S	Hubbard et al., 1979; Maynard & Baenziger, 1981 Steer et al., 1978; Steer & Clarenburg, 1979
Mucopolysaccharides:		
Hyaluronic acid	S	Fraser et al., 1981

Abbreviations: K: Kupffer cells, E: Endothelial cells, S: Sinusoidal cells (i.e., no exact discrimination was made between Kupffer and endothelial cells).

Since adsorptive pinocytosis occurs specifically in bristle-coated pits, the pits must contain binding sites or receptors. These binding sites or receptors might already be present in the pits before they bind the ligand. However, it is also possible that certain binding sites or receptors are previously distributed over the plasma membrane and need the trigger of the bound ligand to move into and cluster in the pits.

During the internalization step of phagocytosis (process 3 in Fig. 3), lamellipodia of the plasma membrane evaginate and tightly move around a particle until it is fully enclosed. This mechanism of internalization has not been observed in endothelial cells (Wisse, 1977a). Time-lapse video recording of cultured Kupffer cells has shown that, before any attachment occurs, preexisting lamellipodia are very motile, as if they are seeking for a "victim" substance for phagocytosis (Barelds et al., 1982). The mechanism of phagocytosis is a common characteristic of all macrophages. In Kupffer cells, however, two additional endocytic mechanisms have been described (Wisse, 1977a). The first is the internalization by worm-like structures, i.e., tubular invaginations of a part of the cell membrane in which the fuzzy coat is quite obvious (see Section 1.3). The second additional mechanism is the internalization by so-called fuzzy-coated pinocytosis, during which large fuzzy-coated pinocytic vesicles (diameter: n x 1 μm) are formed. Unlike the bristle coat which is present on the outside, the fuzzy coat covers the inside of a vesicle. As in the case of macropinocytosis in endothelial cells, the actual internalization step of fuzzy-coated pinocytosis has never been observed and the vesicles may also develop from dilatations of worm-like structures. It is unlikely that fuzzy-coated vesicles develop from internalization by phagocytosis, since the fuzzy coat is always absent on those parts of the cell membrane that are involved in phagocytosis (Wisse, 1977a).

An important question is whether the different routes of internalization are really determined by the size of the ligand. Clearly, a large-sized particle of 1 μm will not be internalized by a vesicle of 0.1 μm. Since macrophages are the only cells that are active in the uptake of very large particles (>1 μm) and at the same time are the only cells that perform phagocytosis, it is tempting to speculate that large-sized particles may be taken up by this mechanism only. Indeed, uptake of latex beads of 0.8 μm by Kupffer cells has been described to occur by phagocytosis (Wisse, 1977b). However, endocytosis by invagination instead of evagination of the plasma membrane in Kupffer cells has also been reported for the uptake of 0.8 μm latex beads (Barelds et al., 1982) and for complement-coated red blood cells (Munthe-

Kaas, 1977). Aggeler & Werb (1982), who studied the endocytosis of 0.45 µm latex beads by peritoneal macrophages, observed that these particles were internalized by an invagination of a part of the plasma membrane which was bristle- (i.e., clathrin-) coated. Thus, the vesicles formed during bristle-coated micropinocytosis are obviously not restricted to a diameter of 0.1-0.15 µm and for the internalization of a specific particle macrophages might be able to choose between two different processes, i.e., phagocytosis or bristle-coated micropinocytosis. Since these studies show that the endocytosis of large particles is not restricted to one mechanism only, the term phagocytosis used in this thesis will always refer to the morphological characteristics described in Fig. 3 (process 3) and not to a mechanism by which particles of a certain size or nature are taken up.

The internalization step of endocytosis ends with the formation of an intracellular vesicle. Fluid-phase pinocytic vesicles contain substances in exactly the same concentration as found in the extracellular medium. This endocytic mechanism is thus nonselective and the amount of endocytosed substance will be a linear function of its concentration in the extracellular medium. In contrast, vesicles that arise from bristle-coated micropinocytosis can contain a concentration of substance which is several times higher than that in the extracellular medium. The amount of endocytosed substance will then depend not only on the rate of vesicle formation but also on the number of binding sites and their affinity for the substance. Since the maximum amount of substance that can accumulate is dependent on the definite number of binding sites on the membrane of the vesicle, uptake by this mechanism will show saturation kinetics. It must be emphasized, however, that a certain amount of substance will always enter the cell in the fluid phase, not only via the fluid-phase pinocytic vesicles but also via the fluid which is trapped in the bristle-coated micropinocytic vesicle itself. The vesicle that arises after phagocytosis usually contains only a single particle, while the amount of fluid captured in the vesicle is considered to be relatively small.

Several names have been used for the vesicles developed as a result of endocytosis. The most frequently used terms are pinosome, endosome (Steinman et al., 1983), phagosome (Silverstein et al., 1977) and receptosome (Willingham et al., 1981b). It is not always clear which term belongs to which internalization process. The accurate description of newly formed vesicles is complicated by the fact that rapid fusion of the vesicles with each other or with other organelles might occur.

After formation of the vesicles, the intracellular transport of the vesicles will start as the last step in the endocytic process. As shown in Fig. 3, some fluid-phase pinocytic or bristle-coated vesicles might merely migrate through the cell and excrete or exocytose their contents at the other side. This process has been described for cells of the vascular endothelium and for mesothelial cells. In sinusoidal liver cells, the endocytic vesicles generally fuse with primary lysosomes and this results in secondary lysosomes (Buys et al., 1973;1975; Wisse, 1977b; Nilsson & Berg, 1977; Kooistra et al., 1977; 1980; de Bruyn et al., 1980). Fusion of an endocytic vesicle with a secondary lysosome might also occur. Morphological investigation of Kupffer and endothelial cells revealed that both cell types possess a considerable number of lysosomal structures (see Section 1.3). The lysosomal enzymes degrade the endocytosed substances. Small size degradation products will leave the lysosomes and finally the cells primarily by diffusion. If a substance cannot be degraded by the enzymes, it remains in the lysosome. A lysosome filled with undegradable material is called a residual body.

1.5 QUANTITATIVE DETERMINATION AND CHARACTERIZATION OF THE ENDOCYTIC PROCESS

Several methods can be applied to quantitatively determine the rate of endocytosis and the amount of endocytosed substance. In vivo, a substance can be intravenously injected into a test animal and the rate of its plasma clearance can be assumed to reflect the rate of endocytosis. Injected colloids appear to be cleared from the plasma in an exponential way, indicating a one-compartment clearance system, which proved to be the liver. The use of the plasma clearance method as an expedient for determining the endocytic capacity of liver cells has several disadvantages. When the amount of injected colloid is very low, the rate of endocytosis depends merely on the rate of hepatic blood flow and not on the endocytic capacity of the cells themselves (Benacerraf et al., 1957a,b; Fujii et al., 1979). Moreover, discrimination between attachment of particles to the cell surface and actual internalization is not possible. Finally, the cell type involved in the endocytosis and the mechanism by which endocytosis occurs cannot be identified. Several investigators have reported that the injection of colloidal carbon leads to an inhibition of the clearance of other colloids. Such an inhibitory effect is often assumed to demonstrate the involvement of Kupffer cells in the clear-

ance of a specific colloid. However, it might just reflect some secondary effect, since nothing is yet known about the exact mechanism of the inhibition by colloidal carbon (see also Section 1.6).

A more direct way to quantify endocytosis in vivo involves the determination of the intracellular accumulation of a substance. This might be achieved by cytochemical methods or by autoradiography. Although often applied, light microscopic examination of accumulated substances cannot give exact information about the cell type involved in the endocytosis nor about the amount of substance attached to the cell membrane or the mechanism of endocytosis. More information can be obtained from ultrastructural studies. At the ultrastructural level, ligands which are not themselves electron dense can be visualized by (immuno-) cytochemical staining, by autoradiography or by labelling with an electron dense particle which does not influence the mechanism and rate of endocytosis. Disadvantages of studies on endocytosis at the ultrastructural level are their time consuming characteristics and the difficulties in accurate quantification.

In the work described in this thesis, quantification and characterization of endocytosis were based on a completely different approach. The recent development of methods for the isolation and purification of the main liver cell classes (see Chapter 2) opened up the possibility for a qualitative as well as quantitative analysis of endocytosed substances present in these cell types. This was achieved in two ways: 1) a substance was injected in vivo and detected in Kupffer and endothelial cells after their isolation and purification; 2) isolated and purified Kupffer and endothelial cells were incubated with a substance in vitro and the amount of endocytosed substance was then determined. The good viability and integrity of isolated Kupffer and endothelial cells permitted the performance of these in vitro experiments immediately after the cell isolation; therefore, no culture period to allow recovery of the cells from trauma was necessary.

Both approaches provide information on the endocytic rate and capacity, the endocytic mechanisms and the differences in endocytosis between Kupffer and endothelial cells.

The in vitro system is especially suitable for investigating receptor mediated endocytosis. By inhibition studies with several ligands of a similar structure, the specificity and affinity of a receptor can be established. Secondly, in the in vitro system, discrimination between the binding of a ligand and its actual internalization is possible because of the energy and temperature dependency of the internalization as contrasted with the binding

process. Thirdly, the in vitro system offers the possibility to easily discriminate between adsorptive and fluid-phase pinocytosis. To achieve this, uptake should be determined at various substance concentrations to establish whether saturation occurs. Discrimination between adsorptive pinocytosis and phagocytosis by means of inhibitors might not be so easy (see Chapter 5) and additional ultrastructural studies are necessary (see Section 1.4).

1.6 PRESENT CONCEPT OF THE RETICULOENDOTHELIAL SYSTEM AND ITS RELATION TO THE CLEARANCE CAPACITY OF THE LIVER

The definition of the reticuloendothelial system (RES) was first formulated by Aschoff in 1924. Based on the uptake of intravenously injected vital dyes, which Aschoff claimed entered cells by the process of phagocytosis, he defined the RES as the collection of all sinusoidal lining cells in the body that are capable of phagocytosis.

Since Aschoff's data were based only on light microscopic observations, he was not able to discriminate between phagocytosis and pinocytosis. He probably was not even aware that there might exist different endocytic mechanisms and meant by "phagocytosis" the process of cellular uptake in general. However, ultrastructural studies proved that phagocytosis, as presently defined (see Section 1.4), is not involved during the uptake of a dye.

Nevertheless, phagocytosis is still one of the most important features on which the present concept of the RES seems to be based. It was generally concluded that the RES was represented only by macrophages, cells which are specialized in the uptake of substances by phagocytosis. It should be obvious that if — as originally — the definition is based on the clearance of injected substances, the RES can only be composed of those macrophages that line the blood vessels and not, for instance, of alveolar macrophages of the lung, lamina propria macrophages of the gut or lymph-sinus lining macrophages of the lymph nodes. Several investigators, however, thought it necessary to broaden the definition of the RES. Altura (1980) noticed that the macrophages lining the bloodstream share several common functions with other cells and he therefore considered all monocytes and macrophages as representatives of the RES. In this broader concept, only part of the RES was involved in the clearance of substances from the bloodstream, whereas it also became involved in lipid metabolism, protein metabolism, iron metabolism,

tumor growth, shock, radiation injury, infection and a variety of immunological processes (Altura, 1980). Obviously, it is not possible to test the overall function of this RES simply by its capacity to clear substances from the blood. In an attempt to again make a distinction between the selective population of macrophages lining the bloodstream ("the" RES) and the total assemblage of macrophages in the body, the latter collection was named by Van Furth the Mononuclear Phagocyte System (MPS) (1972). Cells within this system are assumed to be linked not only by their phagocytic capacity but also by morphologic similarity, characteristic enzymatic activity and common lineage from a bone marrow precursor cell. However, the assumption that all macrophages in the body are derived from a pluripotent stem cell of the bone marrow is still heavily debated (van Furth, 1980; Wisse, 1980). Moreover, the functional capacity of the MPS can again not be tested by its capability of clearing substances from the blood. Meanwhile, the term RES according to Altura's definition is still used as frequently as is the term MPS.

Although according to Altura's definition of the RES and Van Furth's of the MPS, more macrophages are included in both systems than just the ones lining the blood vessels, the functional capacity of the RES is still determined by its activity in the clearance of intravenously injected substances. The most frequently used functional RES test is the "basic colloid clearance method" which is based on the injection of a foreign colloid and the determination of its plasma clearance rate. Various colloids and other substances used to assess the RES clearance function are listed in Table III. Characterization of the RES colloid clearance rate indicated it to be exponential . There was a saturation effect at increasing amounts of administered test substance and an inhibition or blockade in the clearance of one substance after previous injection of another type of colloid (Saba, 1970).

Under normal physiological conditions, most injected RES test substances are cleared primarily by the liver (85-90%) (Saba, 1970; Altura, 1980). Since the clearance is assumed to be performed by macrophages, the macrophages of the liver, the Kupffer cells, are thought to carry out 85-90% of the RES clearance function. Therefore, under normal physiological conditions, a "RES clearance test" might be regarded as being synonymous to a "Kupffer cell clearance test". Thus, such a clearance test gives no information on the functional capacity of other macrophages lining the blood vessels.

TABLE III

SUBSTANCES USED TO ASSESS THE RES CLEARANCE FUNCTION

Substance	Reference
RE test lipid emulsion (colloidal)	Saba & di Luzio, 1969; Antikatzides & Saba, 1977; Saba & Antikatzides, 1979; Lorenzen & Saba, 1979; Saba & Cho, 1979; 1980; Kaplan, 1980; Kaplan & Saba, 1981
Colloidal carbon	Benacerraf et al., 1957b; Jenkin & Rowley, 1961; Degrê & Rollag, 1979; Loegering, 1981; Schlick & Friedberg, 1981; Cornell, 1982
Colloidal or aggregated albumin	Benacerraf et al., 1957b; Liu, 1979; Lahnborg et al., 1981; Toki et al., 1981; Cornell, 1982
^{99m}Tc-sulphur colloid	Marshall & Ludbrook, 1975; van Vliet et al., 1981
Gelatin colloid	Boisvieux et al., 1979
Chromium phosphate particles	Benacerraf et al., 1957b
Saccharated iron oxide	Benacerraf et al., 1957b
Fibrin monomers	Cornell, 1982
Red blood cells	Benacerraf et al., 1957b; Halpern et al., 1957; Lawley, 1980; Grover & Loegering, 1981; Henderson et al., 1981; Gordon et al., 1981
Bacteria	Jenkin & Rowley, 1961; Podoprigora & Zaitsev, 1979

In 1957, Benacerraf (Benacerraf et al., 1957b) listed the following characteristics which a good RES test substance should have to be used to accurately determine the phagocytic activity of the RES:

1) The particles should be phagocytosed by the cells of the RES in contact with the blood.
2) They should not normally cross the capillary barrier.
3) They should be homogenous in size.
4) They should be stable in the blood at the concentration used.
5) The substance should be nontoxic for the cells and for the organism.
6) The substance should be accurately measurable in the blood and in the tissues by chemical or physical methods.
7) The test particle should not be taken up by cells other than those investigated. That is, they should be specifically cleared from the blood by the RES.

Saba (1970) suggested an additional criterion, which is that the clearance rate of the substance should depend primarily on the phagocytic activity of the cells.

A further evaluation of these criteria is necessary to get an impression of whether the frequently used RES test substances fulfil these criteria. The characteristic mentioned under point 1 will be discussed in relation to point 7.

The criterion mentioned under point 2 implies that the test substance should be of such a size that it would not be distributed over a volume greater than that of the plasma. If a substance is distributed over a greater volume, this will be reflected in the clearance rate of the substance, which will at first be nonexponential. The exponential clearance rate of most substances used to test the functional capacity of the RES indicates that these substances fulfil the criterion.

With regard to criteria 3 and 4, it should be emphasized that, although a substance might be prepared in such a way that it is very homogeneous in size, the injection of such a substance might lead to aggregation into clumps of various sizes. For instance, the test substances dextran sulphate (Bradfield, 1980) and colloidal carbon (Bloch & McCuskey, 1977) aggregate in the blood and the extent of aggregation might depend on the rate of injection of certain substances (Bloch & McCuskey, 1977). Aggregation might influence the rate of clearance of a substance and aggregated clumps may be cleared by a mechanism other than that involved in the clearance of the substance

itself. Certain colloids might be suitable as a marker for phagocytosis simply because of the fact that they aggregate after contact with the blood.

The toxicity of some RES test substances (point 5) has been discussed by Saba (1970). Toxicity of biologically inert substances like colloids may be related to contamination with endotoxin (Saba, 1970).

Test substances which are readily degraded by the cells do not satisfactorily fulfil the criterion mentioned under point 6, as was pointed out under Section 1.5. Variable degrees of aggregation will probably also interfere with the accurate determination of substance concentration in the blood.

The criterion added by Saba is a very important one. The rate of colloid clearance from the blood might not reflect the functional phagocytic activity of the RES cells themselves. Various other factors might act as rate limiting ones. Firstly, the amount of substance adhered to the cells might be falsely interpreted as uptake by the cells (see also Section 1.5.). Secondly, the rate of clearance might depend solely on the rate of blood flow (see also Sections 1.1. and 1.5.). Finally, the rate of clearance might depend on the presence of certain serum or plasma factors. The importance of plasma factors or "opsonins" in the clearance of various substances has been described by several authors. Certain substances are endocytosed only when they are covered by opsonins, indicating that the cells involved possess plasma membrane components that are able to recognize these opsonins. Kupffer cells, e.g., are known to endocytose red blood cells which are covered with IgG or with IgM-C_3 (Munthe-Kaas, 1976). The importance of a certain opsonin, fibronectin, in the clearance of foreign particles has been extensively investigated by Saba and DiLuzio (1969). They showed that the concentration of opsonins becomes a rate limiting factor in the clearance as soon as the dose of substance is so high that a depletion of opsonins occurs. The opsonin fibronectin is probably also the factor which causes the aggregation of certain colloids in the blood (Check et al., 1979; Jones & Summerfield, 1982). Another factor which has been mentioned as being important in the clearance of colloids is the presence of blood platelets (Donald & Tennet, 1975). Other factors such as the concentrations of heparin and calcium or the pH of the plasma are also reported to affect the clearance rate of a substance (Ryder et al., 1975). It can be concluded that it is difficult to be certain that the endocytic capacity of the cells belonging to the RES is the rate limiting factor in the clearance of a specific colloid.

The most difficult criteria for an accurate RES colloid clearance test to fulfil are given in points 1 and 7. Since the clearance test as such can give no information on the mechanism by which a given colloid is taken up nor on the exact cellular localization, it is necessary to first investigate the uptake at the ultrastructural level. The electron microscopic observation that certain injected RES test substances are recovered in the endothelial cells of the liver (Wisse, 1977a) indicates that the test substances are not always cleared exclusively by macrophages (i.e., Kupffer cells) and by the mechanism of phagocytosis. Moreover, several observations indicate the presence of more than one clearance mechanism: a) colloids of different size, charge and chemical composition are cleared at different rates (Halpern et al., 1957; Saba, 1970; Bloch & McCuskey, 1977); b) their organ distribution sometimes varies (Bradfield, 1980); and c) they inhibit each others clearance to different extents (Jones & Summerfield, 1982). The question thus arises of which mechanism is tested for by which test substance and whether one of these mechanisms can be regarded as representing a system which reflects the original concept of the RES, i.e., phagocytosis by macrophages lining the blood vessels.

One of the experimental models for the study of the pathophysiology of the RES is the induction of a RES depression or even blockade by intravenous administration — usually an overload — of particles, leading to an inhibition of endocytosis. Estimations on the extent of RES depression are usually based on plasma clearance tests. Bradfield (1980) emphasized the selective nature of the RES depression, since it occurred only in the liver, not in other organs. Many substances give rise to RES depression, the extent of which depends on the nature of the substance. Colloidal carbon is the most commonly used substance for inducing RES depression.

There are various theories to explain RES depression. The classical theory of Benacerraf et al. (1957b) assumes a physiological saturation of the cells. This might be induced by either saturation of the attachment of particles to the cell membrane (Normann, 1973), an intracellular overload, or a secondary inhibitory effect of the particle on the internalization rate. Another theory assumes that RES depression is based on depletion of opsonins (fibronectin) in the plasma (Saba & DiLuzio, 1969). The latter theory gave rise to the clinical administration of fibronectin to patients who were likely to show RES depression as a result of surgical trauma (Saba et al., 1978; Scovill et al., 1978). Saba (1970) mentioned that one type of colloid can induce a state of RES depression relative to the subsequent clearance of that parti-

cular colloid, while the clearance of a dissimilar colloid is less affected. Saba suggested that this phenomenon might be explained by the existence of a multiple opsonic system.

Theoretically, the altered clearance rate during RES depression might be mediated by any of the rate limiting factors mentioned above. It is obvious that a slowed rate of blood flow may also lead to depression of the RES clearance. The blood flow might be influenced by the injected substance itself, e.g., due to extensive aggregation or by secondary physiological factors. Finally, the size of the cell population participating in the clearance of the RES is of great importance. Donald & Tennet (1975) mentioned that, under certain circumstances, the clearance capacity of only a small selected population of Kupffer cells may be determined, while, under other conditions, e.g., a larger dose of test substance, the cell population participating in the clearance becomes larger and even other cell types such as endothelial cells might become involved in the clearance (Hausmann et al., 1976). On the other hand, induction of RES depression might decrease the number of cells, e.g., because the injected substance is toxic to the cells after uptake. In case of cell loss, the depressed state of the RES will last until the population of cells is restored (Souhami et al., 1977; Bradfield, 1980). Although Kupffer cells are capable of self-replication (Bouwens & Wisse, 1982), abnormal cell loss might induce migration of monocytes from the bone marrow to achieve repopulation of the macrophages in the liver (Jones & Summerfield, 1982).

Bradfield (1980) suggested that more than one mechanism of RES depression might exist. This is emphasized by the fact that recovery from depletion of serum opsonins takes only a few hours (Saba & DiLuzio, 1969), whereas Halpern et al. (1957), using a different experimental setup, were able to induce a blockade that was not dependent on opsonins and which lasted for several days. This long lasting blockade might reflect the time necessary for the repopulation of lost cells. A conclusion here is that, when RES depression is evaluated by clearance characteristics, it is often questionable whether one really measures what is intended.

The data presented have illustrated that it is very difficult, if not impossible, to properly explain the current concept of the RES. Since the original one was based on false assumptions and definitions, this has led to the evolution of a new concept that finally bears only a resemblance to the original. It is now clear that the extended new concept of the RES includes a variety of physiological phenomena brought about by an illdefined, heterogeneous population of cells. This makes it difficult to rely on a colloid clear-

ance assay alone for the evaluation of the functional capacity of the RES as a whole. Furthermore, as has been discussed in this section, the colloid clearance test has some other serious disadvantages. If this way of determining the functional capacity of the RES is indeed not accurate, a reevaluation of an immense amount of work will be necessary. Otherwise, a complete revision of the concept of the RES would be indicated.

1.7 OUTLINE OF THE PRESENT STUDY AND CHOICE OF EXPERIMENTAL APPROACH

In the preceding sections (1.1. to 1.6.), indications of the possible role of endothelial cells in the clearance of RES test substances were presented and the complexity of the mechanisms by which substances might interact with the blood and cells was discussed. Such considerations led to the following basic questions for the study described in this thesis: 1) what is the endocytic capacity of Kupffer and endothelial cells in relation to the functional capacity of the "RES"? and 2) by which mechanisms do these two cell types endocytose various substances which are either used as RES test substances or assumed to be cleared by the RES?.

For the present study, the working definition of the RES will be that it is the collection of all cells participating in the clearance of colloidal and certain other test substances from the bloodstream by endocytic mechanisms. Thus, it was supposed that the cell population contributing to the clearance of the RES test substances was not necessarily represented solely by macrophages and that apart from phagocytosis other mechanisms of internalization might also be involved in the clearance of RES test substances. Other possible functions than clearance were not regarded as being a priori included in the working definition of the RES. It was first established that the cell population(s) involved in the clearance of the RES test substances were located mainly in the liver. The correctness of the definition described above was then tested for experimentally.

The studies were performed at the physiological, biochemical and ultrastructural levels. Since most questions could not be answered only from plasma clearance studies or organ localization of injected substances, it was necessary to apply a liver cell isolation procedure by which in vivo endocytosed substances could be quantitatively investigated at the cellular level and by which endocytosis could also be studied in vitro.

The development of such an isolation procedure and the characterization of the isolated cell populations are described in Chapter 2. Chapter 3 deals with the plasma clearance rates, the organ distribution and cellular localization of various injected substances. The latter was estimated quantitatively as well as studied at the ultrastructural level. The rate of blood flow through the liver and its effect on the rate of clearance performed by the liver cells are described. The very minimum and maximum rates at which Kupffer and endothelial cells might clear an injected substance are given. Chapter 4 includes quantitative and ultrastructural information on the endocytic capacity of the cells in vitro. A comparison with the in vivo results is made. Chapter 5 includes a detailed study of the mechanisms by which Kupffer and endothelial cells endocytose the various substances. This study was based on biochemical and ultrastructural observations in vivo as well as in vitro. From the experimental results, additional information was obtained with respect to the value of the colloid clearance test and the influence of adsorption and aggregation of substances on the outcome of quantitative determinations of clearance experiments. Some information was also obtained on the involvement of opsonins in the clearance and on the induction of RES depression. In the general discussion of the results presented in Chapter 6, special attention is paid to the significance of the findings in relation to the present concept of the RES.

CHAPTER 2

ISOLATION AND CHARACTERIZATION OF KUPFFER AND ENDOTHELIAL CELLS FROM THE RAT LIVER

2.1 INTRODUCTION

The isolation of liver cells by enzymatic digestion of the intercellular matrix enables detailed studies on their biochemical characteristics. A suspension of isolated liver cells may be composed of endothelial, parenchymal, Kupffer and fat-storing cells, some smooth muscle, some bile duct and blood cells. All of these cells, except for the parenchymal ones, are called "nonparenchymal cells", whereas the cells actually lining the sinusoids, i.e., Kupffer and endothelial cells, are "sinusoidal cells". In this thesis, a suspension of isolated liver cells from which the parenchymal cells are removed will for convenience be called a "sinusoidal cell suspension", since these suspensions normally contain more than 75% of sinusoidal cells. Several procedures are presently applied for sinusoidal liver cell isolation, all of which have specific advantages and disadvantages (Knook & Sleyster, 1977; van Berkel, 1982). Each isolation procedure has a different effect on the composition of the cell suspension (i.e., yield, purity, etc.) as well as on the characteristics of the individual cells (i.e., ultrastructural integrity, viability, size, density, protein content and function) (Brouwer et al., 1982). Therefore, the choice of an isolation method must be based on the purpose of a specific study.

To achieve the objective of the study, the endocytic capacity of sinusoidal cells had to be determined both *in vivo* and *in vitro*. For quantitative *in vivo* studies, isolation of the cells offers the possibility of determining the amount of a substance endocytosed *in vivo* in a specific cell population. However, one should be aware of the following problems. If the substance is degradable by the cells, degradation of the endocytosed substance can occur during the time required for the isolation. Furthermore, if a substance which is normally avidly endocytosed by the sinusoidal cells is released from cells damaged during the isolation procedure, it may be again endocytosed by other cells and this might lead to an overestimation of the endocytic capacity

of the sinusoidal cells. For in vitro studies on endocytosis, the main problem is the action of proteolytic enzymes used in the isolation procedure on the cell membrane. This may result in destruction of binding sites or receptors necessary for the recognition of the substances presented to the cells.

A suitable isolation method for the study on endocytosis should therefore fulfil the following criteria. For determination of in vivo endocytosis, the method should allow isolation in such a way that no degradation and no redistribution of the endocytosed substances occurs during the isolation procedure. For in vitro endocytosis, isolated cells should still possess binding sites or receptors on their membranes. Apart from these specific criteria, the isolated cells should also be of high viability and good ultrastructural integrity. For separation of sinusoidal cells into Kupffer and endothelial cells, the sinusoidal cell yield must be of sufficient quantity and contamination by parenchymal cells or parts of them (blebs) should be minimal. The cell size, protein content, morphology and specific functional properties should change as little as possible during isolation.

To properly evaluate methods for sinusoidal cell isolation, one of the prerequisites is that a discrimination between Kupffer and endothelial cells must be possible.

In this chapter, several isolation methods were compared and checked as to whether they fulfilled the demands outlined above.

2.2 MATERIALS AND METHODS

2.2.1 Animals

For all experiments, 3 to 4-month-old female Brown Norway/Billingham, Rijswijk (BNBi/Rij) rats with an average body weight of 150 g were used. The animals were maintained under "specific pathogen free" conditions as described by Solleveld (1978). The mean liver weight of the rats was 4.8 g. Before perfusion of the liver, the animals were placed under ether anaesthesia.

2.2.2 Sinusoidal cell isolation procedures

2.2.2.1 Isolation with pronase at 37°C (warm pronase isolation method)

a) Isolation of sinusoidal cells by means of selective destruction of parenchymal cells by pronase E (Merck) was performed as described earlier (Knook

& Sleyster, 1976). The liver was perfused in situ (10 ml per min) at 37°C with Gey's balanced salt solution (GBSS) for 2 min and subsequently perfused with GBSS containing 0.2% pronase for 1 min. After excision of the liver and removal of Glisson's capsule, the liver was minced and incubated at 37°C in GBSS with 0.2% pronase for 60 min under vigorous stirring. The pH was kept at 7.4 with 1N NaOH. The suspension was filtered through nylon gauze.

b) Sinusoidal cells were harvested from the filtered suspension by centrifugation (10 min; 300 x g). Sinusoidal cell suspensions were freed of erythrocytes by density centrifugation in GBSS with a lowered NaCl concentration and containing 17.5% metrizamide (15 min; 400 x g). After centrifugation, the sinusoidal cells present in the top layer of the metrizamide solution were washed with GBSS at 300 x g for 10 min. The sinusoidal cells in the pellet were diluted in 5 ml GBSS. All centrifugation steps were performed at room temperature.

2.2.2.2 Isolation with pronase at 10°C (cold pronase isolation method)

The liver was perfused in situ (10 ml per min) with GBSS at 37°C for 3 min and subsequently at 10°C for 10 min with GBSS containing 0.2% pronase E and followed by GBSS containing 1.3% bovine serum albumin (GBSS-BSA) at 10°C for 2 min. This BSA prevented the cells from the clumping which normally occurs at low temperatures. After excision of the liver, Glisson's capsule was removed. The cells were brought into suspension by shaking the liver briefly in ice-cold GBSS-BSA and scraping the tissue with a spatula. The pH was adjusted to 7.4 with 1N NaOH. The cell suspension was then filtered through nylon gauze. All further steps were performed as described under Section 2.2.2.1b, with the exception that GBSS containing BSA was used and that the temperature was kept at 4°C.

2.2.2.3 Isolation with thermolysine (thermolysine isolation method)

Isolation with thermolysine, an enzyme which like pronase E destroys parenchymal cells, was performed exactly as was the warm pronase isolation described under Section 2.2.2.1. Instead of pronase, 0.035% thermolysine (Boehringer) was used.

2.2.2.4 Isolation with pronase plus collagenase (pronase-collagenase isolation method)

Isolation with pronase plus collagenase was performed as described by Knook et al. (1982). In brief, the liver was first perfused in situ (10 ml per

min) with GBSS for 5 min. This was followed by a perfusion of 6 min with GBSS containing 0.2% pronase E. During the perfusion, the liver was excised and placed on a sieve. It was then connected to a circulation perfusion system containing 0.05% collagenase (type 1, Sigma Chemical Co.) and 0.05% pronase E dissolved in GBSS and perfused for 30 min. All media were kept at 37°C. After the perfusion, the paste-like liver substance was incubated in GBSS containing 0.05% collagenase and 0.02% pronase E (37°C, 30 min) under vigorous stirring. The pH was kept at 7.4. After the suspension was filtered through nylon gauze, the cells were further purified at room temperature as described under Section 2.2.2.1b.

2.2.2.5 Isolation with collagenase (collagenase isolation method)

Isolation of sinusoidal cells with collagenase was based mainly on the method for parenchymal cell isolation described previously (van Bezooijen, 1977). The liver was first perfused in situ at 37°C for 1 min (15 ml per min) with a special Ca^{++} free medium ("perfusion medium") supplemented with 0.15% hyaluronidase (type 1, Sigma Chemical Co.) and 0.05% collagenase ("dissociation medium"). The liver was then perfused for 15 min with "perfusion medium" to eliminate Ca^{++} and subsequently with recirculating "dissociation medium" for 20 min. During the perfusion, the liver was excised and placed on a sieve. After the perfusion, the liver was minced and incubated for 20 min at 37°C in "dissociation medium" by shaking. After the suspension was filtered through nylon gauze, sinusoidal cells were separated from parenchymal cells by differential centrifugation (0.5 min, 50 x g; three times) during which the cells were gradually introduced into GBSS. The sinusoidal cells were further purified as described under Section 2.2.2.1b. In some experiments, trypsin inhibitor (Soybean, Calbiochem) was added to reduce the effect of the proteolytic activity of the enzyme preparation on the cell membrane. However, this resulted in a very low yield of especially Kupffer cells and a protective effect on the cell membrane could not be determined.

2.2.3 Separation of Kupffer and endothelial cells

The Kupffer and endothelial cells in the sinusoidal cell suspension were separated by centrifugal elutriation in a Beckman JE-6 elutriation rotor at 4°C with GBSS (or GBSS-BSA) as the elutriation fluid (Knook & Sleyster, 1976). At a constant rotor speed of 2500 rpm, three successive fractions were collected : lymphocyte, endothelial cell and Kupffer cell fractions at

flow rates of 13.5, 23.5 and 40.0 ml per min, respectively. With cells isolated by the cold pronase method (Section 2.2.2.2), a Sanderson chamber instead of the conventional separation chamber was used in the elutriator rotor to achieve higher purities. In this case, a rotor speed of 3200 rpm was used and the three fractions were eluted at flow rates of 17.5, 32.5 and 52.5 ml per min, respectively. The cells present in the fractions were pelleted by centrifugation (10 min, 300 x g), resuspended in 5 ml GBSS, counted in a hemocytometer and characterized as described below.

2.2.4 Cell culture

For the culture of purified endothelial cells, the cells were suspended in Dulbecco's modification of Eagles' medium with L-glutamine containing 20 mM HEPES and 10 mM $NaHCO_3$, pH 7.4. This medium was supplemented with penicillin (100 units per ml), streptomycin (100 µg per ml) and 30% serum (heat inactivated fetal calf or freshly prepared rat serum). Endothelial cells were cultured in plastic Petri dishes coated with collagen (4×10^6 cells per culture dish of 3 cm diameter). The cultures were incubated at 37°C in a humidified atmosphere containing 3% CO_2 in air. The culture of Kupffer cells as done by Brouwer and Knook (1977) was not included in this study.

2.2.5 Fixation for electron microscopy

2.2.5.1 Perfusion fixation

For direct fixation, the liver was perfused <u>in situ</u> with a physiological salt solution for 1.5 min and then with 2% glutaraldehyde in 0.15 M sodium cacodylate buffer (4°C) for 5 min at a perfusion rate of 5 ml per min.

However, to follow the course of the enzymatic digestion of the liver during cell isolation, the livers were first perfused for cell isolation as described in Section 2.2.2. and the enzymatic digestion was stopped at various time points by direct perfusion with glutaraldehyde containing buffer.

After fixation, the liver was removed and cut into thin slices with a razor blade. These slices were further cut into sections of 75 µm with an Oxford vibratome. The sections were postfixed in 1% OsO_4 in 0.15 M sodium cacodylate buffer for 10-15 min and subsequently dehydrated in a graded series of ethanol. Before embedding in Epon LX 122 resin, the sections were rinsed for 2 hours in a mixture of Epon and propylene oxide (1:1). After the embedding medium was hardened, ultrathin sections were cut and con-

trasted with uranyl acetate and lead citrate. The sections were examined in a Philips 300 transmission electron microscope.

2.2.5.2 Fixation of isolated and cultured cells

Isolated and cultured cells were fixed in 2% glutaraldehyde 0.15 M cacodylate buffer for at least 1 hr. Cells were postfixed, dehydrated and embedded as described under Section 2.2.5.1. After fixation and embedding of cultures (de Leeuw et al., 1982b), the cultures dishes were broken away after the hardening of the embedding medium. Ultrathin sections were cut parallel to the bottom of the culture dish. Further steps were performed as described under Section 2.2.5.1.

2.2.6 Characterization of sinusoidal cell suspensions and cell fractions obtained by centrifugal elutriation

Cell viability was estimated from the percentage of cells which were able to exclude 0.25% trypan blue. The results were confirmed by ultrastructural studies of the cells.

The cellular protein content was determined by the method of Lowry et al. (1951), with bovine serum albumin as the standard.

For peroxidase staining, cells were incubated as described by Wisse (1974). The enzymatic interaction of the substrate DAB with H_2O_2 resulted in a brown polymerization product of DAB. All Kupffer cells were positively stained for endogenous peroxidatic activity, which made them easily recognizable under the light microscope. As a result, the percentage of Kupffer cells in each cell fraction could be determined. Endogenous peroxidase could also be demonstrated at the ultrastructural level. In this case, cells were fixed in 2% glutaraldehyde in 0.15 M cacodylate buffer for less than 30 min and incubated with DAB as described above. Longer fixation periods resulted in the loss of endogenous peroxidatic activity.

Discrimination between lymphoid and sinusoidal cells was possible under the light microscope by staining of the cells for esterase activity. For the nonspecific esterase activity, cells of the different cell fractions were fixed in ice-cold 2% glutaraldehyde in 0.15 M cacodylate buffer (pH 7.4) and incubated with 1-naphtylacetate at 20°C for 5-7 min (Emeis & Planqué, 1976). The esterase-positive Kupffer and endothelial cells could be distinguished from nonstaining lymphoid cells (Emeis & Planqué, 1976). Lymphoid and sinusoidal cells could also be discriminated by incubation (in vivo, in situ or in

vitro, with cold pronase isolated cells) with horseradish peroxidase (HRP). After endocytosis by the sinusoidal cells, HRP could be demonstrated cytochemically by the reaction for peroxidatic activity (see also Sections 3.3, 4.3 and 5.3) and the percentage of sinusoidal cells estimated. The percentage of endothelial cells in the different cell fractions was determined ultrastructurally or by subtracting the percentage HRP or esterase negative (nonsinusoidal) and peroxidase positive (Kupffer) cells from the total cell number.

For determination of the activities of the lysosomal enzymes acid lipase, β-N-acetylglucosaminidase, cathepsin D, β-glucuronidase and arylsulfatase B, purified Kupffer and endothelial cells were incubated as previously described (Knook & Sleyster, 1980).

The density of Kupffer and endothelial cells was estimated by suspending the cells in a linear percoll gradient (density 1.0230-1.0945 g per ml). After centrifugation to equilibrium (20 min; 1900 x g), about 20 fractions of equal volume were collected by siphoning the gradient from the bottom of the tube. The density of the fractions was determined with an Abbé refractometer. Cells in each fraction were counted and the number of Kupffer cells was estimated by their positive peroxidatic activity.

Demonstration of factor VIII antigen on endothelial cells was performed on freshly isolated and purified cells. Cells were incubated with rabbit anti-human factor VIII antigen (diluted 1:40 in GBSS) for 30 min at room temperature. After washing of the cells (3 times), they were incubated with anti-IgG-FITC for 30 min (20°C) (Both primary antibody and the FITC-conjugate were kindly provided by Dr. R. Urbascheck, University of Heidelberg, Germany). After again washing, the cells were examined under a fluorescence microscope. As controls, cells incubated with anti-IgG-FITC only were used.

2.2.7 Determination of endocytic capacity

The suitability of various isolation methods to provide cells for determining the endocytic capacity of Kupffer and endothelial cells for substances taken up in vivo before isolation was tested. Rats were injected with radioactively labelled substances. (Dose, time and site of injection were as described in Section 3.2.2). The procedure for cell isolation was started at five min after injection. For in vitro experiments on endocytosis, freshly isolated cells were incubated with various substances. (Incubation time and cell and substance concentration were as described in Section 4.2.3).

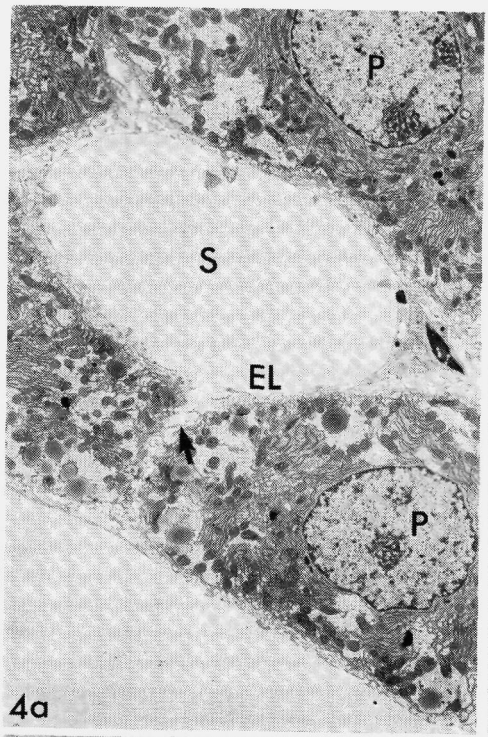
2.3 RESULTS

2.3.1 Morphology of Kupffer and endothelial cells during and after isolation

To follow the ultrastructural changes within the liver during the different cell isolation procedures, the livers were fixed at various time points after the start of the isolation procedures (see Section 2.5.1). A 6 min perfusion with the Ca^{++} containing medium GBSS, which was used during all isolations except for the collagenase method, resulted in no difference in the structure of the liver or in the morphology of the sinusoidal cells as compared with the directly fixed liver (see Fig. 2 and Section 2.2.5). However, when the Ca^{++} free perfusion medium which is employed during isolations by collagenase was used, most of the specific intercellular junctions which are normally present between the parenchymal cells were disrupted within 12 min. These parenchymal cells were no longer attached to each other and seemed to drift apart (Fig. 4). However, Kupffer, endothelial and fat-storing cells possessing intercellular connections different from those between the parenchymal cells (Jones & Summerfield, 1982) were often still attached to each other and to parenchymal cells. Many of the endothelial and fat-storing cells seemed to be torn apart (Fig. 4). This might have been due to the mechanical force exerted by the loose drifting parenchymal cells.

Figure 4 Perfusion of the liver in the absence of Ca^{++}.
The liver was perfused with Ca^{++} free perfusion medium for 12 min and subsequently fixed for electron microscopy (see Section 2.2.5).
a) The connections between the parenchymal cells (P) begin to break (arrow) and cells drift apart. Compare with the normal situation in Fig. 2. The endothelial lining (EL) is torn. S: Sinusoid. (2,790x).
b) A fat-storing cell (F) is stretched due to the mechanical force exerted by the loose drifting parenchymal cells. (4,810x).

Figure 5 Perfusion of the liver with pronase containing medium.
The liver was perfused with GBSS containing pronase and subsequently fixed for electron microscopy (see Section 2.2.5).
a) Perfusion with pronase (37°C, 6 min). The parenchymal cells (P) are still in close proximity to each other, although the specific junctions are dissociated (arrow). The endothelial lining (EL) is disconnected from the parenchymal cells without being torn apart. Kupffer and fat-storing cells are released in a similar way. (5,320x).
b) Perfusion with pronase (10°C, 10 min). The lining of the endothelial cell (E) rolls up and the retracted sieve plates appear as sponge-like structures (arrows). Asterisk: space which originally formed the sinusoid. (5,380x).



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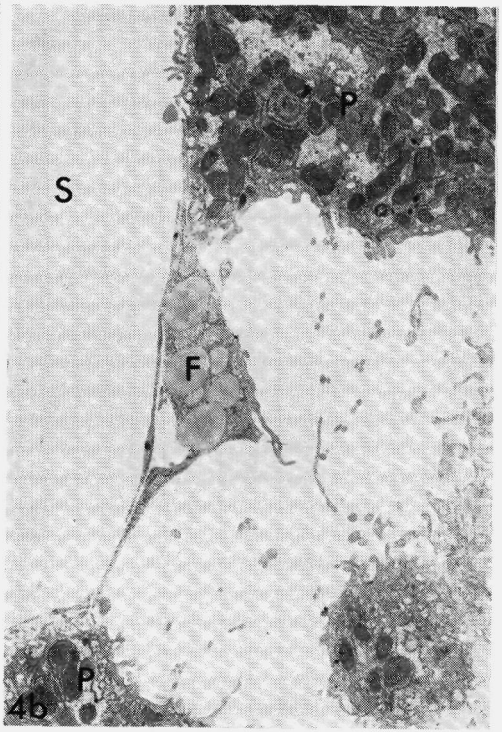

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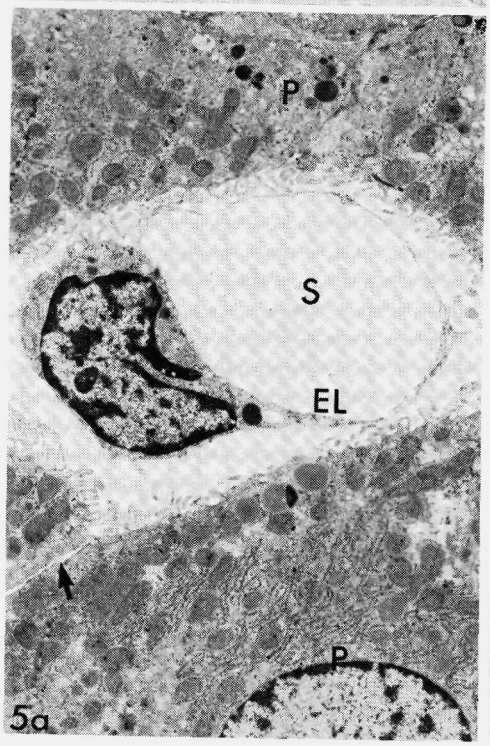

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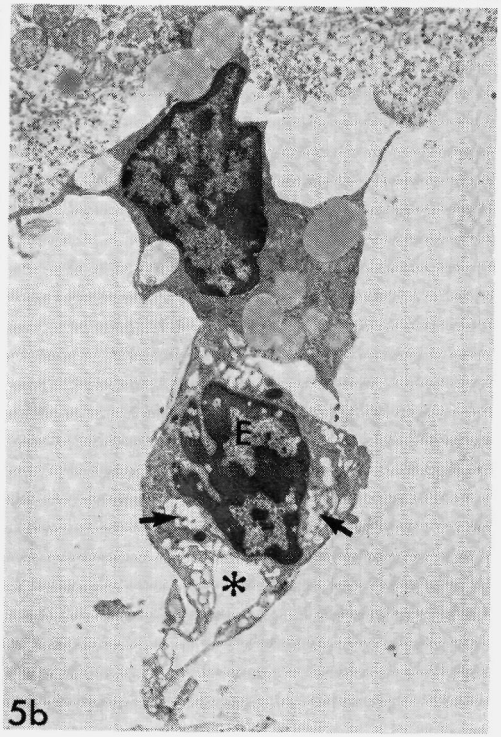

5b

After perfusion of the liver for 6 min with Ca^{++} containing GBSS in the presence of pronase, the sinusoids were completely free of cellular debris. Most of the sinusoidal cells were already loose, without having been torn apart (Fig. 5a). After a 10-12 min perfusion with pronase (37°C or 10°C), the lining of the endothelial cells started to roll up, changing the normally flat and extended cells into completely rounded up balls in which the retracted sieve plates appeared as sponge-like structures (Fig. 5b). Although pronase is supposed to selectively destroy parenchymal cells (Mills & Zucker-Franklin, 1969), perfusion of the liver with pronase for 12 or even 30 min still did not result in visible disintegration of the parenchymal cells. Although Ca^{++} was present in the medium, many intercellular junctions between the parenchymal cells were dissociated, probably due to action of pronase.

Perfusion of the liver with collagenase resulted in a substantial obstruction of sinusoids by cellular debris (Fig. 6). Apparently, no digestion of this debris by collagenase had occurred. This was also seen when the perfusion with collagenase was preceded by a pronase perfusion. When pronase and collagenase were perfused simultaneously, no sinusoidal obstructions occurred. Parenchymal cells lining obstructed sinusoids were still connected to each other and showed a greatly changed morphology (Fig. 6).

After perfusion, mincing and incubation of the liver in a digestion medium, the cells which had survived were mostly floating loose in suspension. After incubation with pronase, most of the parenchymal cells were either digested or no longer viable. This was also observed after the cold pronase isolation where only mincing of the liver but no further enzymatic digestion was applied. Apparently, the destructive effect of pronase on parenchymal

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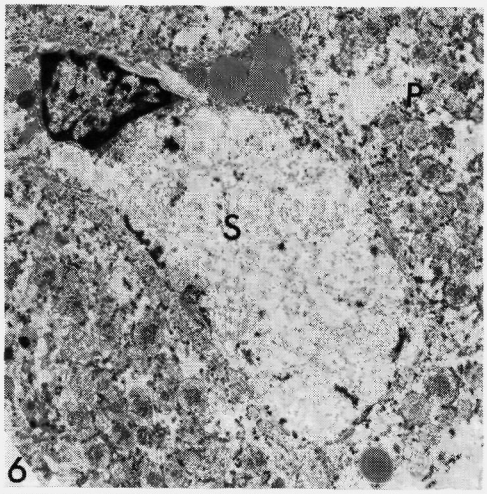
Figure 6 Perfusion of the liver with collagenase containing medium. The liver was perfused with dissociation medium for 6 min and subsequently fixed for electron microscopy (see Section 2.2.5). The sinusoid (S) is obstructed by cellular debris. The ground substance of the cytoplasm of the parenchymal cells (P) has become lumpy. (3,780x).

Figure 7 After digestion of the liver with collagenase (see Section 2.2.2.5), parts of parenchymal cells (blebs) were frequently observed. These blebs sometimes still contained organelles of parenchymal cell origin (arrows). (3,330x).

Figure 8 Kupffer cells isolated by various methods (see Section 2.2.2).
a) After digestion of the liver with warm pronase. (3,200x).
b) After digestion of the liver with collagenase. (4,710x).
c) After digestion of the liver with cold pronase. (5,920x).
Kupffer cells in a) and b) generally contained more ingested cellular debris (arrows) than did the cells in c).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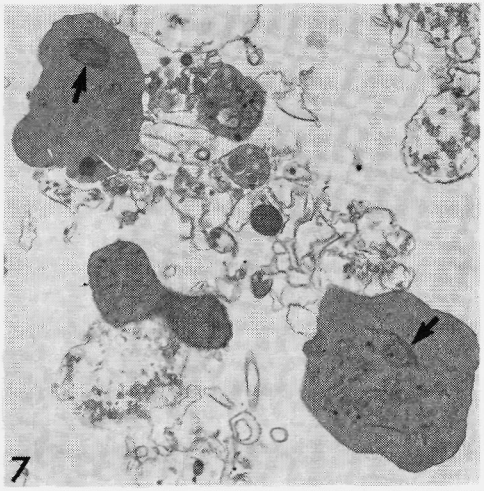
S
6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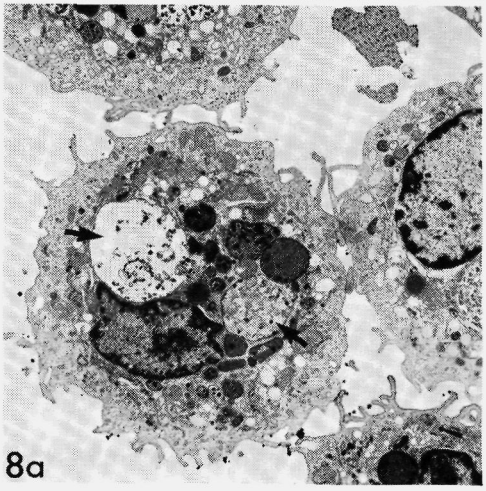
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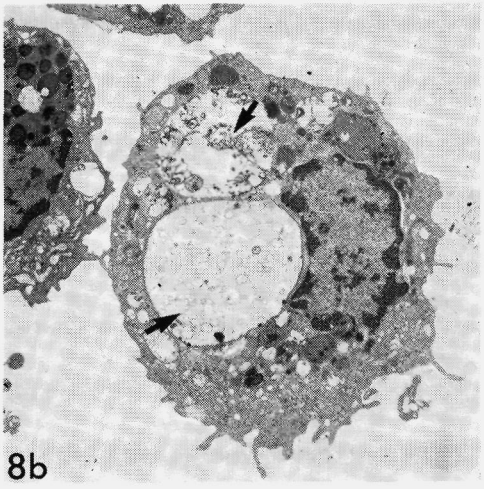
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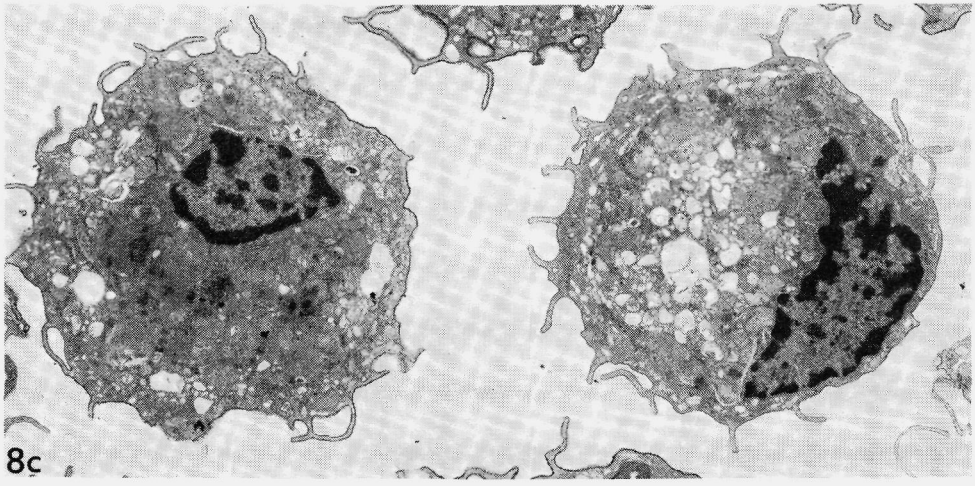
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cells is not manifest until the cells are subsequently treated mechanically. This increased sensitivity to mechanical damage due to proteolytic action also seems to occur with some batches of collagenase (Steffan et al., 1981). With our batches of collagenase, damaged parenchymal cells were not completely degraded. As a consequence, the sinusoidal cell suspension contained a firm amount of small parts of parenchymal cells, designated as blebs (Fig. 7).

During purification of sinusoidal cells by use of a Metrizamide gradient, almost all nonviable cells and erythrocytes were removed. The remaining cell suspension consisted mainly of endothelial and Kupffer cells, with some fat-storing and lymphoid cells (see Section 2.3.2). When cell suspensions obtained by the collagenase method were used, the purified sinusoidal cells still contained blebs of parenchymal cell origin. These blebs excluded trypan blue and were of about the size of the sinusoidal cells. Ultrastructural investigation of the Kupffer cells showed that they had ingested a fair amount of cellular debris during the warm pronase as well as the collagenase incubation (Fig. 8). No ingested debris was observed when Kupffer cells were isolated by the cold pronase method (Fig. 8). The cellular extensions containing the sieve plates of endothelial cells isolated by cold pronase appeared to be less rounded than those of endothelial cells isolated by the warm pronase method (Fig. 9); no other differences in morphology were found.

After separation of Kupffer and endothelial cells by centrifugal elutriation, no changes with respect to morphology and integrity of the cells had occurred. Both cell types still exhibited esterase activity and the Kupffer cells showed peroxidatic activity to be present in the same morphological structures (Fig. 10) as described for perfusion-fixed liver (see Fig. 2).

Isolated and purified Kupffer cells can be easily cultured after both

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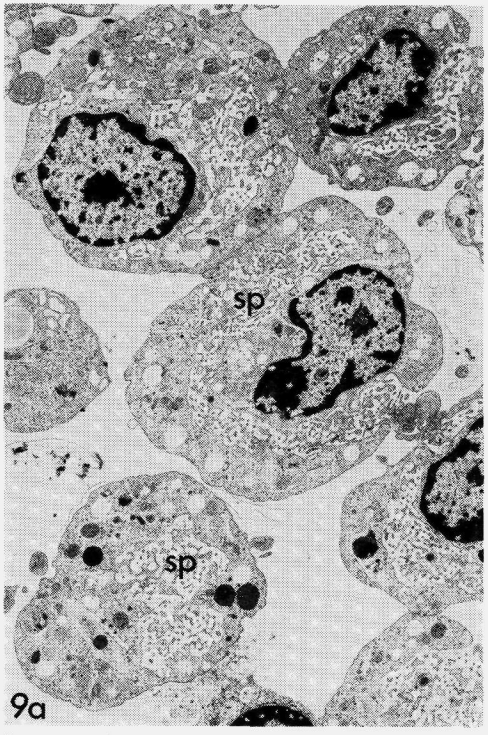
Figure 9 Isolated endothelial cells.
a) After digestion of the liver with warm pronase (see Section 2.2.2.1). (4,400x).
b) After digestion of the liver with cold pronase (see Section 2.2.2.2). (4,320x). Endothelial cells isolated by cold pronase often possess sieve plates (sp) which are less rounded up than in cells isolated by the use of warm pronase.

Figure 10 Isolated Kupffer cell after elutriation (see Section 2.2.3). The cell stains for peroxidatic activity in the RER (arrow) and the nuclear envelope (arrowhead). Compare with the *in vivo* situation (Fig. 2). (11,200x).

Figure 11 Cultured endothelial cells after isolation by the cold pronase method (see Sections 2.2.2.2 and 2.2.4). The cells contain numerous vacuoles (v). (3,230x).



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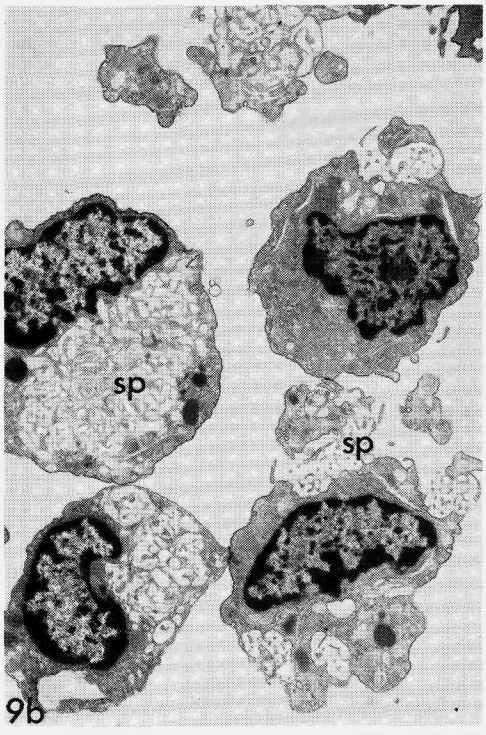

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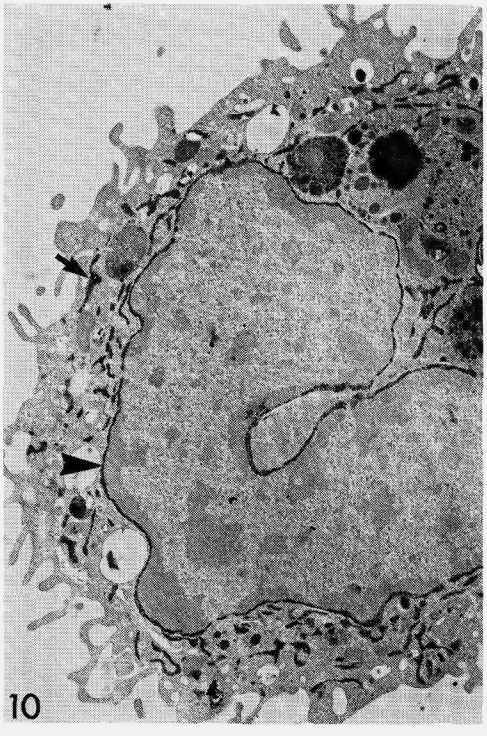

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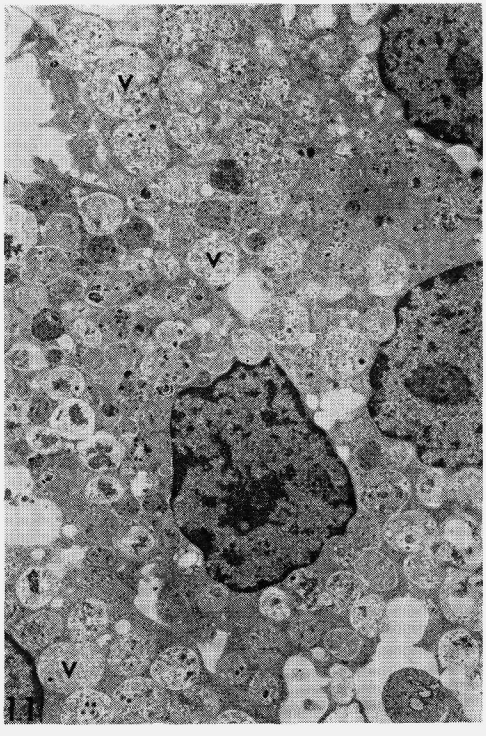

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preparation by collagenase (Caperna & Garvey, 1983) and pronase digestion (Munthe-Kaas, 1976; Brouwer & Knook, 1977). The main characteristics of Kupffer cell cultures have been earlier described elsewhere (Brouwer & Knook, 1977; de Leeuw et al., 1982c).

All attempts to culture purified endothelial cells isolated by the warm pronase isolation method failed. However, endothelial cells isolated by pronase-collagenase (de Leeuw et al., 1982b), or cold pronase could be cultured on a collagen coated surface. Endothelial cells isolated by collagenase only can also be cultured (Steffan et al., 1981). Endothelial cells isolated by the pronase-collagenase or the cold pronase method attached to the culture dishes within a few hours, which is even more rapidly than done by Kupffer cells. After attachment, the cells spread out and formed long thin extensions in which sieve plates could be observed (de Leeuw et al., 1982b). Under the light microscope, where these sieve plates are not visible, the cultured endothelial cells can be easily confused with Kupffer cells. Cultured endothelial cells contain numerous vacuoles (Fig. 11). Cultured cells isolated by the cold pronase method showed the same morphology as those isolated by pronase-collagenase. However, there was a difference in their need for a specific serum. Cold pronase isolated endothelial cells could be cultured in medium supplemented with either heat-inactivated fetal calf serum or freshly prepared rat serum, whereas pronase-collagenase isolated cells survived only when the rat serum was present in the medium (de Leeuw et al., 1982b).

2.3.2 Yield, purity and characteristics of isolated sinusoidal cells

The viability, yield, cellular composition and protein content of sinusoidal cells isolated by five differing methods are given in Table IV. The cell viability varied between 90 and 99%. The highest yield of cells was obtained by the collagenase isolation method and the lowest with the thermolysine method. The yields of Kupffer cells did not differ much for the various isolation methods. The largest number of Kupffer cells, an average of about 46×10^6 cells, was obtained with the pronase-collagenase method. Considering the estimated total amount of Kupffer cells in the rat liver (Knook & Sleyster, 1976), it can be assumed that about 30% of the Kupffer cells were lost during this isolation procedure, whereas about 50-60% of the cells were lost with the other methods. The loss of endothelial cells was comparable.

As mentioned earlier, sinusoidal cell suspensions contained in addition

TABLE IV

YIELD, COMPOSITION AND CELLULAR CHARACTERISTICS OF SINUSOIDAL CELLS ISOLATED BY VARIOUS METHODS

Method	Yield* of sinusoidal cells (10^6)	Contamination by parenchymal cells (10^6)	Composition (%) of of sinusoidal cells**			Viability (%)	Protein content (µg per 10^6cells)
			L	E	K		
Warm pronase	163	0	24	55	21	96	51.7
Cold pronase	203	5.0	22	61	17	97	77.3
Thermolysine	154	7.2	79		21	99	n.d.
Pronase-collagenase	241	0	18	63	19	90	99.3
Collagenase	275	45.9	53	33	14	99	n.d.

Cells were isolated as described in Section 2.2.2. Average of at least 4 experiments.
*The yield represents the number of cells per rat liver.
**The percentages of lymphoid (L), endothelial (E) and Kupffer (K) cells were estimated as described in Section 2.2.6. For thermolysine isolated cells, only the percentage of Kupffer cells was estimated.
N.d.: not determined.

some lymphoid, fat-storing and parenchymal cells and, after collagenase isolation, blebs were also present.

The amount of lymphoid cells was estimated by ultrastructural investigation and by determination of the proportion of esterase negative cells by cytochemical staining. However, the first method is quite laborious and the second resulted in extensive clumping and unclear staining of the sinusoidal cells, by which good judgement was complicated. A more suitable method for discrimination between lymphoid and sinusoidal cells was the estimation of the percentage of cells which endocytosed horseradish peroxidase (HRP) (see Section 2.2.6). Ultrastructural examination revealed that all of the Kupffer and endothelial cells were able to endocytose a considerable amount of HRP, which could be easily visualized light microscopically. All lymphoid cells appeared to be HRP negative. The percentage of lymphoid cells in the sinusoidal cell suspensions varied between 18-24%. Only suspensions isolated by the collagenase method contained as high as 53% lymphoid cells (Table IV). Thus the high yield of cells obtained by this isolation method can be largely ascribed to the amount of lymphoid cells.

The amount of fat-storing cells as light microscopically identified by the presence of lipid droplets was usually very low. The highest amount (about 6%) was found after the pronase-collagenase isolation.

Sinusoidal cells prepared by the collagenase isolation method still contained a relatively high amount of parenchymal cells (Table IV). Prolonged differential centrifugation to remove these parenchymal cells resulted in a considerable loss of sinusoidal cells, especially of Kupffer cells (see also Van Berkel, 1982; Berg, 1982). The warm pronase and the pronase-collagenase methods yielded cell suspensions completely free of parenchymal cells (Table IV). The cell suspension obtained by using collagenase also showed the presence of some blebs; these did not appear in the other cell suspensions. At the ultrastructural level it was observed that most of these blebs showed peroxidatic activity. Therefore, under the light microscope, they may be confused with Kupffer cells. The percentage of peroxidase positive cells given in Table IV might because of that also include some blebs.

Due to the variable cellular composition of the suspensions, the amount of protein per 10^6 cells differed for each isolation method (Table IV). Other factors such as endocytosis of cellular debris by the cells during the isolation procedures might also have influenced the protein content of the cells. This might explain the difference in protein content between warm pronase and pronase-collagenase isolated cells. Parenchymal cells (which would have

increased the protein content) were not present in either of these cell preparations. However, the pronase-collagenase isolated cells had more opportunity to ingest debris during the long liver perfusion of 36 min than did the warm pronase isolated cells (see Section 2.2.2).

2.3.3 Characteristics of purified Kupffer and endothelial cells

When applying the standard procedure for separation of sinusoidal cells by centrifugal elutriation, the relative distribution of the total numbers of Kupffer and endothelial cells recovered in the various fractions collected varies with the isolation method (Fig. 12). When isolated by the warm pronase method, 75% of the Kupffer cells were recovered in the 40 ml per min fraction, whereas when the cold pronase method was employed more than 50% of the Kupffer cells were present in the 13.5 and 23.5 ml per min fractions. Endothelial cells isolated by the warm pronase method were recovered mainly in the 23.5 ml per min fraction, while those isolated by cold pronase were present largely in the 13.5 ml per min fraction. For collagenase isolated Kupffer and endothelial cells, the results averaged those of the warm and cold pronase isolations. Since separation by centrifugal elutriation is based primarily on the size of the cells, these results indicate that after isolation by the cold pronase method the cells obtained are generally smaller than after isolation by the warm pronase method. The different amounts of cell debris ingested by the cells (see Section 2.3.1) isolated by various methods may explain these results.

Due to their heterogeneity and smaller difference in size, Kupffer and endothelial cells isolated by the cold pronase method were difficult to purify by elutriation in the standard separation chamber. Therefore, the Sanderson chamber in which cells which differ little in size can be better separated than in the standard chamber (Brouwer et al., 1983), was used. With this chamber, cold pronase isolated Kupffer and endothelial cells could indeed be obtained in higher purity. Moreover, Kupffer cell fractions elutriated by the Sanderson chamber contained fewer parenchymal cells (<1%) than when the standard chamber (2-4.4%) was used. Kupffer cell fractions obtained by the warm pronase and pronase-collagenase methods were free of parenchymal cells, whereas 6-16% parenchymal cells were present after collagenase treatment. The contamination of the endothelial cell fractions with the peroxidase positive Kupffer cells did not exceed 16%, whereas the Kupffer cell frac-

tions contained 26% or less peroxidase negative cells, as shown in Table V. The viability of the cells was still above 90%, the only exception being the collagenase isolated cells in the Kupffer cell fraction, which were 85% viable.

The protein content of cells in the Kupffer cell fraction was about twice as high as in the endothelial cell fraction (Table V). For the same reasons as discussed for the sinusoidal cell fractions, the protein content of the cells in the purified Kupffer and endothelial cell fractions differed per isolation procedure. The total amount of protein recovered in all fractions after elutriation and subsequent centrifugation averaged 96% for all isolation methods, indicating that almost no cellular protein was lost in the supernatants of the elutriated fractions.

Apart from the cell size, the cell density might also be influenced by isolation, as shown in Fig. 13. After both the warm and cold pronase isolation procedures, the endothelial cells showed a rather heterogeneous density distribution following isopynic centrifugation in a linear Percoll gradient. When isolated by the cold pronase method, Kupffer cells also behaved heterogeneously. Three peaks are found at, respectively, 1.059, 1.068 and 1.076 mg per ml (Fig. 13). After isolation by the warm pronase method, the density of the Kupffer cells was much more homogeneous. Most of the Kupffer cells were found around a density of 1.070. However, the results show that Kupffer and endothelial cells cannot be completely separated on the basis of density, irrespective of the method of their isolation.

Kupffer as well as endothelial cells possess a well developed lysosomal apparatus. The lysosomal enzymes, which are acid hydrolases, are able to degrade various endocytosed substances that enter the lysosomes. The presence of lysosomal enzymes in Kupffer as well as endothelial cells in situ has

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Figure 12 Relative distribution of Kupffer and endothelial cells in elutriated fractions after isolation by various methods (Section 2.2.2). Cells were elutriated in a standard separation chamber (see Section 2.2.3). Fraction numbers: 1) Lymphoid (13,5 ml per min); 2) endothelial (23,5 ml per min); 3) Kupffer (40 ml per min); 4) pellet. The number of endothelial cells was calculated as described in Section 2.2.6. Average numbers of at least 6 experiments.

Figure 13 Isopynic centrifugation of isolated Kupffer and endothelial cells in a linear percoll gradient (see Section 2.2.6).
a) Kupffer cells isolated by the cold pronase method (----) (see Section 2.2.2.2) were distributed over more fractions than were cells isolated by the warm pronase method (——) (see Section 2.2.2.1).
b) After both warm (——) and cold (----) pronase isolation, endothelial cells behaved rather heterogeneously in the gradient.

Figure 12

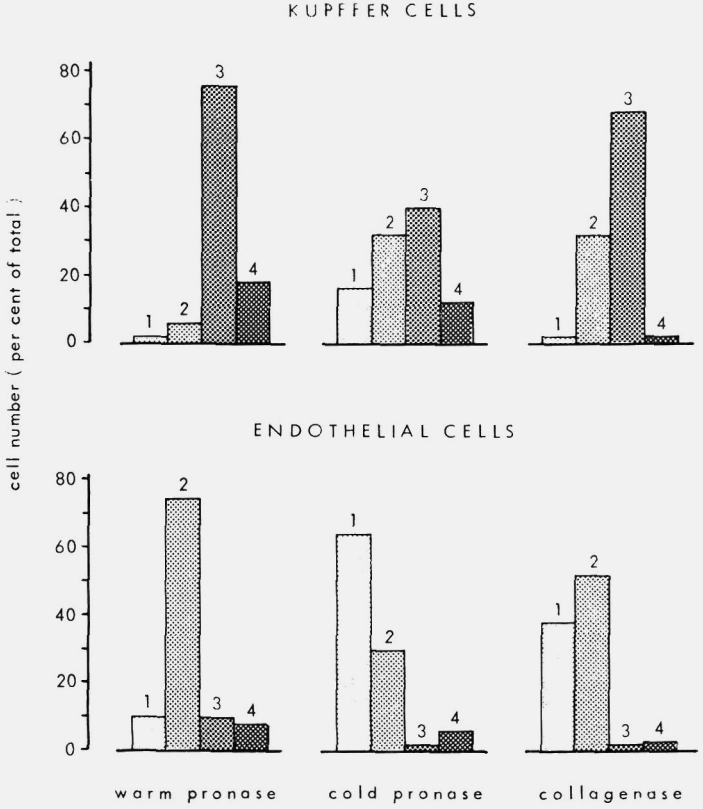

Figure 13

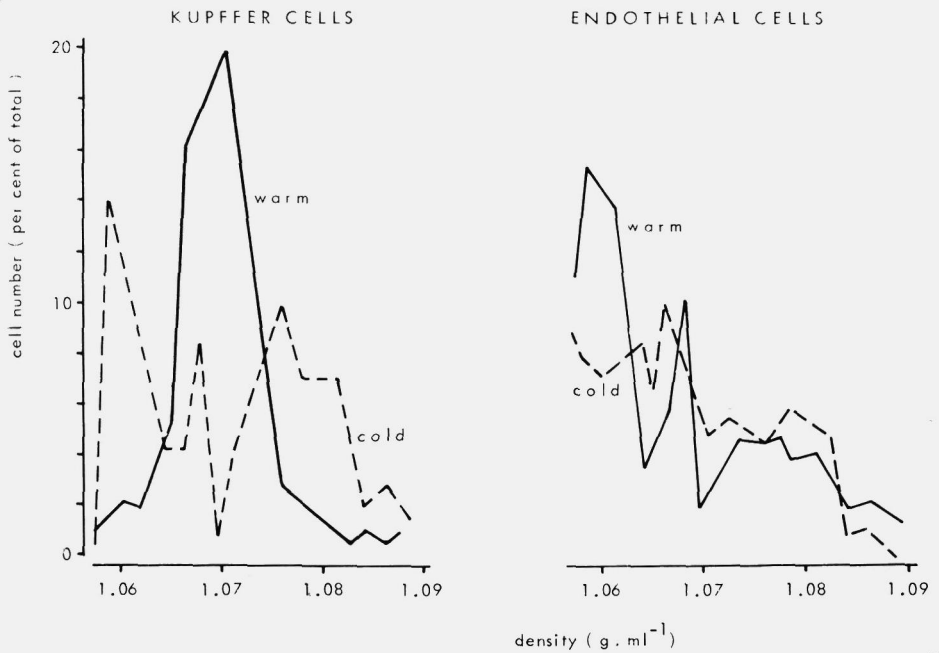

TABLE V

COMPOSITION* AND PROTEIN CONTENT OF PURIFIED KUPFFER AND ENDOTHELIAL CELL FRACTIONS ISOLATED BY VARIOUS METHODS

	Endothelial cell fraction				Kupffer cell fraction			
	Composition			Protein content	Composition			Protein content
Method	L	E	K	(µg per 10^6 cells)	L	E	K	(µg per 10^6 cells)
Warm pronase	23	75	2	44.7	2	24	74	87.4
Cold pronase	19	72	9	51.7	6	18	76	113.9
Thermolysine		90	10	n.d.		19	81	n.d.
Pronase-collagenase		97	3	65.7		24	76	114.2
Collagenase	25	59	16	n.d.	15	7	78	n.d.

Kupffer and endothelial cells were isolated and purified as described in Sections 2.2.2 and 2.2.3, respectively. Cells isolated by cold pronase were separated in a Sanderson chamber. Average numbers of at least 4 experiments.

*The cellular composition is expressed as a percentage of total cell number in each fraction. The percentages of lymphoid (L), endothelial (E) and Kupffer (K) cells were estimated as described in Section 2.2.6. For thermolysine and pronase-collagenase isolated cells, only the percentage of Kupffer cells was estimated.

n.d.: not determined.

been demonstrated by cytochemical staining (Wisse, 1977b; Wilson et al., 1982). In our study, the biochemical determination of lysosomal enzyme activities in isolated and purified Kupffer and endothelial cells was used as an additional characterization of cells isolated by different methods. Tables VI and VII show the activities of five key lysosomal enzymes in Kupffer and endothelial cells prepared by the three different isolation methods employing pronase. A significant difference which was apparent when activities were expressed both per 10^6 cells and per mg protein, was found when the activity of acid lipase in endothelial cells isolated by the warm pronase and the pronase-collagenase methods was compared. The activity of β-acetylglucosaminidase in endothelial cells isolated by use of warm pronase differed significantly from that in endothelial cells isolated by using cold pronase, whereas the activity of cathepsin D in these cells differed significantly from that in the cells isolated by the pronase-collagenase method.

In pronase-collagenase isolated Kupffer cells, the activities of acid lipase and arylsulphatase differed significantly from those in cells isolated by the warm pronase method, whereas the activity of arylsulphatase in these cells was significantly different from that in cold pronase isolated cells.

The absence of a uniform pattern in the differences in enzyme activities for the various isolation methods indicates that this cannot be simply explained by differences in cellular composition or cell size. It might therefore be assumed that during the various isolation procedures the activities of some lysosomal enzymes are either inhibited or stimulated. Despite this, the differences in enzyme activities between Kupffer and endothelial cells are still roughly the same for each isolation procedure when expressed per mg cellular protein, showing the highest activities of acetylglucosaminidase and arylsulphatase in endothelial cells and of acid lipase, cathepsin D and β-glucuronidase in Kupffer cells.

2.3.4 Suitability of cell isolation methods for studies on endocytosis

2.3.4.1 Endocytosis *in vivo*

Isolation and purification of Kupffer and endothelial cells offer the possibility to determine the amount of a substance endocytosed by each of the cell types prior to their isolation. The capacity of Kupffer and endothelial cells to endocytose various substances *in vivo* is discussed in detail in Chapter 3. Since degradation of some endocytosed substances can occur in the

TABLE VI

LYSOSOMAL ENZYME ACTIVITIES IN ENDOTHELIAL CELLS ISOLATED BY VARIOUS METHODS

Enzyme	Warm pronase*		Pronase-collagenase**		Cold pronase	
	activity per 10^6 cells	activity per mg protein	activity per 10^6 cells	activity per mg protein	activity per 10^6 cells	activity per mg protein
Acid lipase[a]	1.3 ± 0.2[θ]	27.1 ± 3.4[θ]	2.8 ± 0.5	48.6 ± 9.8	2.1 ± 1.5	37.3 ± 21.9
β-Acetylglucos-aminidase[a]	13.6 ± 2.0[§]	299.7 ± 73.8[§]	11.7 ± 3.4	202.1 ± 67.2	8.8 ± 2.5	152.1 ± 32.3
Cathepsin D[b]	0.3 ± 0.0[§]	6.4 ± 0.9	0.3 ± 0.1[Δ]	5.1 ± 1.0[Δ]	0.5 ± 0.1	9.5 ± 3.3
β-Glucuronidase[c]	0.9 ± 0.0[§]	19.6 ± 2.7	*n.d.*	*n.d.*	1.3 ± 0.3	23.2 ± 9.4
Arylsulphatase B[d]	7.1 ± 2.2	149.4 ± 40.2	9.3 ± 0.5	159.0 ± 11.6[Δ]	7.1 ± 2.1	125.2 ± 25.5

Cells were isolated and purified as described in Sections 2.2.2 and 2.2.3, respectively. Lysosomal enzyme activities were determined as described in Section 2.2.6. Average numbers of at least 4 experiments. Values are given as ± S.D., for calculation of significant differences.

[a]Expressed as nmoles 4-methylumbelliferone released per min;
[b]Expressed as nmoles tryptophan released per min;
[c]Expressed as nmoles phenolphtalein released per min;
[d]Expressed as nmoles nitrocathechol released per min.
*Results published by Knook & Sleyster (1980).
**Unpublished results of A. Seffelaar.
θ Significant difference ($P < 0.05$) between warm pronase and pronase-collagenase isolation.
§ Idem between warm pronase and cold pronase isolation.
Δ Idem between pronase-collagenase and cold pronase isolation.

TABLE VII

LYSOSOMAL ENZYME ACTIVITIES IN KUPFFER CELLS ISOLATED BY VARIOUS METHODS

Enzyme	Warm pronase*		Pronase-collagenase**		Cold pronase	
	activity per 10^6 cells	activity per mg protein	activity per 10^6 cells	activity per mg protein	activity per 10^6 cells	activity per mg protein
Acid lipase[a]	6.2 ± 1.1$^{\theta}$	53.1 ± 15.0$^{\theta}$	9.7 ± 3.6	86.0 ± 28.1	7.8 ± 5.0	55.6 ± 36.3
β-Acetylglucos-aminidase[a]	16.4 ± 2.3	144.3 ± 18.7$^{\theta}$	18.7 ± 3.7	170.8 ± 19.8$^{\Delta}$	16.4 ± 5.3	118.6 ± 33.6
Cathepsin D[b]	2.0 ± 0.6	17.4 ± 5.1	1.8 ± 0.4	16.2 ± 2.9	2.6 ± 1.1	17.6 ± 5.4
β-Glucuronidase[c]	3.2 ± 0.6	28.4 ± 5.9	n.d.	n.d.	4.5 ± 1.6	30.0 ± 9.7
Arylsulphatase B[d]	5.3 ± 2.0$^{\theta\S}$	42.7 ± 19.8$^{\theta\S}$	8.6 ± 1.3	78.7 ± 16.6	10.2 ± 3.9	71.0 ± 20.1

Cells were isolated and purified as described in Sections 2.2.2 and 2.2.3, respectively. Lysosomal enzyme activities were determined as described in Section 2.2.6. Average numbers of at least 4 experiments. Values are given as ± S.D., for calculation of significant differences.

[a]Expressed as nmoles 4-methylumbelliferone released per min;
[b]Expressed as nmoles tryptophan released per min;
[c]Expressed as nmoles phenolphtalein released per min;
[d]Expressed as nmoles nitrocathechol released per min.
*Results published by Knook & Sleyster (1980).
**Unpublished results by A. Seffelaar.
θ Significant difference ($P < 0.05$) between warm pronase and pronase-collagenase isolation.
§ Idem between warm pronase and cold pronase isolation.
Δ Idem between pronase-collagenase and cold pronase isolation.

cells during their isolation and substances released from damaged cells may be endocytosed anew during the isolation procedure, we tested whether various isolation methods were suitable for the determinations of the endocytosis of the degradable substances colloidal albumin and modified orosomucoid.

When sinusoidal cells were isolated by the warm pronase method, only minor portions of the radioactive substances colloidal albumin and modified orosomucoids present in the liver at the start of the isolation procedure could be recovered in the sinusoidal cell preparation (Table VIII). About 90% of the total amount of colloidal albumin and 60% of the total amount of modified orosomucoids were lost in trichloroacetic acid (TCA) soluble form, indicating extensive degradation of the two substances during the isolation procedures (Table VIII). This is probably not due to degradation by the cells only. Based on the total estimated numbers of sinusoidal cells present in the liver (Knook & Sleyster, 1976), it can be assumed that about 50-60% of the cells are damaged and lost during isolation. As long as pronase is present in the isolation media, substances released from the damaged cells will also be degraded by this proteolytic enzyme preparation. In fact, a 60 min incubation of colloidal albumin and modified orosomucoid with pronase under the same conditions as used during the digestion of the liver resulted in the

TABLE VIII

RECOVERY OF COLLOIDAL ALBUMIN AND MODIFIED OROSOMUCOIDS IN THE SINUSOIDAL CELL FRACTION

	Lost in TCA soluble form during isolation		Present in cell fraction	
Isolation method	CA	OR	CA	OR
Warm pronase	89.1	62.7	4.6	1.1
Warm pronase plus chloroquine*	74.1	45.9	15.7	7.6
Collagenase plus chloroquine*	32.8	54.1	23.2	8.1
Cold pronase	<1	<1	38.5	30.2

Values for the amounts of colloidal albumin (CA) and modified orosomucoid (OR) are given as percentages of the total amount present in the liver at the start of the isolation procedure. Isolation methods were performed as described in Section 2.2.2. Average numbers of at least 2 experiments.
* 0.4 mM chloroquine was added to all media during the entire isolation procedure.

complete degradation of colloidal albumin and a 50% degradation of the orosomucoid. Injection of 0.01 mM chloroquine, an inhibitor of lysosomal degradation, did not significantly change the number of TCA soluble counts lost during the cell isolation. Addition of at least 0.4 mM chloroquine to the isolation medium proved to be necessary to achieve any effect on the rate of degradation (Table VIII). An increase in the amount of chloroquine in the isolation medium to 1 mM did not lead to a further decrease in the rate of degradation. A higher concentration of chloroquine could not be used, since this would have a damaging effect on the cells (van Berkel et al., 1981a). The incomplete inhibition of the degradation could be due to the action of pronase on substances released from damaged cells, but also to incomplete inhibition of the lysosomal degradation by chloroquine. When cells were isolated by collagenase, an enzyme which does not degrade the modified orosomucoid and degrades colloidal albumin at a very slow rate only, a considerable amount of both substances was still lost in a TCA soluble form, in spite of the addition of chloroquine (Table VIII). This indicates incomplete inhibition of lysosomal degradation by chloroquine.

When cells were isolated by the cold pronase method, less than 1% of the total amount of substances was lost due to degradation (Table VIII). With this isolation method, the recoveries of colloidal albumin and modified orosomucoid were increased to 38.5 and 30.2%, respectively. Assuming a cell recovery of 40-50%, it follows that about 77-96% of the colloidal albumin and 60-75% of the modified orosomucoid was present in the sinusoidal cells prior to the isolation. The remainder might have been present in the parenchymal cells (Hubbard et al., 1979; Praaning-van Dalen et al., 1981).

After elutriation of cold pronase isolated sinusoidal cells, colloidal albumin and the modified orosomucoid were found to be present in Kupffer as well as endothelial cells (see Section 3.3.4). When isolated with warm pronase and elutriated in the presence of chloroquine, the amount of colloidal albumin found in the Kupffer cells was 60% of that found after the cold pronase method; the amount found in endothelial cells was the same for both isolation methods. However, when elutriation was performed without chloroquine, the amount recovered in the endothelial cells was also decreased. Chloroquine also did not inhibit degradation of modified orosomucoid in either cell type. When isolated with warm pronase and elutriated in the presence of chloroquine, endothelial and Kupffer cells contained only 30% of the amount present in cells obtained with the cold pronase method.

2.3.4.2 Endocytosis in vitro and integrity of the cell membrane

Detailed results for the in vitro endocytosis by Kupffer and endothelial cells isolated by various isolation methods are given in Chapter 4. Cells treated with warm pronase, thermolysine or pronase-collagenase were not able to endocytose any of a variety of substances when freshly isolated or after a short incubation for recovery. Cells isolated by collagenase treatment showed endocytosis for certain substances only (see Chapter 4). The best results were obtained with cells treated with cold pronase, as will be more fully discussed in Chapter 4.

The results showed that after intensive contact with proteolytic enzymes the cells were no longer capable of endocytosing substances which they normally take up in vivo. Moreover, endothelial cells isolated by the warm pronase method were unable to attach to culture dishes, in contrast to pronase-collagenase or cold pronase isolated cells. The findings indicate that pronase

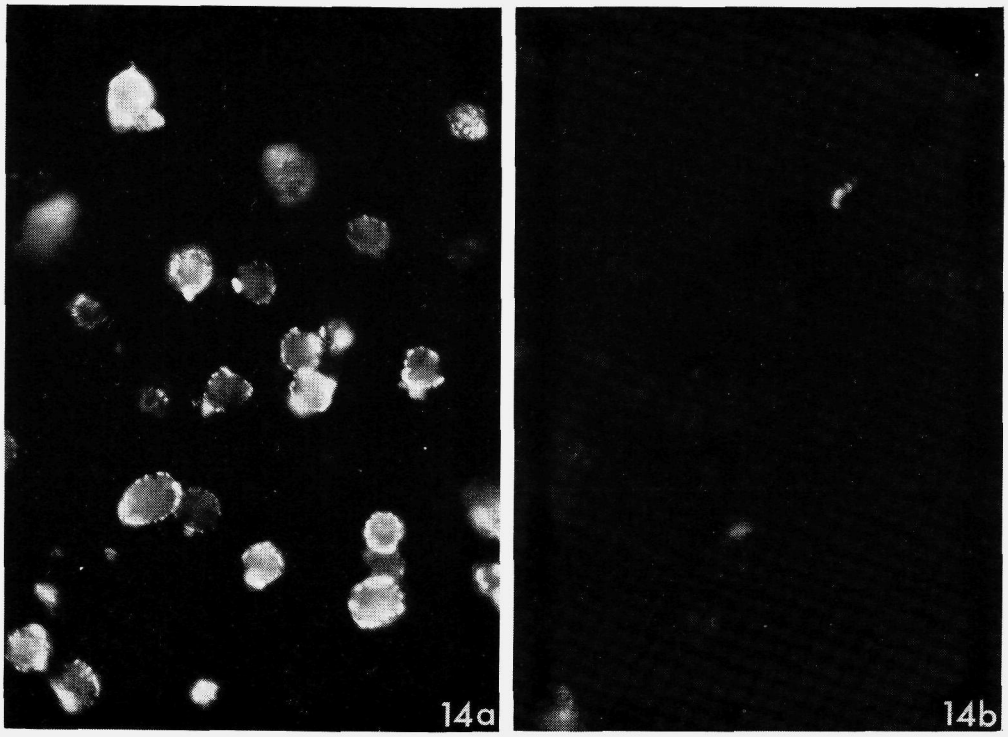

Figure 14 a) Staining of purified endothelial cells for the presence of Factor VIII antigen (see Section 2.2.6). (1,180x).
b) Control. (1,180x).

induced changes in the molecular composition of the cell membrane such as destruction of factors necessary for attachment (Tarone et al., 1982). The relatively low pronase concentration used in the pronase-collagenase isolation method or the reduced activity of pronase at a low temperature might have had a less destructive effect. Therefore, cells isolated by the cold pronase method might possess a more intact cell membrane than those isolated by the warm pronase method.

An additional test for the presence of specific membrane components of cold pronase isolated endothelial cells was the demonstration of the presence of factor VIII antigen. Endothelial cells throughout the body are specialized in the synthesis of this antigen (Jaffe & Nachman, 1975) and its demonstration on the cell membrane might be used for identification and characterization of endothelial cells (Macarak et al., 1978; Jaffe & Mosher, 1978).

When freshly cold pronase isolated and purified endothelial cells were incubated for indirect immunofluorescence to demonstrate factor VIII antigen, most of the cells showed a bright fluorescence, whereas all controls were negative (Fig. 14). The identity of the endothelial cells was confirmed by electron microscopy. In the purified Kupffer cell fraction, only a few cells stained positively for factor VIII antigen and these might have been contaminating endothelial cells.

2.4 DISCUSSION

To study the endocytic capacity of Kupffer and endothelial cells in vivo and in vitro, a suitable isolation method had to be developed. Cell preparations obtained by different isolation methods were compared with respect to their morphology, cellular composition, characteristics and their suitability for the study of endocytosis. This chapter includes the results.

Examination of the various steps of the isolation procedures revealed that each procedure has its specific disadvantages: loss of parts of cells occurred during a Ca^{++} free perfusion and obstruction of sinusoids was observed when perfusion was carried out in the presence of only collagenase.

A problem during isolation by use of warm pronase or collagenase was the ingestion of cellular debris by the Kupffer and probably also endothelial cells, which could lead to a redistribution of enzymes and other substances. Furthermore, after isolation with collagenase, a selective loss of large Kupffer cells during differential centrifugation to eliminate the parenchymal cells

and contamination of the sinusoidal cell preparation by blebs represent serious problems. These blebs can be of the same size as the sinusoidal cells (Nagelkerke et al., 1982); this could lead to an overestimation of the sinusoidal cell yield and, due to the peroxidase positive staining of the blebs, to an overestimation of the percentage of Kupffer cells. In addition to an ultrastructural investigation of the sinusoidal cell preparation, the amount of blebs can be determined biochemically by assessing enzymes which are present only in parenchymal cells and not in sinusoidal cells (Nagelkerke et al., 1982). Another isolation problem was the proteolytic action of warm pronase on the cell membranes, which probably affected the capacity of the cells to endocytose certain substances in vitro. Although Kupffer cells isolated by warm pronase could be cultured and were able to regain their endocytic capacity during a 24 h culture period (Brouwer & Knook, 1977), endothelial cells had lost the capacity to attach to culture dishes, probably due also to the action of pronase. Collagenase, although to a lesser extent than pronase, also seemed to act on the cell membrane in such a way that certain substances could not be effectively endocytosed. Finally, isolation methods which involved digestion of the liver at 37°C appeared to be unsuitable for the study of in vivo endocytosis, since undesired degradation of the substances occurred. Moreover, redistribution of endocytosed substances during the digestion period might also influence the results.

Several criteria were compared for each cell isolation procedure. The viability of the cells obtained by all procedures was above 90% as judged by the trypan blue exclusion test. Although trypan blue exclusion is a useful method for estimating the minimum percentage of dead cells, such an estimation should be combined with an observation on the ultrastructural integrity of the cells. Although cell isolation methods resulted in sinusoidal cells with a good ultrastructural integrity, one cell type could be manifested by different morphological appearances, e.g., different amounts of ingested cellular debris and different cell sizes. Additional criteria for the characterization of the isolated cells included the demonstration of certain enzymes, i.e., peroxidase, esterase and lysosomal hydrolases, and the determination of cellular protein content and density. The results showed that all enzymes tested were present in the cells after their isolation, irrespective of the isolation method. However, some shifting was noted in the activity of lysosomal hydrolases, the amount of cellular protein and the density of the cells.

An important criterion which determines whether a further purification of Kupffer and endothelial cells can be accomplished is a good yield of si-

nusoidal cells. In this respect, each isolation method met the requirements. Another demand which should be fulfilled is the possibility to discriminate between Kupffer and endothelial cells, to be able to determine the purity of the isolated cell fractions. Under normal circumstances, Kupffer and endothelial cells cannot be distinghuished light microscopically. The positive reaction for peroxidatic activity in Kupffer cells could be perfectly used to estimate the percentage of Kupffer cells in the cell suspensions obtained by each isolation method.

The final criterion designating the most useful isolation method was the suitability of the method for the study of in vivo and in vitro endocytosis. The main problem in determinations of in vivo endocytosis, the degradation of the endocytosed substances during the cell isolation procedure, was completely solved by performing the isolation with pronase at 10°C, a temperature at which no intracellular degradation of substances occurred. At 10°C, pronase still has some proteolytic activity, since it could be used for the isolation of Kupffer and endothelial cells at this temperature. Moreover, a control experiment showed that at 10°C pronase degraded colloidal albumin at a rate which is about 17% of that found at 37°C (results not shown). Since endocytosis is also greatly reduced at 10°C (Weigel, 1980), isolation at this temperature also solves the problem of redistribution of the substances and other materials during the isolation procedure.

The main problem in in vitro endocytosis was the action of pronase and — to a lesser extent — collagenase on the cell membrane, which resulted in a loss of the capacity of the cells to bind the substances offered. It appeared that when isolated at low temperature the binding capacity of the cells was still present. Endothelial cells isolated by this method were also able to attach to culture dishes and stained positively for the presence of factor VIII antigen, indicating a preserved molecular composition of the cell membrane. Since the cells isolated by this method also satisfied the other criteria tested for, this method was used for the further study on in vivo endocytosis of degradable substances and on endocytosis in vitro.

For obvious reasons, it is very important that cells obtained by a specific isolation method are identified and characterized very accurately. To achieve this, the establishment of fixed criteria by which Kupffer as well as endothelial cells can be characterized is necessary. The percentage of Kupffer cells is often estimated by determining the proportion of cells able to ingest particulate materials such as latex beads or colloidal carbon (Lukomska & Olszewski, 1978; Barranger et al., 1978; Ullrich et al., 1979; Kolb et al.,

1980a,b). Unfortunately, these estimations are nearly always based on light microscopic observations which cannot prove the exclusive ingestion by Kupffer cells. The study described in this chapter shows that also endothelial cells are able to ingest a colloidal material like colloidal albumin. Therefore, the exclusive ingestion of a substance by Kupffer cells should always be checked on purified cell preparations or at the ultrastructural level. Kupffer cells are also often "purified" by "selective" adherence of cells in a sinusoidal cell suspension to certain substrates (Munthe-Kaas et al., 1975; Lukomska & Olszewski, 1978; Ullrich et al., 1979). Apart from the fact that in this way Kupffer cells are able to ingest a considerable amount of cellular debris derived from cells which do not attach (Brouwer & Knook, 1977), it was evidenced in this chapter that under certain circumstances endothelial cells are also able to adhere even faster than Kupffer cells (de Leeuw et al., 1982c). Therefore, Kupffer cells cannot be identified simply by their capacity to attach to culture dishes. The best way to identify Kupffer cells appeared to be the cytochemical staining for peroxidatic activity. However, this method might not be suitable for species other than the rat. For instance, in mice and humans, some endothelial cells also show peroxidatic activity, while a number of Kupffer cells do not (Stöhr et al., 1978, de Leeuw et al., 1982a). When isolated from animals other than the rat, the best way known at present, is to discriminate between cells by ultrastructural examination.

Endothelial cells of the liver differ in various respects from other endothelial cells of the body (see also Section 1.3). It was of interest therefore to investigate whether liver endothelial cells — like other endothelial cells throughout the body — might also be identified by the presence of factor VIII antigen. The results show that, when isolated with cold pronase, this means of identification is indeed possible.

It was shown that each isolation method described in this chapter had its own problems. However, each also has its advantages, depending on the cellular requirements demanded and the specific aim of a study. For instance, when a high yield of pure Kupffer cells is needed, the best way to isolate them is by the pronase-collagenase method. This method is also suitable for the isolation of fat-storing cells (Knook et al., 1982). The reason why so few of these cells are found with other isolation methods is still unknown.

When the capacity of Kupffer and endothelial cells to endocytose in vivo or in vitro is to be determined, the cold pronase isolation method appears to be the most suitable.

CHAPTER 3

ENDOCYTIC CAPACITY OF RAT LIVER KUPFFER AND ENDOTHELIAL CELLS *IN VIVO*

3.1 INTRODUCTION

The functional capacity of the reticuloendothelial system (RES) is often assessed by determining the rate of plasma clearance of intravenously injected colloidal or particulate materials which are generally believed to be phagocytosed by the Kupffer cells (see Section 1.6). Localization of RES test substances in the liver is frequently determined by the use of light microscopy or by a radioactive liver scan. These methods, however, cannot prove the actual localization in the Kupffer cells; they demonstrate at the most that the substances are present in sinusoidal cells. Two other methods indicate the role of sinusoidal cells in the removal of various substances. The first method is based on the intravenous injection of substances which can be subsequently detected in the sinusoidal cells after their isolation (Nilsson & Berg, 1977; Drevon et al., 1977; Furbish et al., 1978; Schlesinger et al., 1978; Steer et al., 1978,1979; Ose et al., 1979). The second method is based on the *in vitro* incubation of isolated sinusoidal cells with several substances which can be subsequently detected inside the cells (Nilsson & Berg, 1977; Drevon et al., 1977; Ose et al., 1979; Steer & Clarenburg, 1979; Ullrich et al., 1979). Again, these methods do not demonstrate the exclusive involvement of the Kupffer cells.

There are several indications that not only Kupffer but also endothelial cells are involved in the endocytosis of various substances from the circulation. Electron microscopic studies, e.g., have demonstrated the clearance by endothelial cells of several colloidal substances (Wisse, 1972,1977b). The well-developed endocytic system (see Section 1.3) and the high levels of lysosomal enzyme activities in endothelial cells (see Section 2.3.3) also suggest a role of these cells in endocytosis and degradation.

The development of a procedure for the preparation of highly purified

and intact Kupffer and endothelial cells (see Chapter 2) made it possible to estimate the endocytic capacity of each of these cell types for substances taken up in vivo before isolation. In this chapter, the role of Kupffer and endothelial cells in the removal of several intravenously injected substances known to be cleared by the RES will be described. The test substances included polyvinylpyrrolidone as a suitable marker for fluid-phase endocytosis (Roberts et al., 1977; Duncan & Lloyd, 1978), the clinically important blood constituent heparin, colloidal albumin (which is used to determine RES function in humans (Iio et al., 1974)) and three types of modified orosomucoid which are known to be endocytosed by liver cells through specific carbohydrate recognition systems (Ashwell & Morell, 1974; Hubbard et al., 1979).

A second method for demonstrating and estimating the endocytic capacity of both Kupffer and endothelial cells is based on the injection of specific substances which can be localized in the cells at the ultrastructural level. The substances used included horseradish peroxidase, an enzyme which can be visualized by cytochemical staining, and the particulate substances colloidal silver, colloidal carbon and latex beads of different sizes.

3.2 MATERIALS AND METHODS

3.2.1 Test substances

^{125}I-labeled polyvinylpyrrolidone (PVP) and ^{35}S-labeled heparin were purchased from the Radiochemical Centre. ^{125}I-labeled colloidal albumin was prepared as follows: Bovine serum albumin (fraction V, Sigma Chemical Co.) was labeled with ^{125}I by the method of Helmkamp et al. (1960) or with 0-(4-Diazo-3,5-di)[^{125}I](iodobenzoyl)sucrose (DDIBS) by the method of de Jong et al. (1981) and heat-aggregated according to the method of Benacerraf et al. (1957a). Asialo- (ASOR), agalacto- (AGOR) and ahexosaminoorosomucoid (AHOR) were kindly provided by Dr. C. Steer, N.I.H., Bethesda, U.S.A. and labeled with ^{125}I by the chloramine T-method (Greenwood et al., 1963). Horseradish peroxidase (HRP) was obtained from Sigma Chemical Co. and cytochemically visualized by peroxidase staining (see Section 2.2.6). Colloidal carbon (Ø 0.05-0.07 μm) was obtained from Faber Castell-Higgins, colloidal silver iodide (Ø 0.04-0.08 μm) (Neo-Silvol) from Parke-Davis and latex beads of various diameters (monodispersed) from Polysciences Inc.

3.2.2 Injection of test substances and plasma clearance

The radioactively labeled substances were injected intravenously (femoral vein or jugular vein) into 3- to 4-month-old female BN/BiRij rats (see Table IX). Under mild ether anesthesia, at least five blood samples were taken from the tail vein at evenly spaced time points after injection to determine the half-life and the clearance rate of each radioactively labeled substance from the blood. The plasma clearance rate of these substances was determined for each separate experiment because an exact calculation of the average concentration of the substance in the plasma during the time interval between injection and sacrifice of the animals was required for further calculations. The time interval between injection and the death of the animal

TABLE IX

DOSES OF TEST SUBSTANCES AND TIME INTERVAL BETWEEN INJECTION AND PERFUSION OF THE LIVER

Test substance	Dose (µg per 100g rat)	Time interval	Detection	Cell isolation method
PVP	154.5	24 h	R	P 37°
Heparin	153.1	30 min	R	P 37°
CA	405.6	5.4 min	R	P 4°
CA,low dose	20.0	5.8 min	R	-
CA-DDIBS	402.5	15.6 min	R	P-C
AGOR	24.0	5.0 min	R	P 4°
AHOR	22.7	5.8 min	R	P 4°
ASOR	17.9	5.4 min	R	P 4°
HRP	391.3	55 sec	U	-
CS	19,333	5 min	U	-
CC	7,000	5 min	U	-
Latex	0.1*	5 min	U	-

Anesthetized rats were injected with the test substance as described in Section 3.2.2 (dose as indicated). The time interval indicates the period between injection and death. The cell isolation methods are described in Section 2.2.2. Abbreviations: CA: colloidal albumin; CS: colloidal silver; CC: colloidal carbon; R: radioactivity; U: ultrastructural; P 37°: warm pronase; P 10°: cold pronase; P-C: Pronase-collagenase; -: no cells isolated. For further abbreviations, see Section 3.2.1. * expressed as % solids in the plasma.

was chosen to be sufficiently long that the terminal rate of clearance could be determined (see also Section 3.2.5) and that a detectable amount of each test substance had accumulated in the liver cells, taking care that no extensive degradation and excretion occurred prior to the liver cell isolation procedure. For PVP, a time interval of 24 h was chosen because its rate of excretion from the liver is very slow (Munniksma et al., 1980). For DDIBS labeled colloidal albumin, also a longer time interval than for ^{125}I-labeled colloidal albumin was chosen, since the DDIBS label which accumulates in the liver is not degraded and is also excreted very slowly (De Jong et al., 1981).

For ultrastructural studies, electron dense particles were injected. After the time intervals indicated in Table IX, the livers were fixed by perfusion with 2% glutaraldehyde and processed for electron microscopy as described in Section 2.2.5. In the case of HRP, fixed liver slices were incubated for peroxidase staining (see Section 2.2.6) before further processing.

3.2.3 Isolation and purification of Kupffer and endothelial cells

The various methods used for the isolation of sinusoidal cell are described in Section 2.2.2. Sinusoidal cells were further separated into Kupffer and endothelial cells by centrifugal elutriation (see Section 2.2.3). The viability of the cells and the composition of the cell fractions were established as described in Section 2.2.6.

3.2.4 Determination of radioactivity

Radioactivity was measured in samples of plasma, organs and purified liver cell suspensions. ^{125}I was directly counted in a γ-scintillation counter. Samples containing ^{35}S were solubilized in 1 ml soluene for 1 to 2 h at 65°C and were counted in a Mark II liquid scintillation counter after the addition of 15 ml scintillation fluid (composition: 4 g 2.5-diphenyloxazol and 50 mg 2.2'-p-phenylen-bis-(4-methyl-5-phenyloxazol)).

3.2.5 Calculations

If a substance was cleared from the plasma by a first order process, the average plasma concentration during the time interval could be easily

calculated. Since the clearance is then an exponential function, it can be expressed by the following equation:

(1) $C_t = C_0 e^{-KT_1}$

C_1 is the concentration of the substance in the plasma (% of dose per ml plasma) at time point T_1; C_0 is the concentration at time point T_0 and K is the rate constant of the clearance curve. This rate constant gives the slope of the curve and can therefore be expressed by:

(2) $K = \frac{\ln C_0 - \ln C_1}{T_1 - T_0}$

This rate constant is usually called the "phagocytic index" (Benacerraf, 1957a). This term, however, will not be used here, since it can be confused with the term "endocytic index" which will be discussed later.

The average plasma concentration (A) between time points T_0 and T_1 is merely the integral of the clearance curve (equation (1)) divided by this time interval. Thus:

(3) $A = \frac{1}{T_1 - T_0} \int_{T_0}^{T_1} C_0 e^{-KT} dT = \frac{1}{T_1 - T_0} \left[-\frac{1}{K} C_0 e^{-KT} \right]_{T_0}^{T_1} = \frac{1}{T_1 - T_0} \left[\frac{1}{K} C_0 (e^{-KT_0} - e^{KT_1}) \right]$

If $T_0 = 0$, equation (3) becomes:

(4) $A = \frac{1}{T_1 K} C_0 (1 - e^{-KT_1})$

and the equation for the rate constant (2) will become:

(5) $K = \frac{(\ln C_0 - \ln C_1)}{T_1}$

From equations (4) and (5), it follows that:

(6) $A = \frac{1}{\ln C_0 - \ln C_1} C_0 (1 - e^{\ln C_1 - \ln C_0}) = \frac{1}{\ln C_0 - \ln C_1} (C_0 - C_1)$

Thus, only two data items are needed for calculating the average plasma concentration during a certain time interval: the concentration after the chosen time interval and the concentration at time point zero. This method can therefore be easily applied and is the most mathematical way to estimate the average plasma concentration of a first order clearance. A second method is based on the plotting of the clearance curve on a double linear scale. A direct determination of the area under the curve as a percentage of the initial

concentration was obtained by weighing the excised areas. The latter method gave a difference of not more than 3 to 4% when compared with the first. If the clearance of a substance is not a first order process, calculations become much more complicated. In such cases, the second method was used.

The values for the average plasma concentrations were used to calculate the so-called endocytic index. The endocytic index, representing the rate of endocytosis, was defined by Williams et al. (1975) as the volume of external liquid whose contained substance has been internalized by a unit quantity in a unit time. For the liver, the endocytic index (E) was expressed in ml plasma internalized per g liver wet weight per day:

$$(7) \quad E = \frac{S_\ell}{W_\ell} \times \frac{V_\rho}{A} \times \frac{1}{T_1}$$

in which S_ℓ is the amount of the substance present in the liver (% of dose) at time point T_1 (day), W_ℓ is the liver wet weight (g), V_ρ is the total plasmavolume (ml) and A is the average plasma concentration. From equations (1) and (4), it follows that:

$$(8) \quad A = \frac{C_0 - C_1}{T_1 K}$$

Combination of equations (7) and (8) shows that:

$$(9) \quad E = \frac{S_\ell}{W_\ell} \times \frac{V_\rho K}{C_0 - C_1}$$

If the clearance is done solely by the liver, the endocytic index for the liver can be deduced from the clearance curve alone, since then $S_\ell = C_0 - C_1$ and:

$$(10) \quad E = \frac{V_\rho K}{W_\ell}$$

When the plasma volume in this formula is replaced by blood volume, the formula will represent the same one which Benacerraf (1957a) used to calculate what he designated as the "minimal" blood flow in the liver. According to Benacerraf, the calculated minimal blood flow is an underestimation of the real rate of blood flow through the liver. In his view, the formula (10) has to be corrected (about 20%) only by a factor representing the efficiency of the liver in the clearance of a substance in a single pass. However, an additional correction which is not included in formula 10 should be made.

Since in reality not all of a substance is cleared exclusively by the liver, (thus, $S_\ell \neq C_0 - C_1$), formula 10 gives an overestimation of the minimal blood flow. Therefore, formula 9, which includes the correction factor: $\frac{S_\ell}{C_0 - C_1}$ gives a more correct approximation of the minimal blood flow.

3.3 RESULTS

3.3.1 Plasma clearance of radioactively labeled test substances

The curves for the clearance of the radioactively labeled test substances from the plasma are shown in Fig. 15. Immediately after injection, colloidal albumin, AGOR, AHOR and ASOR disappeared from the plasma in an exponential manner, indicating a one-compartment clearance system (see Section 3.2.5). In the cases of heparin and PVP, the clearance rate was not constant until about 10 min and 4 h, respectively, indicating a multicompartment system. The rates of clearance for colloidal albumin, ASOR, AGOR and AHOR were about the same. This can be concluded from the values for the half lives given in Table X. Polyvinylpyrrolidone was cleared very slowly and had a half-life of 18 h. The values for the average plasma concentrations given in Table X were used for further calculations.

3.3.2 Distribution of radioactively labeled test substances in various organs

Table XI shows the distribution of the test substances over the plasma and a selection of various organs generally involved in the clearance of substances from the plasma. The clearance of each test substance by the liver was always higher than that of other organs, except for heparin, which was preferentially taken up by the kidneys. Also, a considerable amount had been excreted in the urine. The amount of heparin not recovered in the organs given in the table was found to be equally divided over the rest of the body by Stau et al. (1973). A ligature around the renal vein did not increase the amount of heparin taken up by the liver (results not shown). In the case of PVP, the remaining amount could be recovered in the bones, skin, intestines, muscles and fatty tissue (Munniksma et al., 1980). Again, about 20% of the substance had already left the body in the urine excreted

TABLE X

DISAPPEARANCE OF RADIOACTIVELY LABELED TEST SUBSTANCES FROM PLASMA AFTER INTRAVENOUS INJECTION

Test substance	Half-life	Average plasma concentration a	b
PVP	17.8 h	23.8	-
Heparin	19.8 min	30.8	-
CA	1.8 min	42.6	44.1
CA,low dose	1.3 min	30.1	31.1
CA-DDIBS	2.3 min	19.8	22.3
AGOR	1.7 min	41.9	41.2
AHOR	1.9 min	40.0	41.5
ASOR	1.1 min	27.8	28.3

Half lives are given as the period of time in which 50% of the substances disappeared from the plasma. In cases where a rapid clearance phase (distribution phase) preceeded a slower phase, the half lives af the latter are given. Average plasma concentrations during the time interval were calculated as described in Section 3.2.5. They are expressed as percentages of the original plasma concentrations which had been calculated by means of the estimated plasma volume of 3.13 ml per 100 g rat (Praaning-van Dalen et al.,1981) and the amount of substance injected. Mean values of at least 4 experiments.
a: calculated by means of plotting the clearance curves (see Section 3.2.5).
b: calculated by means of formula 6 given in Section 3.2.5.
For abbreviations, see Table IX and Section 3.2.1.

during the 24 h time interval.

Since PVP is taken up by fluid-phase endocytosis only, its uptake represents the actual amount of plasma taken up by the liver. With an average plasma concentration of 24% of the injected dose, the uptake of 3.4% of the injected dose by the liver indicates that 14.3% of the total plasma volume per day is cleared by the liver.

The absolute amount of a test substance that accumulates in the liver depends not only on the specific rate of uptake by the liver and other organs but also on experimental variables such as the chosen time interval and the administered dose. If a dose is administered at a concentration such that saturation of the uptake by the liver occurs, the plasma clearance curve will show a different slope and the half-life of the substance in the plasma will increase. The percentage of the dose injected taken up by the liver will de-

TABLE XI

DISTRIBUTION OF RADIOACTIVELY LABELED TEST SUBSTANCES IN VARIOUS ORGANS

Test substance	Liver	Spleen	Kidneys	Lungs	Plasma
PVP	3.4	0.4	0.7	0.3	13.8
Heparin	10.8	0.4	16.9	0.8	16.9
CA	61.4	0.7	1.0	2.1	14.3
CA,low dose	89.6	0.9	0.9	1.0	4.7
CA-DDIBS	54.3	0.9	1.8	0.8	1.2
AGOR	62.3	0.6	7.2	0.5	11.7
AHOR	56.6	0.6	4.4	0.2	12.0
ASOR	80.0	0.0	0.4	0.1	3.3

The amount of substances accumulated in the organs was determined after the time intervals given in Table IX and expressed as percentages of the injected amounts. The amount of substance present in the liver was determined after perfusion for cell isolation. Mean values of at least 4 experiments. For abbreviations, see Table IX and Section 3.2.1.

crease as compared with lower doses. If other organs take over the clearance the clearance rate will no longer be of the first order.

The substances ASOR, AGOR, AHOR and colloidal albumin were all cleared by a first order process at the concentration tested. The low dose of colloidal albumin was cleared at a slightly faster rate and the percentage found in the liver was higher than found for the higher dose. As will be explained below, the rate of clearance of the low dose of colloidal albumin by the liver closely reflects the rate of plasma flow through the liver and therefore also reflects the maximally possible clearance rate of the liver. The clearance rates of ASOR, AGOR and AHOR are of the same order of magnitude. This indicates that the uptake by the liver was not yet saturated.

Since PVP is taken up by fluid-phase endocytosis only, no saturation in the uptake by the liver occurs irrespective of the amount injected (Munniksma et al., 1980). For heparin which is not cleared by a first order process, the saturation point in the uptake by the liver was determined in a separate set of experiments. As shown in Fig. 16, the uptake of heparin by the liver obeyed Michaelis-Menten kinetics. From this curve, an apparent K_M of 54.9 µg and a V_{max} of 13.3 µg per g liver weight per 30 min was calculated.

Therefore, the saturating dose of heparin would be about 164.7 µg per g liver weight (i.e., 3 x K_M), which corresponds to 532 µg per 100 g rat. This means that with the injected amount of 153 µg of heparin per 100 g rat (Table IX), the uptake by the liver was not yet saturated.

The clearance capacity of the liver for each of the substances can be compared under circumstances at which no saturation in the uptake occurs and where the effect of interexperimental variables such as the average substance concentrations in the plasma and the time interval (see Tables IX and X) are taken into account. For this purpose, the rate of uptake by the liver was expressed by the endocytic index (see Section 3.2.5). If a substance enters all liver cells only by the fluid-phase process, the value for the endocytic index will indicate the exact amount of fluid the liver is able to take in, independent of the substance concentration in the extracellular medium. In the case of adsorptive uptake, however, the calculated endocytic index can be much greater than the actual amount of fluid ingested. The data in Table XII show that the endocytic index for the uptake of the marker for fluid-phase endocytosis, PVP, by the liver is indeed very low as compared to that for the other test substances. The endocytic index for heparin is about a hundred times larger. The endocytic indices found for AGOR, AHOR and the high doses of colloidal albumin are of the same order of magnitude and were found to be more than 1500 times higher than for PVP. The highest indices were found for the uptake of the low dose of colloidal albumin and ASOR. With these two substances, almost all that had been cleared from the plasma (more than 80%) was found in the liver (see Table XII). When expressed per min and with the assumption that, in 3-month-old female BN/Bi

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Figure 15 Clearance curves for various test substances, given in percentage of the initial plasma concentration. Test substances were injected into rats as described in Section 3.2.2. The clearance curves for ASOR, AGOR, AHOR and CA appeared to be linear on a logarithmic scale (correlations of the lines were between 0.935 and 0.995). For the plotting of these lines, the slope K (see Section 3.2.5) was calculated by means of the time intervals (Table IX) and the percentages of substance present in the plasma at the start (i.e., 100%) and the end of the time interval (see Table XI). For PVP and heparin, the mean of at least 4 clearance curves is given.

Figure 16 Lineweaver-Burk plot of the uptake of heparin by the liver at 30 min after injection. Varying concentrations of heparin were intravenously injected into rats. After a 30 min time interval, the amount of heparin present in the liver was determined (correlation of the line: 0.9891).

Figure 15

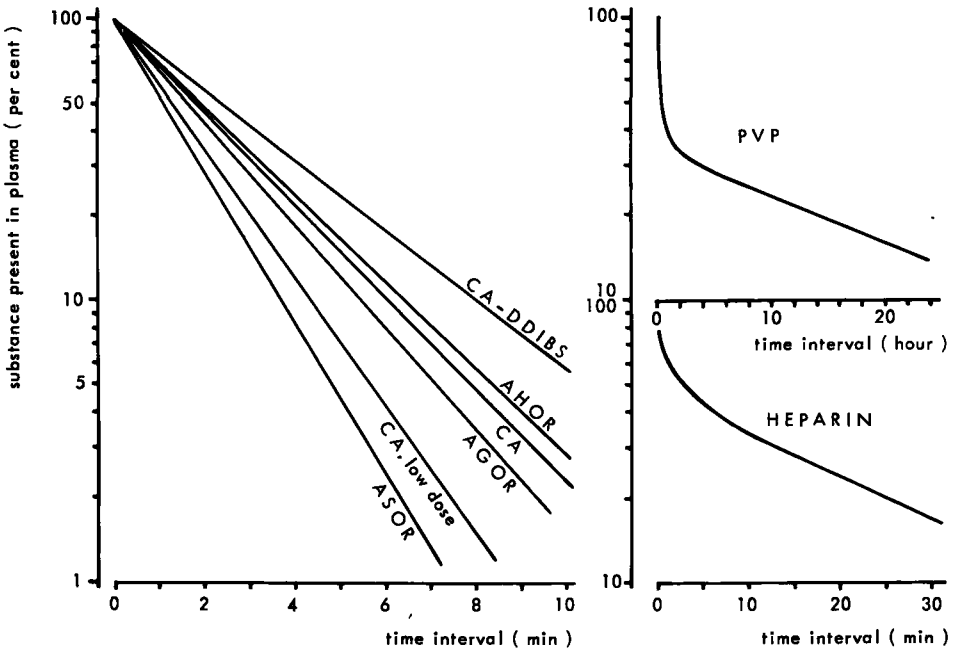


Figure 16

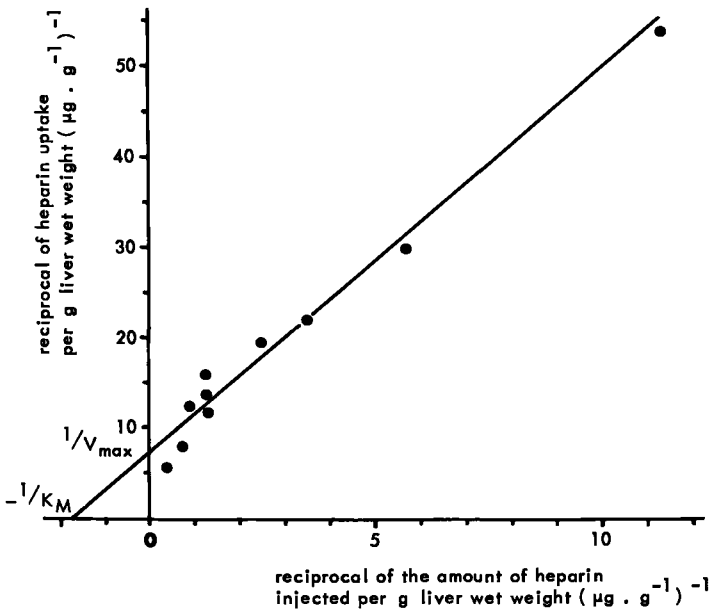


TABLE XII

ENDOCYTIC INDICES FOR THE UPTAKE OF RADIOACTIVELY LABELED TEST SUBSTANCES BY THE LIVER

Test substance	Clearance by liver*	Endocytic index a**	Endocytic index b***
PVP	3.9	0.14	-
Heparin	13.0	16.3	-
CA	71.6	372	360
CA,low dose	94.0	714	693
CA-DDIBS	55.0	245	218
AGOR	70.6	414	422
AHOR	64.3	339	328
ASOR	83.6	740	730

The endocytic index, given in ml plasma per g liver weight per day, was calculated from data given in Table IX (time interval), Table X (average plasma concentration) and Table XI (percentage of dose present in the liver). The average liver weight for a 100 g rat was determined to be 3.23.
*The clearance by the liver is given as a percentage of the total amount of substance cleared from the plasma at the specific time interval (calculated from Table XI).
**calculated from average plasma concentration given in Table X under a.
***calculated from average plasma concentration given in Table X under b.
For abbreviations, see Table IX and Section 3.2.1.

rats, 1 ml blood corresponds to 0.54 ml plasma (Brouwer, personal communication), the endocytic indices for colloidal albumin and ASOR will become 0.921 and 1.003 ml blood per g liver per min, respectively. This is close to the amount for the minimal blood flow (see Section 3.2.5) of 1.13 ml blood per g liver per min estimated by Benacerraf (1957a) in another rat strain. Thus, if the liver is the only organ involved in the clearance and if this clearance is done in a single pass, then the endocytic index actually represents the rate of blood or plasma flow through the liver.

Since the liver cannot take up any substance at a faster rate than at which it is presented to it, the values for the endocytic indices for colloidal albumin and ASOR are very close to the maximally possible value that can be found for the liver. Thus, the value for the endocytic index found for the clearance of an injected substance by the liver is of physiological impor-

tance: whatever soluble substance one might choose for studying the rate of uptake by the liver, the rate will never be slower than found for fluid-phase endocytosis and will never be faster than that of the blood or plasma flow in the liver. Table XII shows that these two extreme rates may differ by a factor of 5000!

3.3.3 Recovery of radioactively labeled test substances in the sinusoidal cell fraction

The amount of test substances present in the liver was never totally recovered in the isolated sinusoidal cell fractions, as shown in Table XIII. The percentage of substances recovered in the sinusoidal fraction varied from 2% for ASOR to 38.5% for colloidal albumin. The exact number of sinusoidal cells present in the whole liver is not known. There are indications that about 50-60% of the total number of sinusoidal cells is lost during isolation (see also Section 2.3.2). This would imply that the actual contribution of the sinusoidal cells to the uptake of heparin, colloidal albumin, AGOR and AHOR by the whole liver is more than 50%. In the cases of heparin and colloidal albumin, the remaining amounts were demonstrated to be present in the parenchymal cells (Praaning-van Dalen et al., 1981). Also in the cases of AGOR and AHOR, the parenchymal cells may contribute to the uptake, which is probably due to the incomplete degalactosylation of both substances (Hubbard, 1979). Only in the cases of PVP and ASOR is the contribution of the parenchymal cells to the uptake likely to be greater than that of the sinusoidal cells.

3.3.4 Localization of radioactively labeled test substances in Kupffer and endothelial cells

The rates of *in vivo* endocytosis by Kupffer and endothelial cells which were determined in these cell types after their isolation and purification are given in Table XIV. The rates of endocytosis of ASOR by purified Kupffer and endothelial cells were not determined since the amount recovered in the sinusoidal cell fraction was very low (Table XIII). The localization of the test substances can be considered to be exclusively intracellular, as membrane bound substances were eliminated by the proteolytic enzymes during cell isolation. In the case of heparin, which is known for its capacity to bind to endothelial cell membranes (Busch et al., 1979), the extent of binding was

TABLE XIII

RECOVERY OF RADIOACTIVELY LABELED TEST SUBSTANCES IN THE SINUSOIDAL CELL FRACTION

Test substance	Percentage in sinusoidal cell fraction
PVP	14.5
Heparin	22.2
CA	38.5
CA-DDIBS	35.9
AGOR	25.1
AHOR	35.3
ASOR	2.0

Values are given as percentages of the total amounts of test substance present in the whole liver. Mean values of at least 4 experiments. For practical reasons, the higher dose of CA was chosen for cell isolation experiments (see Table IX). The methods used for cell isolation are given in Table IX. For abbreviations, see Table IX and Section 3.2.1.

tested for by means of an additional treatment of the endothelial cells with trypsin. No difference in the amount of heparin in endothelial cells was found after trypsin treatment.

The results in Table XIV are again expressed by an endocytic index, given this time in µl plasma per 10^6 cells per day. A ratio between the endocytic indices found for endothelial and Kupffer cells is also given to emphasize the differences in the selectivity of the cells for taking up a specific test substance.

Surprisingly, the endothelial cells were very active in endocytosing most of the substances. As shown by the ratios, the endothelial cells endocytosed colloidal albumin and AHOR in about the same amount but AGOR and heparin even more avidly than did the Kupffer cells. Since endothelial cells contain about half as much cellular protein as Kupffer cells, all ratios would be as much as twice as high if expressed per mg cellular protein. The endocytic index for ^{125}I-labeled colloidal albumin was slightly higher than for DDIBS-labeled colloidal albumin. A similar difference was also observed for the uptake by the whole liver (Table XII). Since DDIBS is undegradable and

TABLE XIV

ENDOCYTIC INDICES FOR THE UPTAKE OF RADIOACTIVELY LABELED TEST SUBSTANCES BY KUPFFER AND ENDOTHELIAL CELLS *IN VIVO*

Test substance	Endothelial cells (E)	Kupffer cells (K)	Ratio (E/K)
PVP	0.53	1.61	0.33
Heparin	126	53.9	2.34
CA	3710	3620	1.03
CA-DDIBS	1700	1410	1.21
AGOR	2620	1050	2.50
AHOR	2870	2310	1.24

The endocytic index, given in µl plasma per 10^6 cells per day, was calculated from the uptake in ng per 10^6 cells (not shown) and from data given in Table IX (time interval) and Table X (average plasma concentration). The methods used for cell isolation are given in Table IX. For abbreviations, see Table IX and Section 3.2.1.

cannot leave the cells, these results strongly indicate that ^{125}I labeled colloidal albumin was also not degraded during the cold pronase isolation and purification of the cells (see also Section 2.3.4).

It should be mentioned that the difference in endocytic capacity between Kupffer and endothelial cells might be changed when a saturating dose of substance is injected. Hubbard et al. (1979), e.g., found in a semiquantitative study a higher ratio in the *in vivo* uptake between endothelial and Kupffer cells when a high dose of AGOR or AHOR was used.

As mentioned earlier, the magnitude of the endocytic index again gives an indication of the mechanism by which a substance enters the cell. Heparin is taken up at a much higher endocytic index than is the marker of fluid-phase endocytosis, PVP (Table XIV). This finding and the assessed saturation in the uptake by the liver (Fig. 16) indicate that heparin is adsorbed to the cell membrane prior to its internalization. All other substances are taken up with an even higher endocytic index than heparin. Analogous to the situation for the whole liver (Table XII), the endocytic index for colloidal albumin in the isolated cells might also be a factor of 1.9 higher (i.e., 7000 µl per 10^6 cells per day) if the lower dose of colloidal albumin had been used. This endocytic index most probably represents the maximum possible rate of endo-

cytosis in Kupffer and endothelial cells in vivo, since it corresponds to the maximum rate of uptake by the whole liver (Table XII), which depends on the rate at which the injected substance is delivered to the cells by the blood.

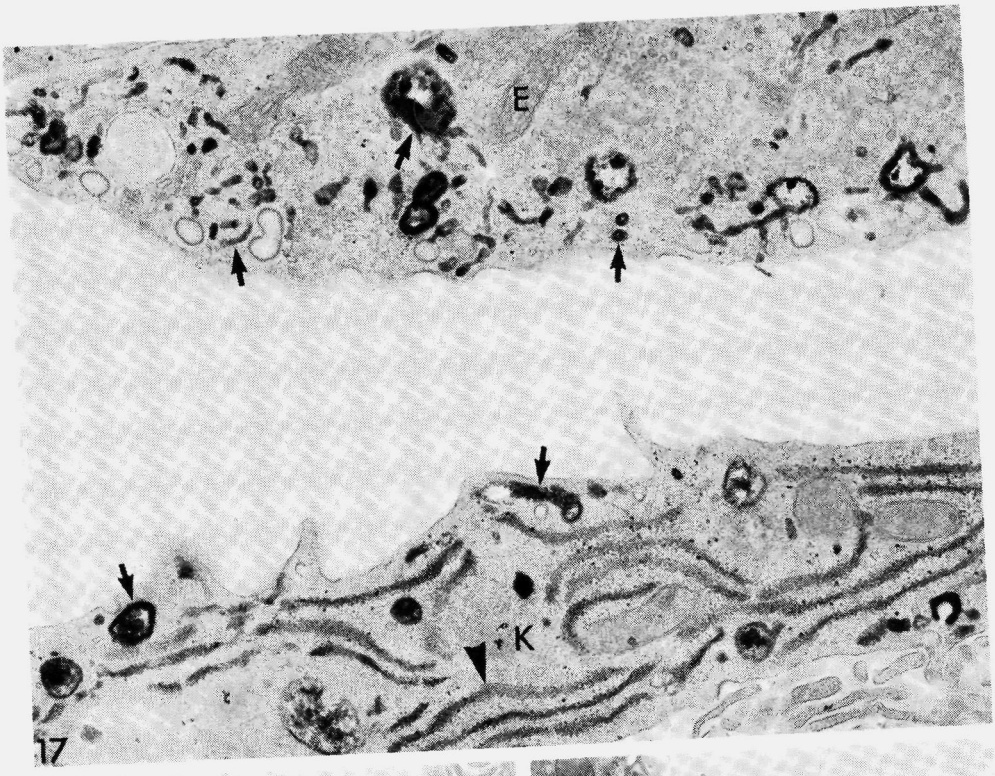
3.3.5 Localization of test substances in Kupffer and endothelial cells as revealed by electron microscopy

An ultrastructural study enabled a semiquantitative determination of the capacity of Kupffer and endothelial cells to endocytose HRP, colloidal silver, colloidal carbon and latex beads in vivo. A comparison of the endocytic capacities of Kupffer and endothelial cells based on these determinations is given in Table XV. This table shows, as a confirmation of the results obtained with the uptake of radioactively labelled substances, that the endothelial cells were active in the endocytosis of most of the substances. The relative amounts taken up by endothelial or Kupffer cells were dependent on both the nature and the size of the test substance.

During an incubation time of 55 sec, endothelial cells had visibly taken up more HRP than had the Kupffer cells (Fig. 17). Colloidal silver was taken up by endothelial cells in about the same amounts as found for Kupffer cells (Fig. 18). Colloidal carbon, however, was found mainly in Kupffer cells. Only very small quantities were detected inside endothelial cells (Fig. 19). The observed difference in the amounts of uptake of colloidal carbon and colloidal silver by endothelial cells might be due to the tendency of colloidal carbon to form larger aggregates in the blood than colloidal silver (Figs. 18 and 19). Latex beads with diameters of 0.1 and 0.23 µm were found primarily in Kupffer cells, but were sometimes also associated with endothelial cells (Fig. 20). Latex beads with diameters of ≥ 0.29 µm were seen exclusively in Kupffer cells. None of the test substances was ever observed inside parenchymal cells.

Figure 17 In vivo endocytosis of HRP by an endothelial (E) and a Kupffer (K) cell at 55 sec after intravenous injection of the ligand. HRP positive vesicles (arrows) are more abundant in endothelial than in Kupffer cells. The Kupffer cell also shows endogenous peroxidatic activity (arrowhead). (22,100x).

Figure 18 In vivo endocytosis of colloidal silver by an endothelial (a) (8,640x) and a Kupffer (b) (7,070x) cell at 5 min after intravenous injection of the ligand. Vesicles containing the ligand (arrows) are seen in both cell types. Adsorbed colloidal silver is present on the membrane of the Kupffer cell (arrowheads).



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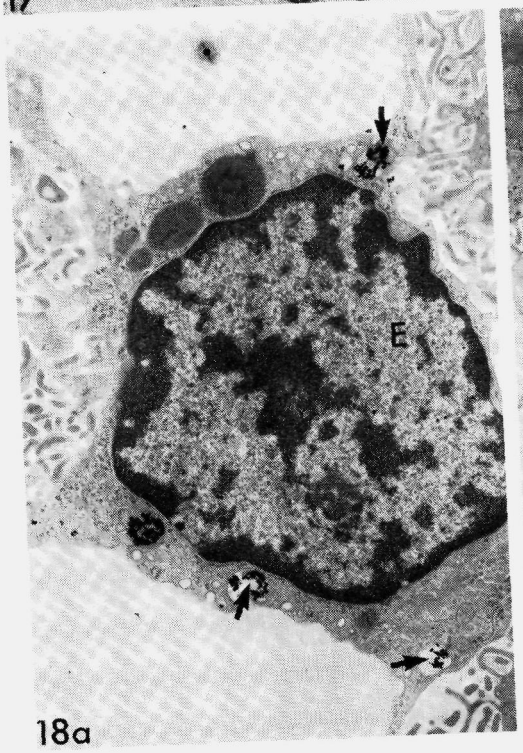

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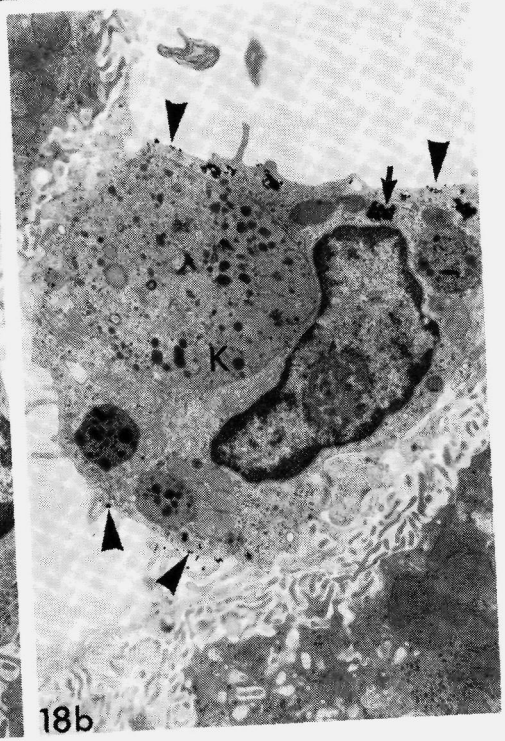

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TABLE XV

IN VIVO ENDOCYTOSIS OF SUBSTANCES BY KUPFFER AND ENDOTHELIAL LIVER CELLS AS REVEALED BY ELECTRON MICROSCOPY

Test substance	Endothelial cells (E)	Kupffer cells (K)	Relative amounts in E versus K*
HRP	+	+	E > K
Colloidal silver	+	+	E = K
Colloidal carbon	+	+	E << K
Latex beads 0.1 µm	+	+	E << K
0.23 µm	+	+	E << K
0.29 µm	-	+	
0.48 µm	-	+	
0.65 µm	-	+	

Rats were injected with the test substances as described in Table IX. The substances were localized in fixed livers (see Section 3.2.2) HRP: horseradish peroxidase; +: occurs; -: does not occur. *For the semiquantitative determinations, not only the number of substance-filled vacuoles per cell type but also the relative frequency at which this cell type contained endocytosed substance was taken into account. About 100 cells were examined for each substance.

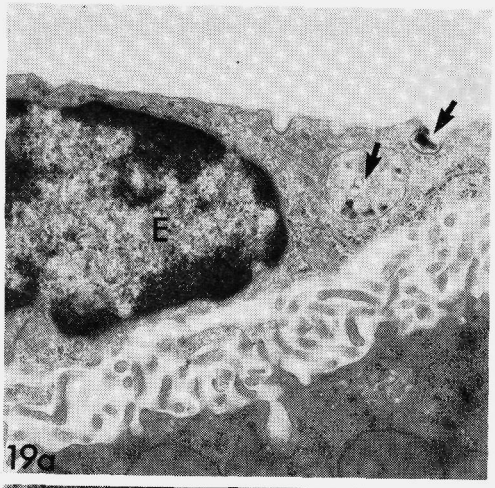
Figs. 18-20 also demonstrate that colloidal carbon, colloidal silver and latex beads are adsorbed to the Kupffer cell membrane to a greater extent than to endothelial cells. Nevertheless, endothelial cells endocytosed about as

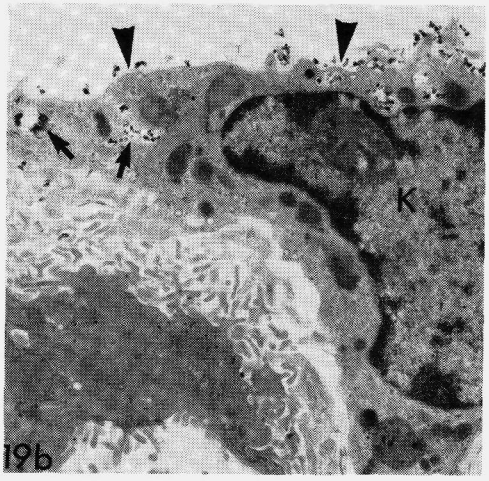
Figure 19 In vivo endocytosis of colloidal carbon by an endothelial (a) (15,200x) and a Kupffer (b) (6,620x) cell at 5 min after intravenous injection of the ligand. Vesicles containing the ligand (arrows) are more abundant in Kupffer than in endothelial cells. Adsorbed colloidal carbon is present on the membrane of the Kupffer cell (arrowheads).

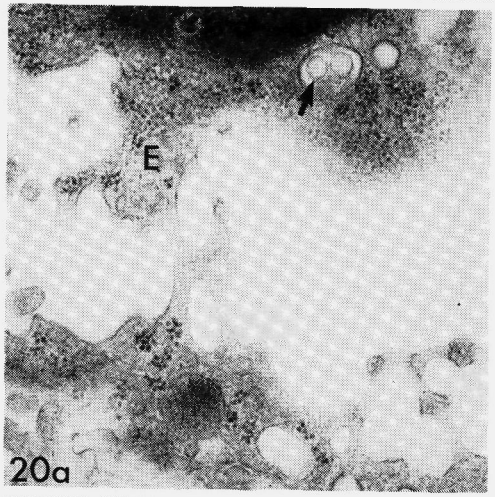
Figure 20 In vivo endocytosis of latex beads by endothelial (E) and Kupffer (K) cells at 5 min after intravenous injection.

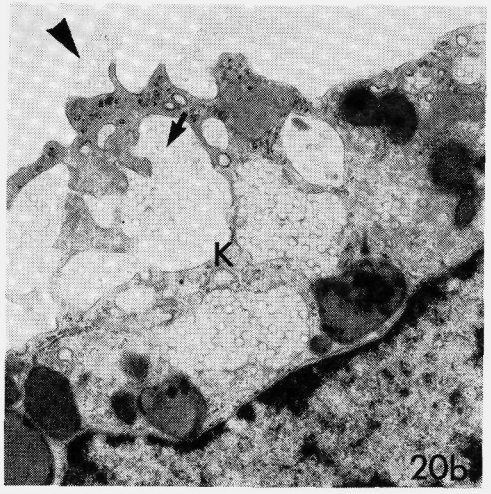
a),b) Latex beads of 0.1 µm (arrows) are present mainly in vesicles of Kupffer cells (12,000x), but are also found in endothelial cells (33,300x).

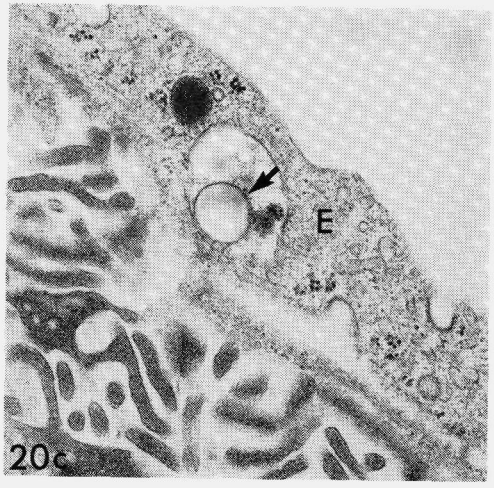
c),d) Latex beads of 0.23 µm (arrows) are also found in both cell types, although mainly in Kupffer cells. Adsorbed latex beads are present on the membrane of the Kupffer cells (arrowheads). (c: 27,400x; d: 17,500x).

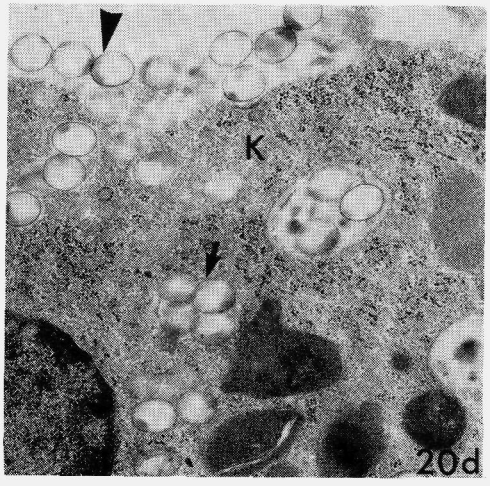

E
19a


K
19b


E
20a


K
20b


E
20c


K
20d

much colloidal silver. This might indicate that colloidal silver remains for a relatively longer time at the Kupffer cell membrane and that the internalization process in Kupffer cells is slower than in endothelial cells.

Although all cells of a certain type seemed to be equally active in the endocytosis of HRP, this was not observed for the uptake of the other materials. This was especially true for the uptake of latex particles by Kupffer cells. Kupffer cells loaded with latex were found, while no particles were observed in other cells. A similar heterogeneity in the uptake of latex by Kupffer cells has been described by Sleyster and Knook (1982).

3.4 DISCUSSION

This chapter deals with the involvement of Kupffer and endothelial cells in the endocytosis of the following substances known to be cleared by the RES.

Polyvinylpyrrolidone is a synthetic, metabolically stable, hydrophilic polymer and has been clinically used as a substitute for plasma proteins. It can be considered as a suitable marker for fluid-phase endocytosis (Roberts et al., 1977; Duncan & Lloyd, 1978; Munniksma et al., 1980). PVP has also been described as a marker for Kupffer cell or RES function (Bradfield & Souhami, 1980; Brouwer & Knook, 1983).

The mucopolysaccharide heparin is used to prevent thrombosis and intravascular coagulation. Heparin, a normal constituent of the blood, is highly negatively charged and therefore readily forms stable complexes with a variety of cationic molecules (Jaques, 1982). Heparin binds to the opsonic protein fibronectin and consequently increases the rate of fibronectin binding to collagen (Ruoslahti et al., 1978; Yamada et al., 1979; Kiebe & Mock, 1982; Del Rosso et al., 1982). In this way, heparin might be involved in the cellular attachment and clearance of various fibronectin-collagen coated substances from the blood (Gudewicz et al., 1980). Impairment of the clearance function of the RES is reported to be induced by heparin (Berghem et al., 1977). However, enhancement of RES activity by heparin has also been described (Kaplan & Saba, 1981). Apart from the reported uptake of heparin by the RES, especially the liver (Losito et al., 1977; Teien, 1977; Shanberge et al., 1978; Dawes & Pepper, 1979) it is reported to be bound to or endocytosed by macrophages (Oh et al., 1973; Fabian et al., 1978; Bleiberg et al., 1981) and endothelial cells (Hiebert & Jaques, 1976; Busch et al., 1979;

Mahadoo et al., 1980) in various locations in the body. Endocytosis of heparin by Kupffer cells has been described by Stau et al. (1973). However, this conclusion was drawn merely from the light microscopic autoradiographic localization which cannot demonstrate the actual presence of the substance in Kupffer cells. Ultrastructurally, accumulation of intravenously injected heparin in Kupffer cells has been described by Hiebert (1981) who, however, also noted the presence of the substance in the liver endothelial cells. Since this study involved only cytochemical observations, no quantitative data on the relative contributions of Kupffer and endothelial cells to the uptake of heparin could be obtained.

Orosomucoid, also called α_1-acid glycoprotein, is a plasma protein which is synthesized by the liver. Orosomucoid has an exceptionally high content of carbohydrates which are present in complex-type oligosaccharide side chains. In the plasma form of orosomucoid, most of these chains are terminated by a sialic acid residue. The orosomucoid present in the liver cells, however, does not contain sialic acid groups. It has been suggested that, before being secreted by the cells, sialic acid residues are attached to the terminal end of the oligosaccharide chains (Nagashima et al., 1980).

Ashwell and Morell (1974) were the first to discover that sialic-free orosomucoid (i.e., asialoorosomucoid or ASOR), which possesses galactose terminated oligosaccharide chains, disappears from the plasma at a much faster rate than does the native sialic rich form. They found that ASOR was rapidly cleared by the liver parenchymal cells and that this was a general phenomenon for glycoproteins with galactose terminated oligosaccharide chains. The physiological importance of this phenomenon is not known. It has been suggested that during cellular damage glycoproteins which are not yet sialilated might be released into the circulation and then be rapidly cleared by the liver. Polymeric IgA might also be cleared in the same way (Stockert et al., 1982). In the plasma of patients with cirrhosis, polymeric IgA is present in an abnormally high concentration.

Removal of the terminal galactose groups from ASOR results in agalactoorosomucoid (AGOR) which possesses terminal N-acetylglucosamine residues. Upon further cleavage of the N-acetylglucosamine, ahexosaminoorosomucoid (AHOR) which has oligosaccharide chains that are terminated by mannose groups is obtained. Both AGOR and AHOR as well as other N-acetylglucosamine or mannose terminated glycoproteins are rapidly cleared from the plasma (Stahl et al., 1976a,b; Achord et al., 1977,1978; Hubbard et al., 1979). Like ASOR, these substances are readily cleared by the liver, but

they accumulate mainly in the sinusoidal cells and not in the parenchymal cells (Schlesinger et al., 1978; Hubbard et al., 1979). In vitro, various macrophages are able to endocytose glycoproteins with terminal N-acetylglucosamine or mannose terminated oligosaccharide chains (Stahl et al. 1978, 1980). Therefore, it has been generally assumed that the clearance by the liver was mainly done by the Kupffer cells. However, Hubbard et al. (1979) demonstrated in a semiquantitative autoradiographic study that endothelial cells also contributed to the uptake of these glycoproteins. As is the case with the glycoprotein clearance system of parenchymal cells, the physiological importance of that of Kupffer and endothelial cells is not fully understood. However, it has been suggested that lysosomal enzymes derived from damaged cells are cleared in this way. Most lysosomal enzymes are glycoproteins containing mannose terminated oligosaccharide chains. When injected, lysosomal enzymes are indeed rapidly cleared by the sinusoidal cells of the liver (Steer et al. 1979; Achord et al., 1978; Schlesinger et al., 1978). It has also been suggested that certain antigen-antibody complexes (Day et al., 1980*) and some bacterial components such as zymosan (Sung et al., 1983) are cleared by the same system.

Horseradish peroxidase (HRP) is still frequently used as a marker for fluid-phase endocytosis (Steinman et al., 1983). However, HRP is a glycoprotein which, like AHOR, possesses mannose terminated oligosaccharide chains and is therefore likely to be cleared in the same way. Clearance studies indicated that HRP is indeed taken up by the liver by the specific clearance system for N-acetylglucosamine/mannose terminated glycoproteins (Rodman et al., 1978) and not by fluid-phase endocytosis. The localization of injected HRP in sinusoidal liver cells has also been described by Straus (1964,1967, 1981). In these studies, no attempt has been made to quantify the relative difference in the uptake between Kupffer and endothelial cells nor was the percentage of sinusoidal cells that were endocytically active described.

Colloids in general are assumed to be cleared by the RES. In the study described in this thesis, three types of colloids were used: colloidal albumin, colloidal silver and colloidal carbon. Colloidal albumin has been clinically used as a test substance for RES function (Ito et al., 1974; Brouwer & Knook,

*These results have been recently redrawn by the authors (1983, J.Biol.Chem. 258, 8519)

1983). The uptake of colloidal albumin by the liver was described by Benacerraf in 1957. Endocytosis of colloidal albumin by isolated and cultured Kupffer cells has been demonstrated by Brouwer & Knook (1977). However, the possible involvement of endothelial cells in the uptake of colloidal albumin has not been included in these studies. After intravenous injection, colloidal silver has been reported to be localized in Kupffer cells (Emeis & Lindeman, 1976). No comparison was made between the uptake by Kupffer and endothelial cells in their study. The plasma clearance of colloidal carbon is still often used as a marker for RES activity (Souich et al., 1981; Grün et al., 1980; Agostini et al., 1980). In addition, colloidal carbon is used to induce impairment of the functional capacity of the RES (i.e., RES blockade) (Rikkers & Newton, 1980; Braatz, 1980; Filkins & Yelich, 1980; Bradfield & Souhami, 1980). The ultrastructural localization of injected colloidal carbon not only in Kupffer but also in endothelial cells has been reported (Widmann et al., 1972; Ogawa et al., 1973; Satodate et al., 1977; Wisse, 1977b).

Endocytosis of latex particles is used often as a marker for the functional capacity of Kupffer cells exclusively, since it is generally thought that endothelial cells do not ingest these particles (Widmann et al., 1972; Barranger et al., 1978). However, the uptake of small latex particles of 0.1 μm diameter by endothelial cells of the rat liver has been reported (Wisse, 1977b).

All test substances described in this chapter were cleared by the liver in considerable amounts. In general, substances that were cleared very rapidly from the plasma were taken up almost exclusively by the liver. In the case of ASOR and the low dose of colloidal albumin, the rate limiting factor in the plasma clearance appeared to be the rate of blood flow through the liver instead of the maximal clearance capacity of this organ. Therefore, conditions under which substances are cleared by the liver at the same rate as that at which they are delivered cannot be applied to determine the functional clearance capacity of the liver or of the RES. Under certain pathological conditions or during aging, the blood flow might be altered (Varga & Fisher, 1978). This would automatically lead to a decreased clearance by the liver, while the actual clearance capacity of this organ is not necessarily decreased.

The specific rates of uptake of the test substances, expressed as the endocytic index, give an indication of the mechanism by which a substance is cleared by the liver. Substances like PVP that enter the liver cells without

prior adsorption to the cell membranes are taken up by the mechanism of fluid-phase endocytosis, which results in a very low endocytic index for the liver. All other radioactively labeled substances showed much higher endocytic indices than the base value found for PVP, indicating that they were adsorbed to the membranes of the liver cells before their internalization. The highest possible endocytic index for the liver that can be obtained for any substance is limited by the rate of plasma flow through this organ. The lowest possible endocytic index for any soluble substance equals the rate of fluid-phase endocytosis. The results given in this chapter show that the minimum and the maximum values of the endocytic index for the liver may differ by a factor of 5000.

Two methods were used to discriminate between the endocytic capacity of Kupffer and endothelial cells in vivo. With the first, the presence of an injected radioactively labeled substance in the cells was demonstrated after they had been isolated and purified. With the second, endocytosed substances were demonstrated in the cells in situ at the ultrastructural level.

With both methods, the uptake of substances by the cells was determined at one time point only. This leaves the possibility of undetected transfer of test substances from one cell type to the other within the time interval between injection and cell isolation. However, a stable intracellular localization of PVP has been demonstrated (Munniksma et al., 1980). The incubation times for HRP, ASOR, AGOR, AHOR, colloidal albumin, colloidal silver, colloidal carbon and latex beads did not exceed 5 min, which seems a very short period for intracellular transfer. With the somewhat longer time interval of 15 min for heparin, a transfer cannot be excluded, but seems unlikely. Moreover, a direct transfer of heparin from sinusoidal cells to parenchymal cells seems unnecessary, as heparin is a molecule which can easily pass the fenestrae of the endothelial cells and the parenchymal cells are thus able to endocytose the substance directly from the medium in the space of Disse.

With the two methods mentioned above, it was shown that endothelial cells are very active in the endocytosis of not only soluble substances but also of colloidal and particulate materials. Endothelial cells are about two to three times more abundant in the liver than are Kupffer cells; as a result, the contribution of endothelial cells to the clearance of heparin, colloidal albumin, AGOR, AHOR, HRP and colloidal silver is very likely to be greater than that of the Kupffer cells. Other studies have also shown uptake of colloids or particles by endothelial cells, e.g., colloidal carbon (see earlier),

colloidal iron (Hausman et al., 1976; Kuchta et al., 1982), nanoparticles of 0.22 µm (Lenaerts et al., 1982), colloidal gold (Wisse, 1977b) and the frequently used RES test substance antimony sulfur colloid (Praaning-van Dalen et al., 1981). Not only endothelial but also parenchymal cells might play a role in the clearance of colloids (Praaning-van Dalen et al., 1981). It seems necessary, therefore, to determine the extent of endothelial cell endocytosis when the function of Kupffer cells or the RES is to be monitored by the use of an arbitrarily chosen test substance.

The preceding discussion might give the impression that there is no substance which is cleared exclusively by the Kupffer cells and may thus serve as a marker for Kupffer cell functions. However, lactate dehydrogenase (De Jong et al., 1982), endotoxin (Praaning-van Dalen et al., 1981) and two types of malate dehydrogenase (Bijsterbosch et al., 1982a) are taken up primarily by Kupffer cells. Moreover, the results presented in this chapter show that only small amounts of colloidal carbon and large latex particles are taken up by endothelial cells under the conditions established.

The ultrastructural studies revealed that all individual sinusoidal cells were active in the endocytosis of HRP. Since HRP is a mannose terminated glycoprotein, a comparable situation can be expected for the endocytosis of AGOR and AHOR. However, the amounts of endocytosed colloidal carbon, colloidal silver and latex beads were not the same in all Kupffer or endothelial cells. This suggests that clearance of certain substances is accomplished by a subpopulation of cells. This is in accord with the existence of a heterogeneous populations of Kupffer cells with respect to their size, location in the liver and endocytic capacity (Sleyster & Knook, 1982). The results discussed here indicate that such a heterogeneous population might also exist for endothelial cells.

The ultrastructural studies also demonstrated a strong adsorption of several test substances to the Kupffer cell membrane. This implies that, if endocytosis is estimated only by the clearance of substances from the circulation, a portion of the "cleared" substance may still be adsorbed instead of endocytosed.

The question can be raised as to what extent the Kupffer and endothelial cells are able to degrade the test substances after endocytosis. PVP, colloidal carbon, colloidal silver and latex beads are inert, undegradable substances. ASOR, AGOR, AHOR and colloidal albumin are degraded very rapidly (see Section 2.3.4). The degradation of HRP seems to be much slo-

wer. The enzymatic acitivity of HRP remains in the cells for at least 24 hours (Fahimi, 1970). The catabolism of heparin is poorly understood (Losito et al., 1977; 1981). Several investigators mention that there is a storage of heparin in the liver (Hiebert, 1981; Jacques, 1982). Others claim that the liver is able to degrade heparin (Day et al., 1962; Friedman & Arsenis, 1974; Stau et al., 1973; Dawes and Pepper, 1979), although at a slow rate. In principle, the endothelial cells possess several lysosomal enzymes such as α-L-iduronidase (unpublished results), arylsulfatase B, β-glucuronidase, β-N-acetylglucosaminidase and β-galactosidase (see Section 2.3.3) which are able to act on mucopolysaccharides in general (Rodén, 1980). The capacity of endothelial cells to degrade the mucopolysaccharide hyaluronic acid after its endocytosis has been discussed by Smedsrød et al. (1982). In view of the capacity of the cells to degrade several test substances quite rapidly, the clearance capacity of the cells should always be determined within a short time interval.

In summary, the assumption that Kupffer cells, as constituents of the RES, are almost exclusively responsible for the clearance of RES test substances or substances known to be cleared by the RES does not hold. Especially colloids are often selectively taken up by the endothelial cells. If the definition of the RES is based on the clearance of certain substances, endothelial and even parenchymal cells can be considered as being part of the RES (Praaning-van Dalen et al., 1981). Since the extent to which these cells contribute to the RES function is completely dependent on the nature of the colloid used, it is very difficult to give a proper definition for the RES.

As a consequence, one should use great caution in the interpretation of experimental data based upon the clearance of arbitrarily chosen RES test substances. The clearance capacity of a specific population of cells should always be assessed by determining the actual amount of substance endocytosed by the cell type under study. Testing of the functional capacity of the RES simply by the determination of the clearance of a test substance, even if this substance is a colloid, includes the danger that: a) the substance is taken up by a cell type other than the one under study; b) only a selective portion of the cells under study are endocytically active; c) a considerable amount of cleared substance is merely adsorbed but not endocytosed; and d) the rate of blood flow through the liver instead of the endocytic capacity of the cells is the rate limiting factor in the clearance.

CHAPTER 4

ENDOCYTIC CAPACITY OF RAT LIVER KUPFFER AND ENDOTHELIAL CELLS IN VITRO

4.1 INTRODUCTION

The involvement of sinusoidal liver cells in the clearance of a variety of intravenously injected substances known to be removed by the RES was described in the previous chapter. Evidence was presented to show that both Kupffer and endothelial cells are able to endocytose most of these substances in vivo.

For a more detailed study on the mechanisms and kinetics of endocytosis, in vitro incubations of isolated cell preparations with the test substances are preferred. The important advantages of an in vitro system include the exclusion of possible physiological influences on liver function, the use of a strictly defined incubation medium and the multiplicity of experiments which can be performed with a cell preparation of a single liver.

For the accurate determination of endocytosis in vitro, the experimental conditions should fulfil certain criteria. Firstly, a cell incubation method by which endocytosis can be quickly arrested and the cells effectively separated from the incubation medium should be applied. Secondly, the rate of endocytosis should be determined during a period at which the internalization of the test substance is linear with time. Thirdly, the amount of substance which is merely bound to the cells should be determined, as it may otherwise be falsely included in the amount present in the intracellular compartment. Finally, the cells introduced into the incubation medium should be intact, pure, viable and still carrying out the functions under study. Their endocytic capacity should be high enough to be quantified and the characteristics of the endocytic process should be comparable to the in vivo situation. These functional characteristics should always be carefully checked.

A variety of methods for isolating intact, pure and viable sinusoidal liver cells was described in Chapter 2. In this chapter, these isolation me-

thods are compared with respect to their suitability for in vitro studies on endocytosis by sinusoidal cells. One isolation method which proved to be superior to the others was used to assess endocytosis by sinusoidal cells under various incubation conditions and to determine the endocytic capacity of purified Kupffer and endothelial cells.

4.2 MATERIALS AND METHODS

4.2.1 Test substances

All test substances used for in vitro incubations with sinusoidal cells have been described under Section 3.2.1.

4.2.2 Isolation and purification of Kupffer and endothelial cells

Sinusoidal cells were prepared by the warm pronase, cold pronase, pronase-collagenase or collagenase method as described under Section 2.2.2. Different batches of collagenase were tested. Sinusoidal cells isolated by the cold pronase method were separated into Kupffer and endothelial cells by centrifugal elutriation as mentioned under Section 2.2.3. Cell viability and peroxidatic activity were determined as described under Section 2.2.6.

4.2.3 In vitro incubation of cells with the test substances

As a standard procedure, isolated cells were diluted to a concentration of 5×10^6 cells per ml in ice-cold GBSS containing 1.3% BSA and 17.5% fetal calf serum (FCS). BSA and FCS were omitted in studies on the endocytosis of colloidal albumin (see also Section 4.3.2). After addition of the test substances at 4°C, the cells were incubated at either 4°C or 37°C in a shaking water bath.

Incubations of the cells with radioactively labeled test substances were terminated by collecting 200 ml samples from the incubation medium and separating the cells from the medium by centrifugation through a mixture of dinonyl and dibutyl phthalate (1:4) (10 sec in a Beckman microfuge, 8700 x g) (Nilsson & Berg, 1977; Ose et al., 1980a). The first sample (0 min incubation time) was taken immediately after adding the test substance at 4°C. The

radioactivity present in the cell pellets and supernatants was counted as described under Section 3.2.4.

Incubations of the cells with electron dense substances were terminated by addition of an excess of ice-cold GBSS to the incubation medium and subsequent centrifugation of the cells (10 min; 300 x g; 4°C). The cell pellet was then fixed by adding it to an isotonic 0.15 M sodium cacodylate buffer containing 2% glutaraldehyde and processed for electron microscopy (see Section 2.2.5). In studies on the endocytosis of HRP, the cells were washed in the cacodylate-glutaraldehyde buffer to remove free HRP. An overnight fixation period (4°C) resulted in the abolishment of the endogenous peroxidatic activity in the Kupffer cells (Fahimi, 1970). The cells were then washed three times in 0.05 M Tris-HCl buffer containing 7% sucrose. After incubation for exogenous peroxidatic activity (see Section 2.2.6), the cells were washed three times in the same Tris buffer and processed for electron microscopy.

4.3 RESULTS

4.3.1 Endocytic capacity of sinusoidal cells isolated by various methods

The endocytic capacity of sinusoidal cells isolated by various methods was quantitatively compared by incubating the cells at 37°C with radioactively labeled colloidal albumin, AGOR and AHOR and by charting the time dependent increase in the "cell associated radioactivity". After an incubation at 37°C, the amount of "cell associated radioactivity" is actually a combination of three different parts: the amount actually endocytosed by the cells, the amount bound to the cell membrane and the amount present in the fluid that is trapped between the cells in the pellet. The amount of trapped fluid will depend on the concentration of cells in the original sample, but will be independent of the concentration or type of substance used. Therefore, at an established cell concentration, the amount of trapped fluid will always represent a fixed percentage of the original amount of fluid in the sample prior to the centrifugation of the cells.

As shown in Fig. 21, for each substance, the highest cell associated amount was observed when the cells had been isolated by the cold pronase method. A linear increase during at least 15 min was observed in the amount

of cell associated radioactivity for each substance (Fig. 21). After that, the increase seemed to level off. In the case of colloidal albumin, even a decrease was noted. When cells were isolated by the warm pronase or by the pronase-collagenase method, the cells showed no increase in cell associated radioactivity within the 30 min incubation time. The same was observed when the cells were previously incubated for two hours at 37°C for recovery from the isolation procedure. In cells isolated by the collagenase method, a relatively high increase in the amount of cell associated colloidal albumin was found (Fig. 21). In contrast, almost no AGOR or AHOR was associated with these cells at any time point, indicating that the suitability of the collagenase isolation method for studies on endocytosis depends on the test substance.

To gain an impression of the amount of bound and trapped radioactivity in the samples, cold pronase isolated cells were incubated with the substances at 4°C. At this low temperature, only binding of the substances to the cells but no uptake should occur (Silverstein et al., 1977). If the cells are also not able to bind a specific substance, as is the case for PVP (see also Chapter 3), the amount of cell associated radioactivity will merely reflect the amount of fluid trapped between the cells in the pellet. It was found that, after a 4°C incubation with PVP, 0.135% of the total amount of fluid in the original sample was trapped between the cells. This happened to be quite comparable with the amount present in the pellets of warm pronase isolated cells incubated with any of the other substances, which amounted to 0.1% of the original sample. These results indicate that no measurable binding of the substances to warm pronase isolated cells had occurred and it can therefore be assumed that the curves for the warm pronase isolated cells (Fig. 21) represent the amounts of trapped fluid in the pellets.

Although the percentage of trapped fluid seems to be very low, it was already too high to determine the amount of fluid-phase pinocytosis. When the cold pronase isolated cells were incubated at 37°C with PVP, no significant increase in the pinocytosed amount was observed as compared with the amount of trapped fluid, even after an incubation period of 2 h. Since cell viability started to decrease after 2 h, longer incubations were omitted. From the cellular composition of the sinusoidal cells (Table IV) and the amount of fluid-phase pinocytosis performed by Kupffer and endothelial cells in vivo (Table XIV), it can be calculated that the maximal expected amount of fluid-phase pinocytosis within 2 h might reach 0.064 µl per 10^6 sinusoidal cells. Since, as already mentioned, 0.135% of the 200 µl sample containing 10^6 cells

is trapped between these cells, the amount of trapped fluid will be 0.27 µl. This exceeds the amount of expected pinocytosed fluid by a factor of at least 4. Ose et al. (1980a), who attempted to quantify the fluid-phase pinocytosis of PVP by parenchymal cells during an incubation period of 1 h, found that the amount of trapped fluid exceeded that of what he considered to be pinocytosed by almost a factor of 5. It might be concluded therefore that the rate of fluid-phase pinocytosis is too slow to be accurately measured within the time period during which isolated Kupffer and endothelial cells remain viable.

For binding experiments, the cold pronase isolated cells were incubated with AGOR, AHOR and colloidal albumin at 4°C for at least 60 min, after which no further increase in the binding was observed. To determine the actual amount of binding, the amount contributed by the trapped fluid was subtracted from the total amount of radioactivity present in the cell pellet (Table XVI). As can be deduced from Table XVI and Fig. 21, the amount of bound plus trapped substance represents about 10-16% of the total amount of cell associated radioactivity found after 15 min incubation at 37°C. Thus, 84-90% of the activity found at 37°C can be regarded as actual endocytosis. Therefore, the amounts of AGOR, AHOR and colloidal albumin which were associated with the cells at 37°C will be hereafter referred to as endocytosis or uptake.

The observed difference between 37°C and 4°C incubations indicates that the endocytic process is strongly temperature dependent. A different situation was found for heparin. When cold pronase isolated cells were incubated with heparin at 37°C, some increase in cell associated radioactivity

TABLE XVI

ESTIMATION OF THE AMOUNT OF CELL BOUND SUBSTANCES

Substance	Total amount (A)	"Trapped" (B)	Bound (A - B)
Colloidal albumin	32.3	26.5	5.8
AGOR	2.7	0.6	1.1
AHOR	3.6	0.6	3.0

Cold pronase cells were incubated with the test substances for 60 min at 4°C as described in Section 4.2.3. The amount of trapped medium was determined by the incubation of warm pronase isolated cells with the substances. Concentration of the substances as described in Fig. 21. Mean values of at least 3 experiments, given in ng substance per 10^6 cells.

occurred during 30 min incubation. However, the same increase was observed at an incubation temperature of 4°C. On subsequent incubation with trypsin, all cell associated heparin could be removed after both 4°C and 37°C incubations. This indicates that only binding and no uptake of heparin occurred at 37°C.

4.3.2 Influence of incubation conditions on endocytic capacity of sinusoidal cells

For a good comparison of the endocytic capacity of cells for different substances, the optimal conditions for the uptake of each substance must be established. As mentioned in Chapter 2, cold pronase isolated cells were prepared in GBSS containing 1.3% BSA. As a standard procedure, BSA and FCS were added to the incubation medium (see Section 4.2.3.), since the cells showed less tendency to clump and remained vital at 37°C for about 2 h in the presence of these supplements. However, BSA appeared to strongly inhibit the endocytosis of colloidal albumin, as is shown in Table XVII. The uptake of colloidal albumin was also inhibited by FCS. Inhibition of the endocytosis of colloidal albumin by FCS has also been reported for cultured Kupffer cells (Brouwer & Knook, 1977). BSA and FCS inhibited the endocytosis of AGOR and AHOR, but not when both BSA and FCS were added simultaneously. As a consequence of these experiments, further incubations with colloidal albumin were done in the absence of BSA and FCS, whereas incubations with AGOR and AHOR were done in the presence of these substances.

Figure 21 Endocytic capacity of sinusoidal cells isolated by different methods (see Section 2.2.2). Sinusoidal cells were isolated by the warm pronase (——), cold pronase (- - -) or collagenase (. . .) method and incubated with the test substances as described in Section 4.2.3. The concentrations of the substances were 75 µg per ml (colloidal albumin), 6 µg per ml (AGOR) and 5 µg per ml (AHOR). Values obtained with cells isolated by the pronase-collagenase method were the same as with the warm pronase method. Mean values of at least 3 experiments.

Figure 22 Saturation kinetics for the endocytosis by cold pronase isolated sinusoidal cells (Lineweaver-Burk plot). Cold pronase isolated cells were incubated at 37°C (see Section 4.2.3) with increasing amounts of test substances. The amount of cell associated radioactivity was determined after a 5 min incubation period. Each point represents the mean of at least two experiments.

Figure 21

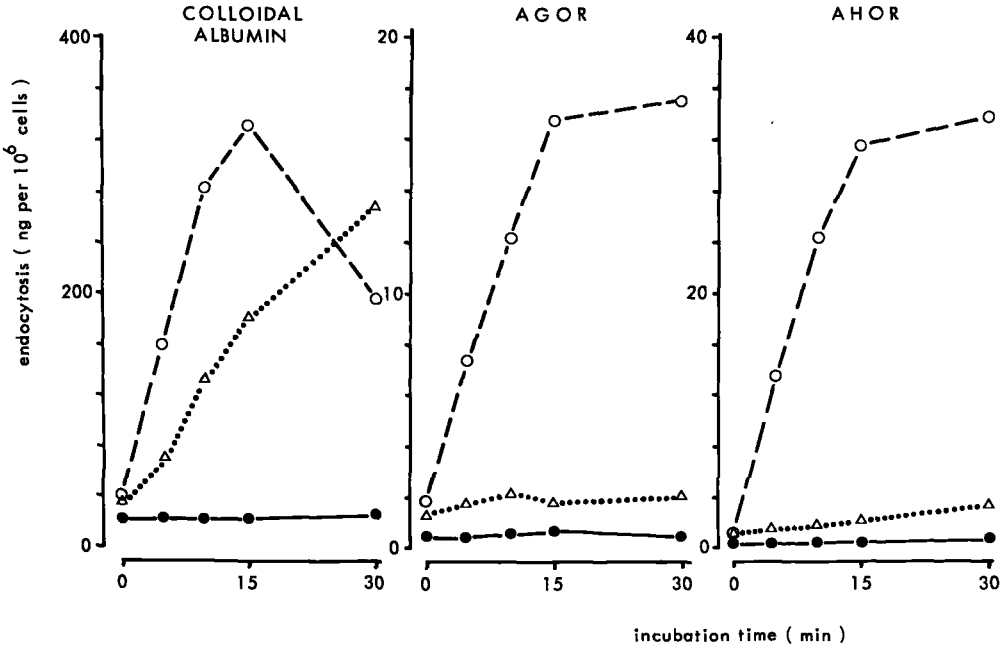


Figure 22

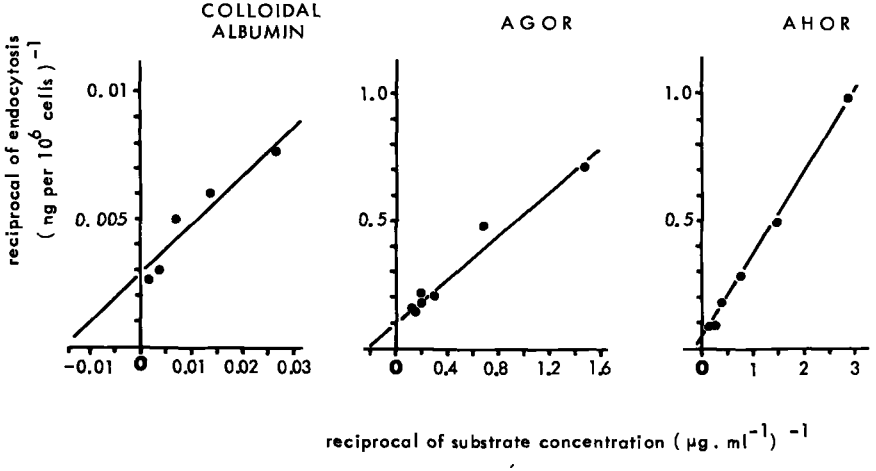


TABLE XVII

ENDOCYTIC CAPACITY OF COLD PRONASE ISOLATED SINUSOIDAL CELLS UNDER VARIOUS INCUBATION CONDITIONS

Condition	Colloidal albumin	AGOR	AHOR
No additions	100 (5)	88 (3)	114 (2)
BSA (1.3%)	9 (2)	49 (3)	n.d.
FCS (17.5%)	38 (4)	78 (2)	59 (2)
BSA + FCS	44 (2)	100 (5)	100 (5)
Cell conc. (10^6 per ml)			
5	100 (5)	100 (5)	100 (5)
10	72 (2)	104 (5)	97 (4)
20	n.d.	n.d.	63 (1)

Cold pronase isolated cells (5×10^6 per ml) were incubated in GBSS with the test substances as described in Section 4.2.3. Values are given as percentages of endocytosis (mean values of the endocytosis found after 5 and 10 min incubation). In parentheses: number of experiments. n.d.: not determined.

The omitting of BSA and FCS in the incubations with colloidal albumin resulted in clumping of the cells. This clumping could be prevented by a preincubation of the cells with DNase. However, the endocytic capacity of cells treated with DNase was reduced to 17% of that of untreated cells, probably due to some proteolytic enzyme activity being present in the DNase preparation. Clumping of the cells could also be prevented by the use of a rather low cell concentration. The optimal concentration at which endocytosis in the samples collected was still assessable and clumping was greatly reduced appeared to be 5×10^6 cells per ml incubation medium (Table XVII). Therefore, this cell concentration was used for the standard incubations.

A characteristic of an endocytic process which involves the binding of a substance to the cell membrane is the occurrence of a saturation effect. Such an effect was observed when cold pronase isolated cells were incubated with increasing amounts of AGOR, AHOR and colloidal albumin during a time period at which uptake was still linear (Fig. 22). On the basis of Lineweaver-Burk plots (Fig. 22), the apparent K_M and V_{max} values were calculated (Table XVIII). From the V_{max} values, the maximum numbers of molecules of a substance taken up per 10^6 cells within a certain time period can be calculated. The numbers of molecules endocytosed per min were comparable for

TABLE XVIII

V_{MAX} AND APPARENT K_M VALUES FOR THE ENDOCYTOSIS OF TEST SUBSTANCES BY COLD PRONASE ISOLATED SINUSOIDAL CELLS

Substances	K_M		V_{max} (per 10^6 cells)		
	µg per ml	nM	ng per 5 min	pmol per min	molecules per min
CA	66.42	22.1	341.97	0.023	1.4×10^{10}
AGOR	4.26	96.6	9.69	0.044	2.6×10^{10}
AHOR	7.04	159.7	23.33	0.106	6.4×10^{10}

Cold pronase isolated cells were incubated with varying amounts of substances (see Fig. 22) as described in Section 4.2.3. K_M and Vmax values were calculated from the Lineweaver-Burk plot given in Fig. 22. CA: colloidal albumin.

each of the three substances (Table XVIII). For a better comparison, the K_M values for each substance were expressed per nM (Table XVIII). In this way, the lowest K_M was found for the uptake of colloidal albumin, indicating that the affinity of the cells for this substance was somewhat stronger than for the other two substances.

4.3.3 Endocytic capacity of purified Kupffer and endothelial cells

Sinusoidal cell preparations obtained by the cold pronase isolation method were further separated into purified Kupffer and endothelial cells to determine the specific endocytic capacity of each cell type. To make possible the direct comparison of the endocytosis of the substances AGOR, AHOR and colloidal albumin by the two cell types, the uptake in Table XIX is expressed by the endocytic index (see Section 3.2.5) in µl of substance containing incubation medium per 10^6 cells per day. For this purpose, the substances were added in an amount which did not result in a saturation in the uptake (see Tables XVIII and XIX).

The purified endothelial cells endocytosed the test substances better than did the Kupffer cells, which is comparable to the *in vivo* situation (see Section 3.3.4). The endocytic index of isolated endothelial cells was of the same order of magnitude for each substance. A comparable situation was found *in vivo* (Section 3.3.4). However, the absolute endocytic capacity of

TABLE XIX

ENDOCYTIC INDICES FOR THE UPTAKE OF RADIOACTIVELY LABELED TEST SUBSTANCES BY ISOLATED AND PURIFIED KUPFFER AND ENDOTHELIAL CELLS

Test substance	Endothelial cells (A)	Kupffer cells (B)	Ratio A/B
Colloidal albumin	569	219	2.6
AGOR	709	77	9.2
AHOR	572	279	2.0

Cold pronase isolated cells were incubated with the substances as described in Section 4.2.3. The values are given in µl per 10^6 cells per day and calculated after 5 and 10 min incubation (mean values for both are given). The amount of substance present in the cell pellets at 0 min incubation time (i.e., substance bound to the cells and present in the trapped medium) was subtracted. Concentration of the substances: colloidal albumin: 77 µg per ml; AGOR: 1.35 µg per ml; AHOR: 1.35 µg per ml. Mean values of at least 3 experiments (3 to 6).

endothelial cells in vivo (Table XIV) was about 5 times higher than in vitro (Table XIX).

The capacity of purified Kupffer cells to endocytose AGOR was smaller than found for the other two substances (Table XIX). This also reflects the in vivo situation, although the absolute endocytic capacity of Kupffer cells in vivo (Table XIV) was about 13 times higher than in vitro.

Since the endocytic capacity of Kupffer cells in vitro is more diminished

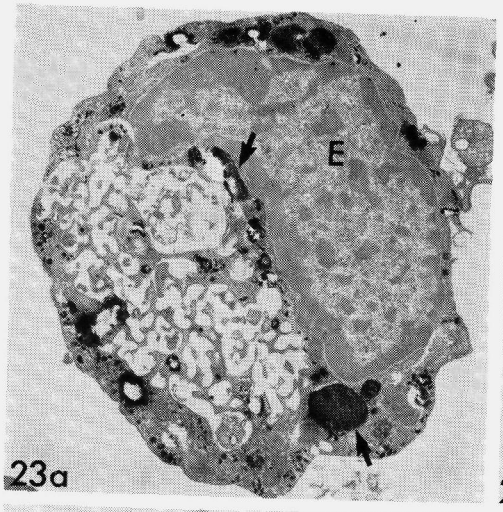
Figure 23 Endocytosis of HRP by cold pronase isolated endothelial cells (E) and Kupffer cells (K) after an incubation period of 10 min at 37°C (a,b,c,d). (For experimental conditions, see Section 4.2.3).

a,b) When a high concentration of HRP (125 µg per ml) was present in the medium, HRP was found (arrows) in a variety of vesicles in both cell types. Generally, the amount of HRP present in endothelial cells exceeded that in Kupffer cells. (a:8,430x; b: 6,930x).

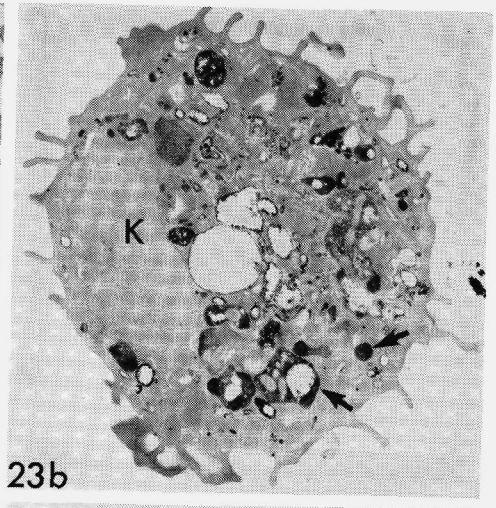
c,d) When the concentration of HRP in the medium was only 12.5 µg per ml, the amount endocytosed by the endothelial cells (E) was considerably higher than in the Kupffer cells (K). (c:10,400x; d:5,540x).

e) After an incubation period of 30 min at 37°C with HRP (125 µg per ml), cells accumulated even more HRP and were still in the process of endocytosis (arrows). (14,500x).

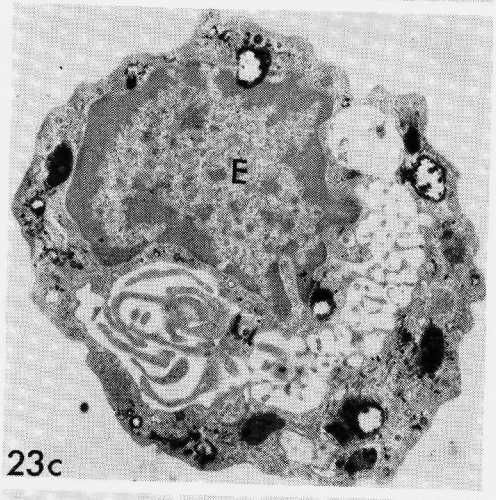
f) When incubated for 30 min at 4°C, the amount of HRP present in the cells is very low. (11,500x). HRP is seen only in small vesicles which are probably not completely internalized by the cells (see inset, arrows). (22,600x).



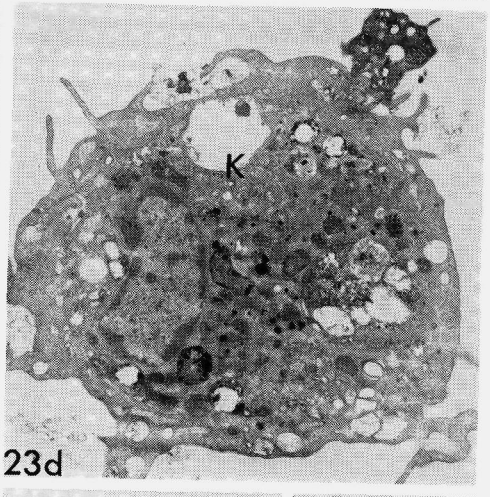
23a



23b



23c



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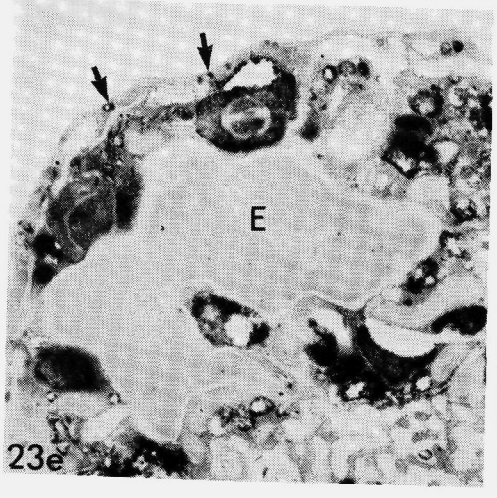
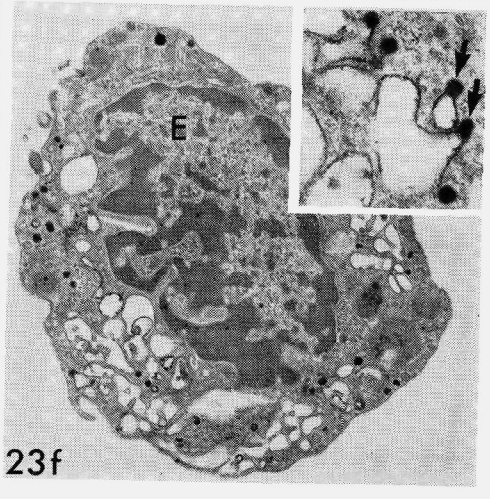


23e



23f

than that of the endothelial cells, the difference in the endocytic capacity between the two cell types in vitro is greater than in vivo, as can be seen from the ratios given in Tables XIV and XIX. It should be emphasized that the values given in these tables were not corrected for the presence of contaminating cell types in the purified fractions. Since the fractions were never 100% pure (see Section 2.3.3), the values might be slightly overestimated (for Kupffer cells) or underestimated (for endothelial cells). Especially the in vitro endocytosis of AGOR by cells in the Kupffer cell fraction might be fully accounted for by the presence of only 11% endothelial cells.

The endocytic capacity of isolated Kupffer and endothelial cells was also investigated at the ultrastructural level by incubating the cells with HRP, colloidal silver, colloidal carbon and latex beads. A comparison of the endocytic capacities of Kupffer and endothelial cells for these substances is given in Table XX.

All substances found to be endocytosed by both cell types in vivo (see Section 3.3.5), were also endocytosed in vitro. The amount of HRP present in endothelial cells appeared to be higher than in Kupffer cells (Fig. 23a,b). A comparable situation was described for the in vivo endocytosis of HRP (see Section 3.3.5). Especially at a low concentration of HRP in the medium the difference in uptake between the two cell types was obvious (Fig. 23c,d). The amount of endocytosed HRP increased for at least 30 min (Fig. 23e).

Colloidal silver was present in endothelial cells in at least equal amounts as in Kupffer cells (Fig. 24a,b), whereas colloidal carbon was present mainly in Kupffer cells (Fig. 25a,b). These results are comparable with the situation in vivo (see Section 3.3.5). Latex beads of 0.1 µm diameter were acti-

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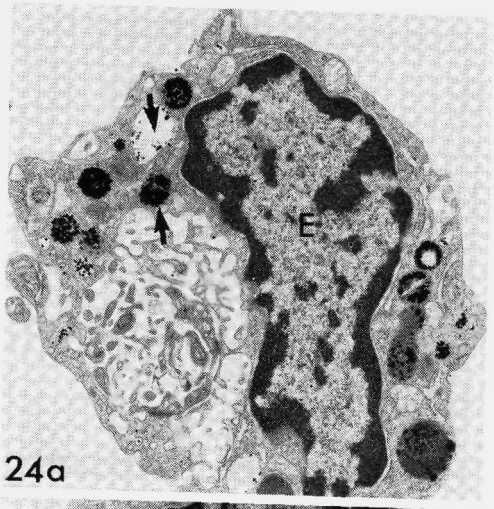
Figure 24. Endocytosis of colloidal silver by a cold pronase isolated endothelial (E; Fig. 24a) (9,900x) and Kupffer cell (K; Fig. 24b) (9,820x) after an incubation period of 30 min at 37°C. (For experimental conditions, see Section 4.2.3).In both cell types, the colloid is present in a variety of vesicles (arrows).

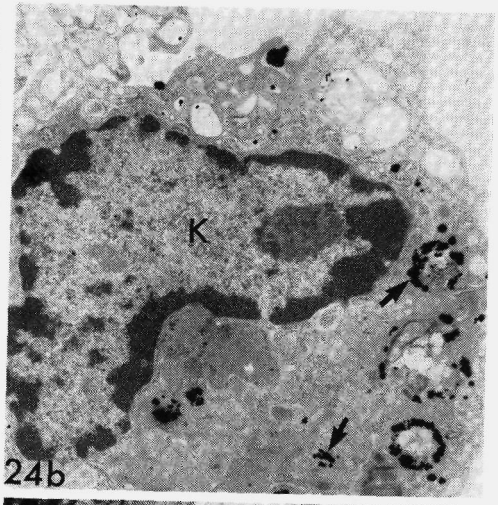
c) An endothelial cell incubated with colloidal silver for 30 min at 4°C. The substance is found mainly in vesicles which are not completely internalized (arrows). (12,826x).

Figure 25 Endocytosis of colloidal carbon by a cold pronase isolated endothelial (E; Fig.25a) (13,600x) and Kupffer cell (K; Fig. 25b) (6,850x) after an incubation period of 30 min at 37°C. (For experimental conditions, see Section 4.2.3). Much more colloidal carbon (arrows) is found in the Kupffer cell than in the endothelial cell.

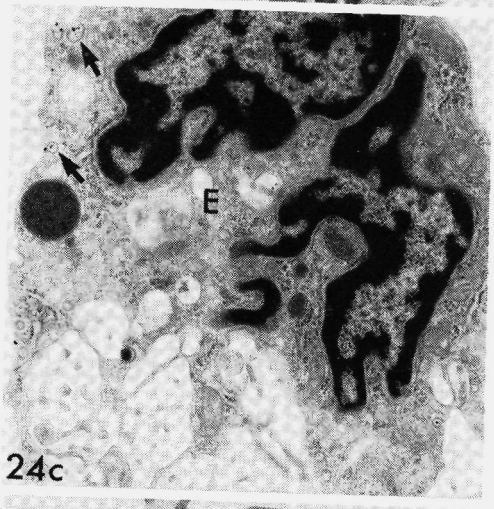
c) A Kupffer cell incubated with colloidal carbon for 30 min at 4°C. Virtually no substance is found inside the cell. (12,600x).



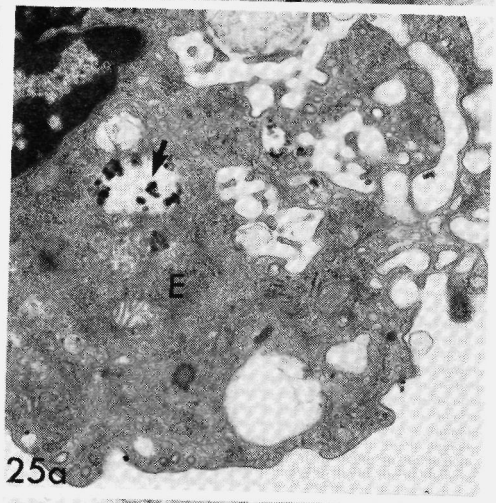
24a



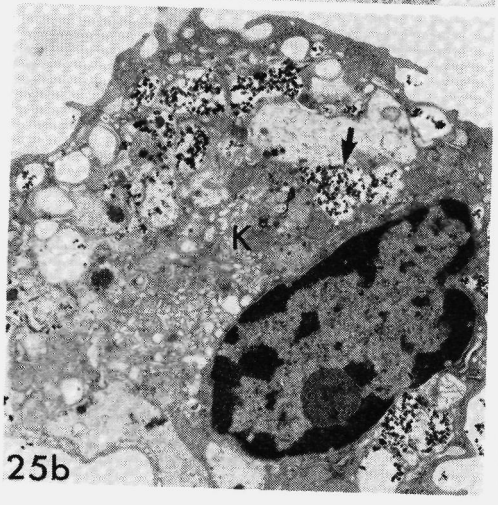
24b



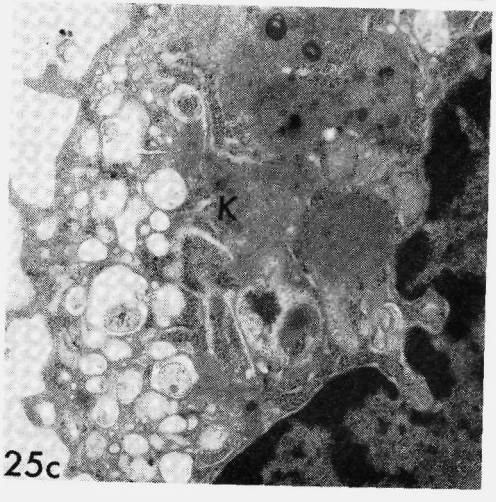
24c



25a



25b



25c

TABLE XX

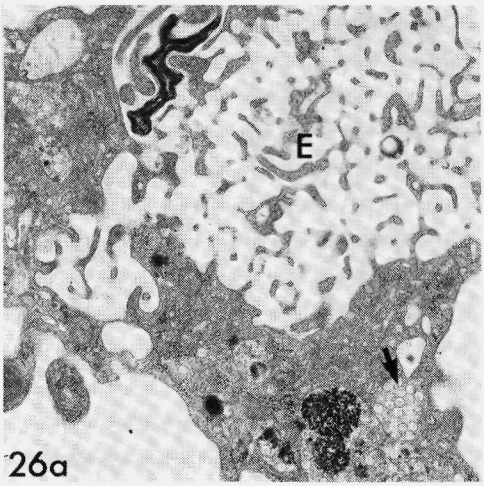
ENDOCYTOSIS OF TEST SUBSTANCES BY ISOLATED KUPFFER AND ENDOTHELIAL CELLS AS REVEALED BY ULTRASTRUCTURAL INVESTIGATION

Test substance	Endothelial cells (E)	Kupffer cells (K)	Relative amounts in E versus K*
HRP	+	+	E > K
Colloidal silver	+	+	E ≥ K
Colloidal carbon	+	+	E < K
Latex beads 0.1 µm	+	+	E ≤ K
0.23 µm	+	+	E < K
0.29 µm	+	+	E < K
0.48 µm	+	+	E < K
0.65 µm	+	+	E < K

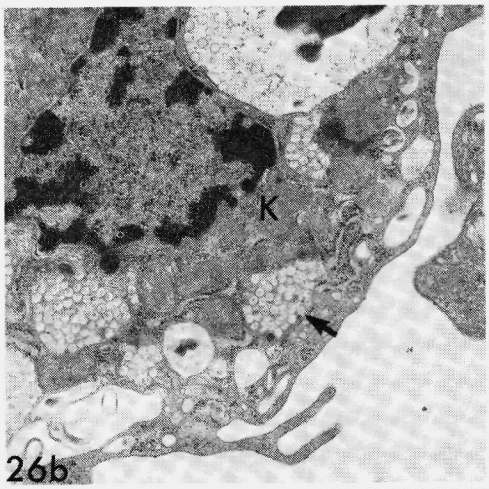
Cold pronase isolated cells were incubated with the substances for 30 min at 37°C as described in Section 4.2.3. Concentration of the substances: HRP: 125 µg per ml, colloidal silver: 2 mg per ml, colloidal carbon: 2.24 mg per ml, latex beads: 0.1% solids. +: endocytosis observed.
* For the semiquantitative determinations, not only the number of substance filled vacuoles per cell type but also the relative frequency at which this cell type contained endocytosed substance was taken into account. About 100 cells were examined for each substance.

vely endocytosed by both Kupffer and endothelial cells (Fig. 26a,b), whereas beads of ≥0.23 µm were endocytosed more actively by Kupffer cells (Fig. 26c). In contrast to endothelial cells *in vivo*, isolated endothelial cells were more active in the uptake of 0.1 µm and 0.23 µm latex beads and they were also able to endocytose latex beads of 0.48 and 0.65 µm diameter (Fig. 26d,e).

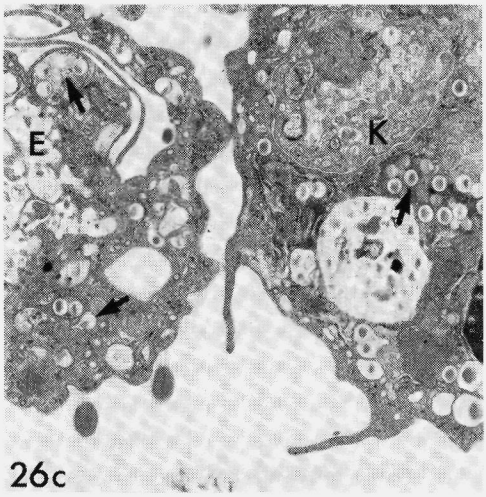
Figure 26 Endocytosis of latex beads by cold pronase isolated endothelial (E) and Kupffer (K) cells after an incubation period of 30 min at 37°C (for experimental conditions, see Section 4.2.3).
a,b) Latex beads of 0.1 µm diameter are actively endocytosed by both cell types (arrows). (12,600x).
c) Latex beads of 23 µm diameter are present in both cell types (arrows) but Kupffer cells generally contain more latex. (8,360x).
d,e) Endothelial cells are also able to endocytose latex beads (arrows) of 0.48 µm diameter (Fig. 26d) (10,400x) and 0.65 µm diameter (Fig. 26e) (10,200x).
f) A Kupffer cell incubated with latex beads for 30 min at 4°C. No uptake of latex is observed. (8,120x).



26a



26b



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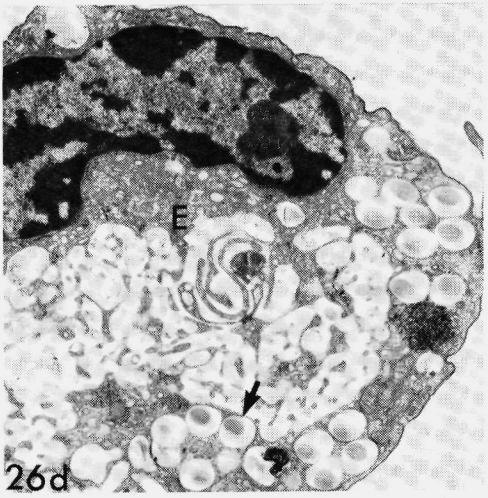

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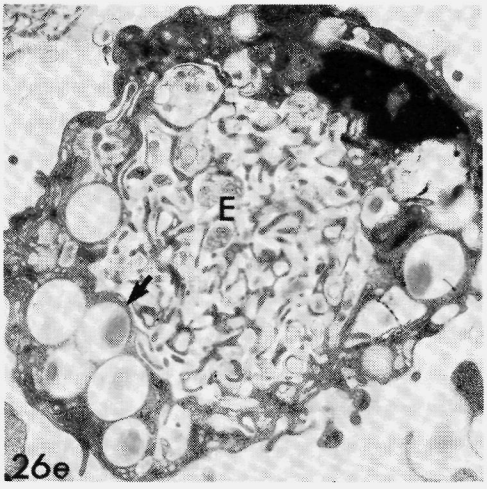

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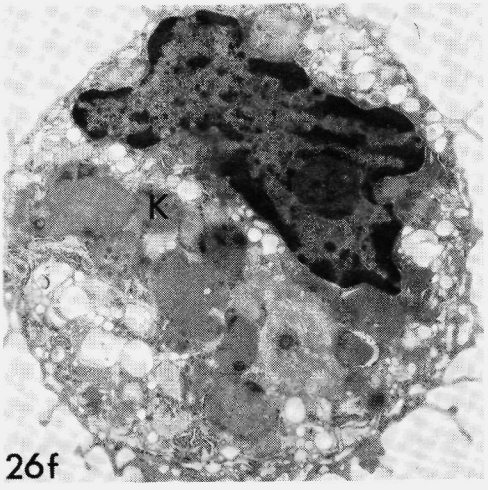

26f

The adsorption of the substances to the Kupffer cell membrane in vitro was less obvious than in vivo (Figs. 24b, 25b, 26b,c). This could be due to the extensive washing of the cells during the processing for electron microscopy. As in vivo, all cells were active in the endocytosis of HRP, but not all were equally active in the uptake of the other substances, especially of the latex particles.

At an incubation temperature of 4°C, the amount of endocytosis for the substances tested was negligible when compared with incubations at 37°C (Figs. 23f,24c,25c,26f).

4.4 DISCUSSION

When sinusoidal cells isolated by various methods were tested for their endocytic capacity in vitro, it appeared that the best retention of this capacity was found in cells isolated by the cold pronase method. Cells isolated by the warm pronase or the pronase-collagenase method had absolutely no endocytic capacity for the various test substances. This latter observation agrees with what has been described by several authors (Munthe-Kaas et al., 1975; Brouwer & Knook, 1977; Steer & Clarenburg, 1979). In addition, cells which were able to endocytose after isolation or after a culture period lost their endocytic capacity following a treatment with pronase or trypsin at 37°C (Stahl et al., 1980; Ose et al., 1980b; Wandel et al., 1981; Brouwer et al., 1982; Rieder et al., 1982). These results indicate that proteolytic enzymes present in the pronase preparation affect the cell membranes in such a way that they are no longer capable of binding certain substances. Some cells, e.g., Kupffer cells, are able to regain their endocytic capacity after isolation using pronase at 37°C (Munthe-Kaas, 1976, Brouwer & Knook, 1977). However, to achieve this, a culture period of at least 24 hours is required. To avoid these problems, sinusoidal cells are often isolated by use of collagenase, which is supposed to have less effect on the membrane properties than has pronase (Nilsson & Berg, 1977; Steer & Ashwell, 1980; Jansen et al., 1980; Maynard & Baenziger, 1981; Summerfield et al., 1982; Zeitlin & Hubbard, 1982). Some authors have reported that collagenase also affects the binding capacity of the cells, though to a lesser extent than pronase (Steer & Ashwell, 1980; Zeitlin & Hubbard, 1982). This might be due to some contamination of the collagenase with proteolytic enzymes (Zahlten et al., 1981). In fact, when 14 different batches of collagenase from three different firms

were tested by us, they were proved to contain nonspecific proteolytic activity. Addition of trypsin inhibitor (see also Section 2.2.2.5) to the collagenase containing media used for cell isolation did not improve the endocytic capacity of the cells (unpublished results). However, some investigators might have used batches of collagenase with very low proteolytic enzyme activities and this might explain why collagenase isolated sinusoidal cells are reported to be able to bind or endocytose, for instance, formaldehyde denatured albumin (Nilsson & Berg, 1977; Eskild & Berg, 1982), lipases (Jansen et al., 1980), HDL (Drevon et al., 1977; Wandel et al., 1981) and galactose coated particles (Kolb et al., 1979; Schlepper-Schäfer et al., 1980).

In our hands, the endocytic capacity of collagenase isolated cells was preserved in a selective way. Of the three substances tested, only colloidal albumin was effectively endocytosed. This selective endocytic capacity might be due to a selective effect of the proteolytic enzymes in the collagenase preparation on different binding sites on the cell membrane.

Apart from the proteolytic effect, the isolation by the use of collagenase might also influence the endocytic capacity through other mechanisms such as a Ca^{++} depletion during the perfusion of the liver, a different cellular composition of the sinusoidal cell preparation and the ingestion of cellular debris (see Chapter 2).

It is not completely clear why freshly cold pronase isolated cells are immediately able to bind and endocytose various substances. It is possible that the proteolytic activity of the pronase preparation might affect the cell membranes much less at 10°C than at 37°C. Stahl et al. (1980) incubated alveolar macrophages with pronase or trypsin at 4°C and found that after rewarming to 37°C the maximum rate of endocytosis by these cells was only slightly slower than before the trypsin treatment. Moreover, the affinity of the cells for the added substances remained exactly the same. These results also indicate that the effect of a proteolytic enzyme treatment on the endocytic capacity of cells is not as dramatic at a low temperature as at 37°C.

Although retention of the endocytic capacity in cells isolated by various methods has been demonstrated, one should be aware that due to the proteolytic enzymes used during the isolation the number of binding sites might vary for each isolation procedure and probably will be smaller than in vivo. Thus, interpretations of calculated numbers of binding sites on isolated cells should be done with great caution. In addition, the number of binding sites, which is commonly determined at 4-10°C (Steer & Ashwell, 1980; Tolleshaug,

1981), might vary at different temperatures (Weigel, 1980). For this reason, no attempt was made here to calculate the number of binding sites on the cells for the various test substances.

When cold pronase isolated sinusoidal cells were incubated with colloidal albumin, AGOR or AHOR, the increase in cellular uptake had already reached a plateau after 15 to 30 min. Such a plateau in the uptake has been observed with various cell types and substances (Nilsson & Berg, 1977; Stahl et al., 1980; Maynard & Baenziger, 1981; Summerfield et al., 1982). This has been generally ascribed to the rapid degradation of the endocytosed substances and excretion of the degradation products from the cells. On the other hand, a plateau might also be found if the endocytic capacity of the cells diminishes after a certain incubation period. The latter is probably not the case for cold pronase isolated cells, since it was shown at the ultrastructural level that they were still able to endocytose HRP after an incubation period of 30 min (Fig. 23e). This is likely to occur for the other substances as well. Since the capacities of Kupffer and endothelial cells to very rapidly degrade AGOR, AGOR and colloidal albumin had already been demonstrated in vivo (see Section 2.3.4.1), the lesser increase in the amount endocytosed in vitro was probably due to degradation. This indicates that the cold pronase isolated cells may also be useful for studies on intracellular digestion.

Under optimal incubation conditions, the specificity of the endocytic process in cold pronase isolated sinusoidal cells was demonstrated by the dependence of the uptake on temperature and substance concentration. For the uptake of colloidal albumin, an apparent K_M of 66.4 µg per ml and a V_{max} of 342 ng per 10^6 cells per 5 min were found. For warm pronase isolated and subsequently cultured Kupffer cells, K_M and V_{max} values for colloidal albumin of 250 µg per ml and 17.2 µg per mg cellular protein per 30 min, respectively, have been reported (Brouwer & Knook, 1982). The latter value corresponds to about 315 ng per 10^6 cells per 5 min, which is very similar to that found for the cold pronase isolated sinusoidal cells.

For the uptake of AGOR, the apparent K_M and V_{max} were found to be 96.6 nM and 4.4×10^{-2} pmoles per 10^6 sinusoidal cells per min, respectively. Maynard & Baenziger (1981) reported a K_M of 140 nM for collagenase isolated sinusoidal cells. From their results, a V_{max} of 3.6×10^{-2} pmoles per 10^6 cells per min can be calculated. Summerfield et al. (1982) reported a K_M

for collagenase isolated endothelial cells of 290 nM and a V_{max} of 16×10^{-2} pmoles per 10^6 cells per min can be calculated from their results.

The K_M and V_{max} values found for AHOR in this study were 160 nM and 10.6×10^{-2} pmoles per 10^6 sinusoidal cells per min, respectively. Maynard & Baenziger (1981) reported a K_M of 110 nM and a V_{max} of 4.8×10^{-2} pmoles per 10^6 collagenase isolated sinusoidal cells per min can be calculated from their results. Steer et al. (1979) reported a K_M for collagenase isolated sinusoidal cells of 360 nM and a V_{max} of 2.1×10^{-2} pmoles per 10^6 cells per min can be calculated from their results. These different K_M and V_{max} values indicate the variability which can occur when different isolation methods and different rat strains and sexes are used.

With the cold pronase isolated sinusoidal cells, no uptake of PVP could be assessed due to the slow process of pinocytosis for this substance. Also, no uptake of heparin could be demonstrated. The same findings were made with collagenase isolated cells (Dr. F. Nagelkerke, Rotterdam, personal communication). It is known that heparin binds to a variety of substances in the blood (see Section 3.4) and it is possible that the uptake of heparin in vivo is mediated by a factor which is not present in the FCS used in our in vitro study or in the human serum used by Nagelkerke.

After isolation by the cold pronase method, sinusoidal cells were further purified into Kupffer and endothelial cells to determine the difference in endocytic capacity between the two cell types. Colloidal albumin, AGOR and AHOR were endocytosed much better by endothelial cells than by Kupffer cells. In contrast, the difference in the endocytic capacity between the two cell types for AHOR and colloidal albumin in vivo (see Table XIV) was smaller. The uptake of AGOR by isolated Kupffer cells was very moderate, if occurring at all. This phenomenon has also been described for collagenase isolated cells by Summerfield et al. (1982), although these authors published completely opposite results in a subsequent article (Parise et al., 1982). The uptake of AGOR and AHOR by warm pronase isolated and subsequently cultured Kupffer cells resulted in endocytic capacities of 340 and 370 ml per g cellular protein per day, respectively, which corresponds to about 37 and 41 µl per 10^6 cells per day (Brouwer et al., 1982). Compared with the values in Table XIX, these cultured Kupffer cells endocytosed AGOR at the same moderate rate and their capacity to endocytose AHOR was at least 6 times less than that of cold pronase isolated Kupffer cells.

The endocytic capacity of isolated endothelial cells seems to be more preserved than that of Kupffer cells when compared with the in vivo situa-

tion. At a concentration of 2 µg AGOR per ml, Summerfield et al. (1982) found an uptake of 5 ng per 10^6 cells per 5 min by collagenase isolated endothelial cells. This corresponds to an endocytic index of 720 µl per 10^6 cells per day, which is very comparable to the index of 709 found for cold pronase isolated endothelial cells (Table XIX). Endocytosis of AGOR by endothelial cells in vivo was nevertheless more than 3 times higher (Table XIV). A difference of the same order of magnitude between in vivo and in vitro endocytosis has also been reported for the uptake of formaldehyde denatured albumin by collagenase isolated sinusoidal cells (Nilsson & Berg, 1977).

Although the in vitro system might not completely reflect the in vivo situation, ultrastructural demonstration of the endocytic capacities of purified Kupffer and endothelial cells for HRP, colloidal silver, colloidal carbon and latex beads has shown that the mechanisms of internalization and the intracellular fate of the substances remains qualitatively equivalent to the in vivo situation, as will be discussed in more detail in Chapter 5. As in vivo, isolated endothelial cells were able to endocytose both of the colloids as well as latex beads of 0.1 µm diameter. This is of practical importance, since the uptake of latex beads or colloids is often used to estimate the percentage of Kupffer cells in isolated cell preparations (Barranger et al., 1978; Lukomska & Olszewski, 1978; Ullrich et al., 1979; Kolb et al., 1980b). Especially, identification of Kupffer cells by the uptake of latex beads in vitro is very dubious, since endothelial cells in vitro appeared to be able to also endocytose larger latex beads of up to 0.65 µm diameter. In vivo, uptake of latex beads as large as 0.31 µm has been observed in endothelial cells from the mouse liver (De Leeuw et al., 1982a), but not in those cells from the rat (see Section 3.3.5).

The in vitro system described in this chapter makes it possible to study the different stages in the endocytic process and the intracellular digestion of various substances in Kupffer and endothelial cells. The fact that cold pronase isolated cells are active in endocytosis while warm pronase isolated cells are not might be helpful in obtaining more information on the mechanism by which substances are endocytosed (see Chapter 5). Besides, the large number of cells that can be isolated from a single liver makes it possible to study several aspects of the endocytic mechanism in the same experiment.

CHAPTER 5

ENDOCYTIC MECHANISMS IN KUPFFER AND ENDOTHELIAL CELLS

5.1 INTRODUCTION

Evidence has been presented in the preceding chapters which shows that both Kupffer and endothelial cells have the capacity to endocytose various RES test substances in vivo (Chapter 3) and in vitro (Chapter 4). The mechanisms by which the cells take up these various substances have not yet been extensively discussed. As pointed out in Section 1.4, the different endocytic mechanisms can be distinguished at three levels: 1) the events that occur at the cell membrane, i.e., binding of the test substance or not; 2) the mechanism of internalization, i.e., pinocytosis versus phagocytosis and 3) the intracellular transport of the endocytosed material. The first two steps determine the initial rate at which a substance is endocytosed, whereas the third step determines the fate of a substance in the cell. It has been discussed earlier that most test substances are endocytosed very effectively when compared with the marker for fluid-phase pinocytosis, PVP, and that their uptake shows saturation kinetics (see Sections 3.3 and 4.3). These observations indicate a high affinity binding of the test substances to the cell membrane and a rapid subsequent internalization. Specific recognition sites may be present on the cell membrane for the binding of the substances.

The various endocytic processes in Kupffer and endothelial cells will be described in more detail in this chapter. To obtain a better impression of the individual steps involved in the uptake of the various test substances, the process was followed at the ultrastructural level, by the use of isolated cells. The results obtained were supplemented by those found in vivo or during a perfusion of the liver in situ. In addition, the effect of inhibitors of various specific cellular processes on in vitro endocytosis was studied semi-quantitatively (i.e., at the ultrastructural level) as well as quantitatively. For the in vitro experiments, cells isolated by the cold pronase method were used, since they were most suitable for studies on endocytosis (see Section 4.3).

5.2 MATERIALS AND METHODS

5.2.1 Reagents

The test substances used were described under Section 3.2.1. The following inhibitors of endocytosis were used: mannan (Baker's yeast), antimycin A, cytochalasin B, colchicine, chloroquine (all obtained from Sigma Chemical Co.), EDTA (Sodium salt) (Fluka AG), sodium fluoride (UCB) and dimethylsulphoxide (DMSO) (BDH).

5.2.2 Isolation and incubation of sinusoidal cells

Sinusoidal cells were prepared by the cold pronase method as described under Section 2.2.3. Incubations of the cells with the test substances were performed as outlined in Section 4.2.3.

5.2.3 *In situ* perfusion of the liver with electron dense substances

Livers were perfused through the portal vein (5 min, 37°C) with GBSS (usually containing 1.3% BSA and 17.5% FCS) containing either HRP (125 µg per ml), colloidal silver (2 mg per ml), colloidal carbon (2.24 mg per ml) or latex beads of various sizes (0.1% solids). Perfusion with HRP was also done for shorter time intervals, sometimes followed by a further perfusion with HRP for 45 sec at 4°C. After perfusion with the test substances, the livers were perfused with a physiological salt solution (1.5 min, 4°C), then perfused with 2% glutaraldehyde (4°C) and processed for electron microscopy (see Section 2.2.5). In case of HRP, fixed liver slices were incubated for peroxidase staining (see Section 2.2.6) before further processing.

5.3 RESULTS

5.3.1 Ultrastructural studies on endocytic mechanisms

Ultrastructural studies on the three successive steps of the endocytic process were mainly done *in vitro* by the use of cold pronase isolated cells. Additional results were obtained *in vivo* (see also Chapter 3) or during per-

fusion of the liver in situ.

The first step of endocytosis concerns the events at the cell membrane. It appeared that the extent of adherence of colloidal silver, colloidal carbon and latex beads to the cell membrane was greater in Kupffer cells than in endothelial cells. This was observed in vivo (see Section 3.3.5) as well as in the in situ situation. Adherence in vitro was probably disrupted due to extensive washing of the cells during processing for electron microscopy. Binding of HRP was difficult to demonstrate under all three experimental conditions (i.e., in vivo, in situ and in vitro). This was probably due to rapid subsequent internalization of the substance. However, when in situ experiments with HRP were performed at 4°C and the cells were immediately fixed, the internalization step was inhibited and binding could be demonstrated (Fig. 27). In Kupffer cells, bound HRP was present only in invaginations of the cell membrane (Fig. 27b). In endothelial cells, it was also present on the other parts of the cell membrane (Fig. 27a).

The next step in endocytosis is internalization. Under all three experimental conditions and for all substances tested, only one mechanism of internalization was observed in endothelial cells, namely, by bristle-coated pits (Figs. 27a,28). After incubation with HRP, all bristle-coated pits present were actually filled with the substance. In endothelial cells, bristle-coated pits have a diameter of about 0.15 µm. One would suppose that the diameter of these pits would limit the size of a particle that can enter the cell by this mechanism. Nevertheless, latex beads of wider diameter than the pits could be taken up by endothelial cells (see also Chapter 4). We observed that several pits were acting together when these larger beads were internalized (Fig. 28c). This phenomenon will be referred to as "multiple-coated pits".

In Kupffer cells, two mechanisms of internalization were observed. Colloidal silver, colloidal carbon and latex beads of $\leq$ 0.23 µm diameter were internalized by both bristle-coated pits, sometimes multiple ones, and phagocytosis (Fig. 29). Latex beads of $\geq$ 0.29 µm diameter were taken up only by phagocytosis. The phagocytosis of latex beads occurred by engulfment of one or a few particles with lamellipodia. During the phagocytosis of colloids, lamellipodia formed elongated vesicles by enclosing parts of the cell membrane to which several colloid particles were attached (Fig. 29a). HRP was internalized by Kupffer cells solely by bristle-coated pits (Fig. 27b). Uptake in Kupffer cells by so called worm-like structures (see Section 1.3) was not observed for any of the substances.

The internalization step results in the formation of a vesicle which con-

tains the substance. In the case of internalization by coated pits, a bristle-coated micropinocytic vesicle having the same diameter as that of the pit appeared to be formed (Figs. 28,29). When large particles (i.e., latex) were taken up by bristle-coated micropinocytosis or phagocytosis, the cell membrane of the vesicles enclosed the particles more tightly than in case of the smaller colloidal particles (Fig. 29). This indicates that the amount of extracellular fluid captured within the vesicles varies with the size of both the vesicle and the endocytosed substance.

After the formation of the vesicles, the substance will be further transported into the cell. In both cell types and under all three experimental conditions, large smooth-walled vesicles (average diameter of 0.7 µm) containing internalized substances were observed after incubation periods of 5 to 30 min (Figs. 28,29). In Kupffer cells, large vesicles possessing a fuzzy-coated membrane were sometimes observed (Fig. 29c). Both in vivo and in situ, the large vesicles (which might have been derived from the smaller vesicles by fusion) were observed at the periphery of the cell but also frequently — in contrast to the smaller newly internalized vesicles — deeper inside the cell. In the cases of colloidal silver and carbon, these vesicles were either completely filled with the substance or only a rim of substance was observed along the inside of the vesicle wall (Figs. 28,29).

For HRP, a more detailed time course study of the intracellular transport was made in vitro as well as in situ. Isolated cells were incubated with HRP at 37°C for only 30 seconds, after which they were chilled (4°C) and extracellular HRP was washed away. The cells were then postincubated for various time intervals. It appeared that the 30 second incubation time was too short to detect any HRP inside Kupffer cells. Endothelial cells, however, had already taken up detectable amounts of HRP which were present only in bristle-coated vesicles (about 0.15 µm; Fig.30a) by that time. After a post-

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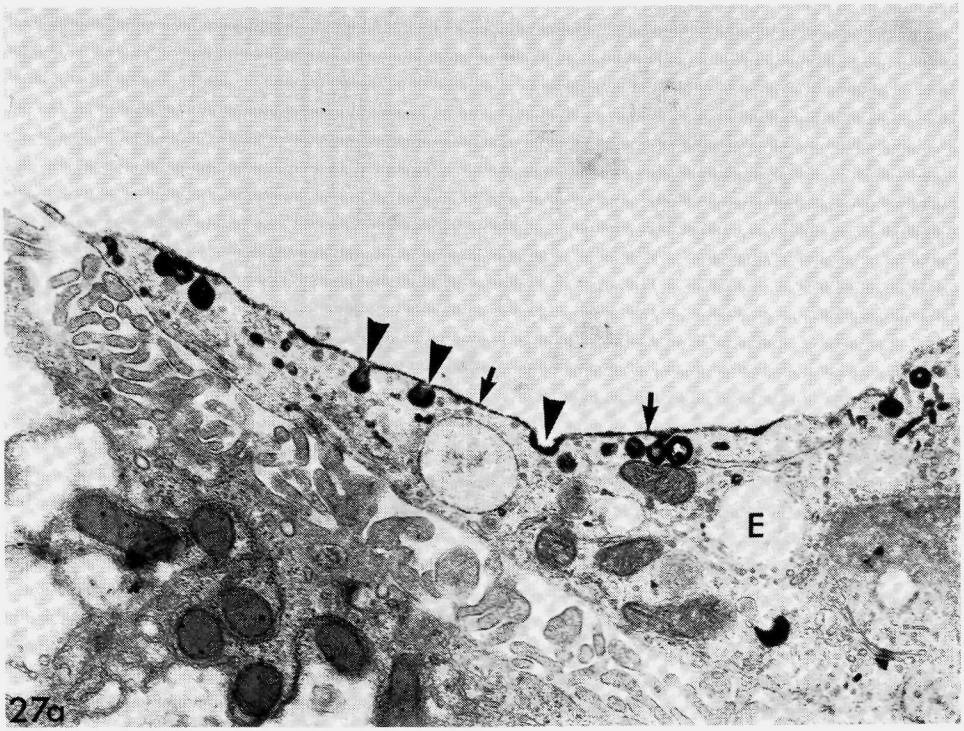
Figure 27 Binding of HRP to the membrane of sinusoidal cells. HRP (125 µg per ml) was perfused through the liver in situ (see Section 5.2.3) for 15 sec at 37°C and subsequently for 45 sec at 4°C. After a 30 sec postperfusion with GBSS (4°C), the liver was directly fixed.
a) At the cell membrane of the endothelial cell (E), HRP is evenly distributed (arrows) and present in invaginations (arrowheads), the bristle-coated pits. (18,000x).
b) On the membrane of a Kupffer cell (K), bound HRP is found exclusively in bristle-coated invaginations of the membrane (arrows). The Kupffer cell is also stained for endogenous peroxidatic activity. (20,000x).



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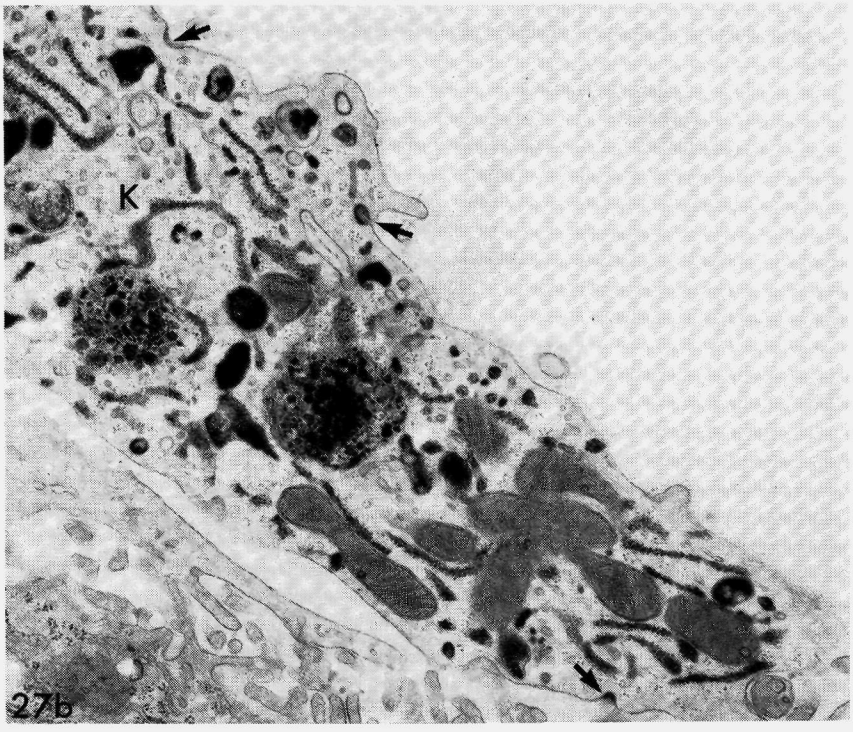


27b

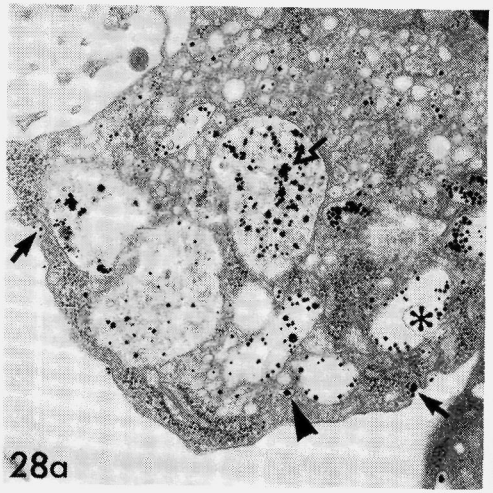
incubation of 15 seconds, almost all of these small vesicles had fused to form larger smooth walled ones (about 0.7 µm; Fig.30b). The HRP was present only along the wall of such vesicles. After a 10 or 30 min period of postincubation, the HRP was found to be distributed all over the vesicle (Fig.30c).

When HRP was added for 15 seconds during an *in situ* perfusion, the same situation as in isolated endothelial cells was found, i.e., most of the HRP was present in bristle-coated vesicles (Fig. 31a). After a perfusion of 2 minutes with HRP, much larger vesicles were also observed (Fig. 31b). Long thin tubules filled with HRP were frequently observed (Fig. 31b) both *in situ* and *in vivo*. These structures were seldom seen *in vitro*. Some *in situ* observations suggest that these tubules may fuse with larger vesicles (Fig. 31b). The presence of endocytosed substance in tubular structures was found only for the uptake of HRP.

To approach the question of whether sites of internalization and types of vesicles were specific for a certain substance, all possible combinations of two test substances were added simultaneously to isolated sinusoidal cells.

Figure 28 Bristle-coated pits and intracellular vesicles observed during the uptake of substances by endothelial cells.
a) colloidal silver, *in vitro* (see Section 5.2.2). (17,700x).
b) colloidal carbon, *in vivo* (see Section 3.2.3). (24,300x).
c) latex beads (Ø 0.23 µm), *in situ* (see Section 5.2.3). (33,800x).
Under all three experimental conditions, all substances are internalized by bristle-coated pits (arrows). Multiple-coated pits are formed during internalization of latex beads. The internalization process results in formation of bristle-coated micropinocytic vesicles (arrowheads). Large smooth-walled vesicles in which substance is present along the border of the vesicle wall (asterisks) or distributed over the vesicle content (open arrow) may have been formed from the smaller vesicles by fusion.

Figure 29 Bristle-coated pits, phagocytosis and intracellular vesicles observed during uptake of substances by Kupffer cells.
a) colloidal silver, *in situ* (see Section 5.2.3). (18,300x).
b) colloidal carbon, *in vivo* (see Section 3.2.2). (24,800x).
c) latex beads (Ø 0.23 µm), *in vivo* (see Section 3.2.2). (15,900x).
inset) Latex beads (Ø 0.65 µm), *in vitro* (see Section 5.2.2). (12,200x).
Under all three experimental conditions, all substances are internalized by bristle-coated pits (arrows) or phagocytosis. During phagocytosis of colloids, elongated vesicles are formed by lamellipodia (open arrows). During phagocytosis of latex beads, lamellipodia move tightly around the particle (inset). Internalization by bristle-coated pits results in formation of bristle-coated micropinocytic vesicles (arrowheads). Large smooth-walled vesicles (open arrowheads) may have been formed from the smaller ones by fusion. On some occasions, a large fuzzy-coated vesicle containing endocytosed substance is observed (asteriks).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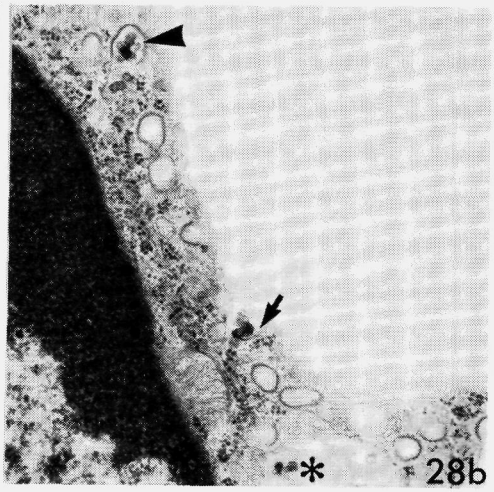
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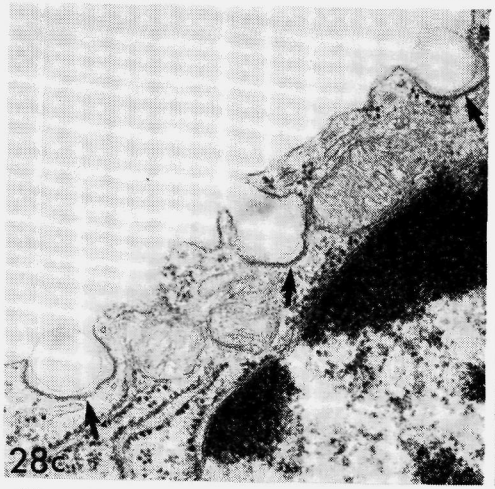
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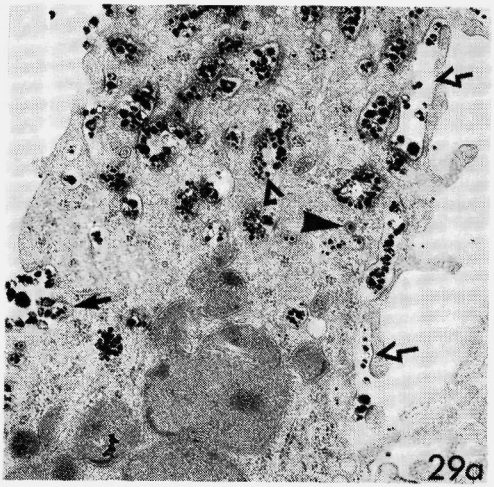
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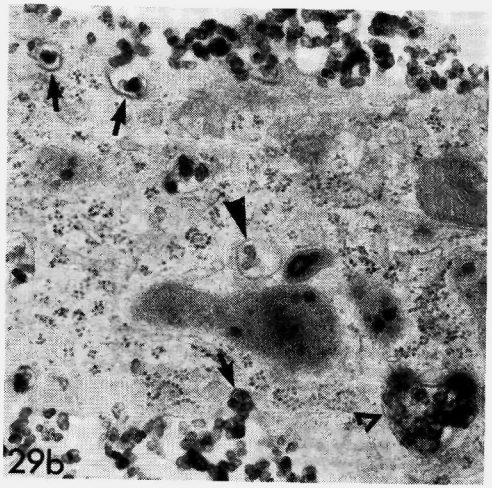
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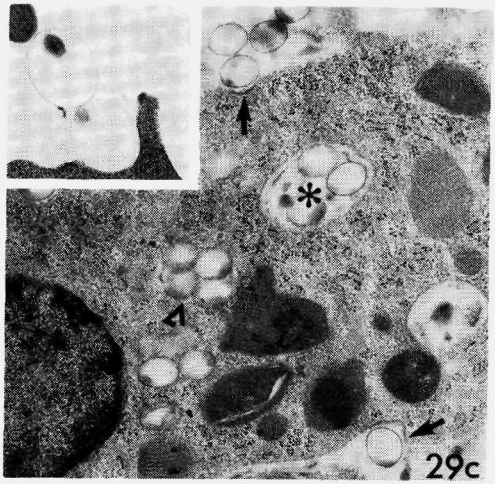
29c

For any combination of the test substances, large smooth-walled vesicles containing both substances were formed, indicating that their intracellular transport occurred, at least for some time, in the same organelles (Fig. 32).

When colloidal carbon or latex beads of 0.65 µm diameter were injected and the liver was perfused 5 min later with latex beads of 0.23 µm diameter, endothelial cells appeared to have internalized these small beads more efficiently as compared with a perfusion with 0.23 µm latex beads only. Kupffer cells were also still active in the endocytosis of these small beads (Figs. 33,34). Although colloidal carbon and the large beads were introduced into the cells earlier, Kupffer cells were still in the process of internalization of aggregated clumps of colloidal carbon or some of the large beads, while some small beads were already present in intracellular vesicles (Fig. 34).

A striking finding was that, during incubation with HRP, several Kupffer cells were active in phagocytosing each other or other cell types (Fig. 35). This "cannibalism" was observed in vivo as well as in vitro.

5.3.2 Inhibition of endocytic processes in vitro

The effects of various compounds on the endocytic process were studied with isolated cells both semiquantitatively (i.e., ultrastructurally) and quan-

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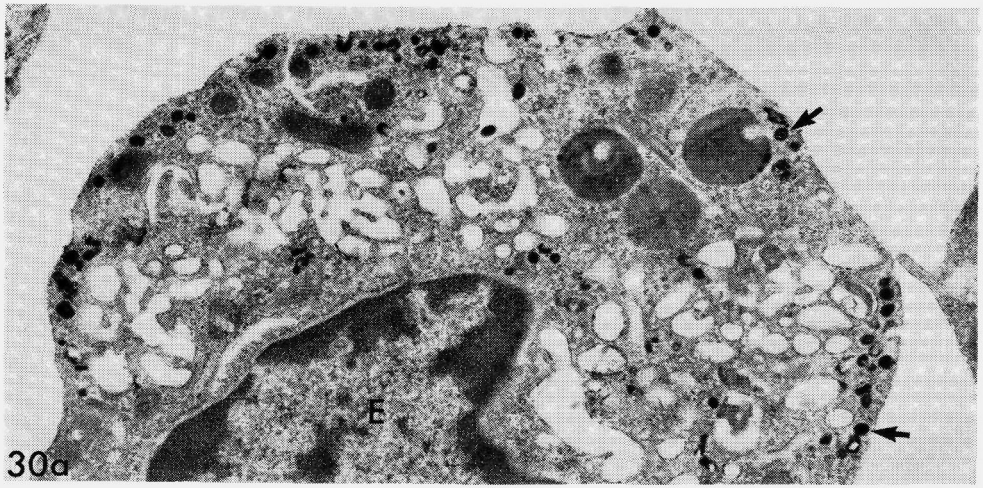
Figure 30 Intracellular transport of HRP after endocytosis by isolated cells (see Section 5.2.2).
a) After incubation with HRP (125 µg per ml) at 37°C for 30 sec, most of the HRP is observed in bristle-coated micropinocytic vesicles (arrows). (15,600x).
b) Cells were incubated with HRP as in a). They were then chilled and extracellular HRP was washed away. After a subsequent postincubation (15 sec, 37°C, without HRP), HRP is observed mainly in large smooth-walled vesicles (arrows), bound to the border of the vesicles. Most bristle-coated vesicles have disappeared. (8,430x).
c) Cells were treated as in b); however, they were postincubated for 30 min. Most of the HRP is present in large smooth-walled vesicles (arrows) in which it is evenly distributed. (10,400x).

Figure 31 Intracellular transport of HRP after endocytosis by endothelial cells in situ (see Section 5.2.3).
a) After perfusion of the liver with HRP (125 µg per ml) for 15 sec (37°C), HRP is observed mainly in bristle-coated micropinocytic vesicles (arrows). (8,430x).
b) After perfusion of the liver with HRP for 2 min (conditions as in a), the substance is observed in large smooth-walled vesicles (arrows) and tubular structures (arrowheads). Tubules and large vesicles are often found in close association (open arrows) and in continuity with each other (open arrowheads). (10,700x).



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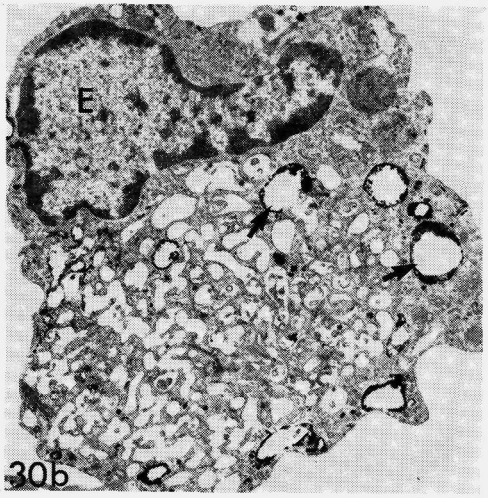

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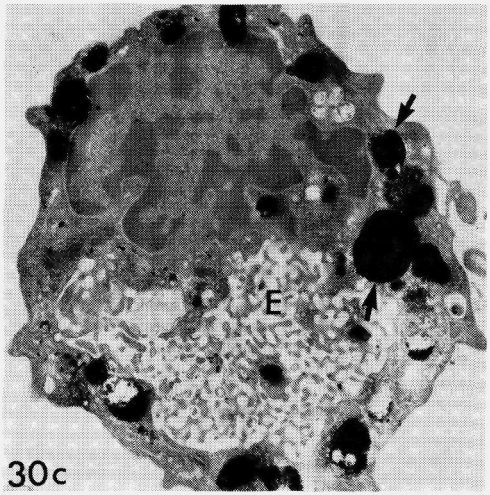

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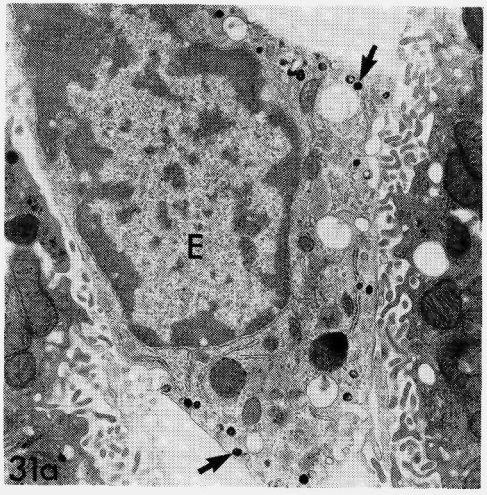

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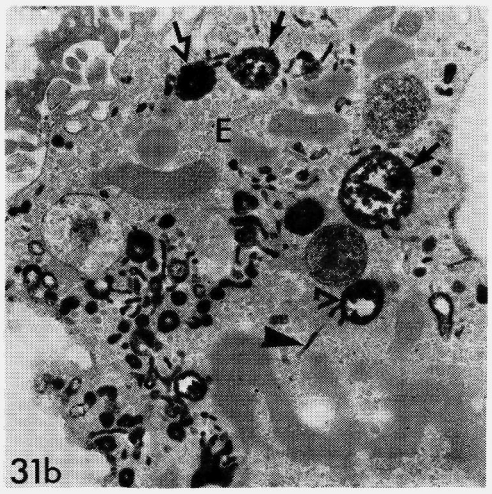

31b

titatively. For the quantitative studies, no discrimination was made between Kupffer and endothelial cells. The number of endothelial cells in the sinusoidal cell preparations used for these studies was about 3-4 times greater than that of Kupffer cells (see Section 2.3.2) and the capacity of endothelial cells to endocytose colloidal albumin, AGOR and AHOR was about 3, 9 and 2 times higher, respectively (see Table XIX). Thus, the contribution of endothelial cells to the amount of endocytosis by sinusoidal cells was 6-36 times greater than that of the Kupffer cells. This means that Kupffer cells accounted for only 3-17% of the amount of substance endocytosed. Therefore, inhibition of endocytosis by a sinusoidal cell preparation mainly reflects inhibition of the process by endothelial cells. Whether inhibitory compounds also affect endocytosis by Kupffer cells may be claimed with certainty in these quantitative studies only if endocytosis by sinusoidal cells would have been less than 3-17% of the control values. This situation, however, was never fully reached.

In contrast to the quantitative studies, the semiquantitative ultrastructural studies allowed discrimination between Kupffer and endothelial cells in the sinusoidal cell preparation. These studies showed that both cell types reacted in the same way when inhibitory compounds were added. Since some of the semiquantitative inhibition studies were performed with HRP, a substance which is endocytosed by the same mechanism as is AGOR and AHOR (see below), it can be presumed that the quantitative inhibition studies on endocytosis of AGOR and AHOR by sinusoidal cells reflect what happens in both endothelial and Kupffer cells.

It is often assumed that serum factors (i.e., opsonins) are required for the endocytosis of a variety of substances, especially colloidal or particulate materials (see also Chapter 1). To maintain a long lasting viability of the

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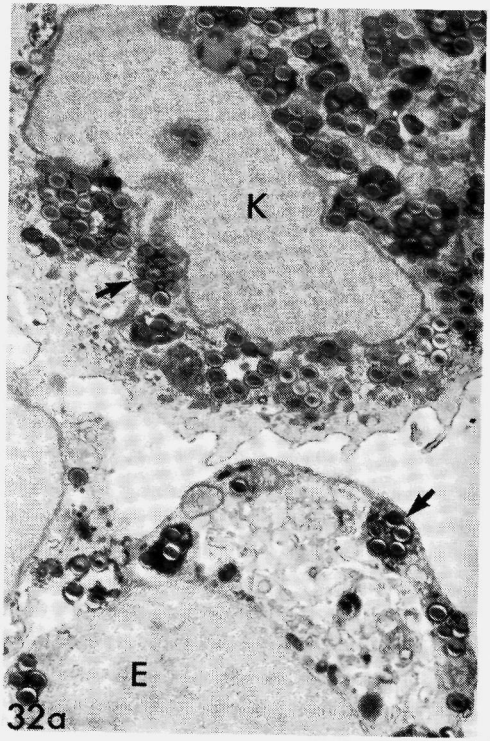
Figure 32 Simultaneous endocytosis of two test substances by Kupffer and endothelial cells after incubation in vitro (see Section 5.2.2).
a) HRP and latex beads (Ø 0.23 µm) in an endothelial (E) and a Kupffer (K) cell. (7,590x).
b) HRP and colloidal silver in an endothelial cell.
c) Colloidal silver and latex beads (Ø 0.23 µm) in a Kupffer cell. (18,300x).
d) Colloidal carbon and latex beads (Ø 0.23 µm) in a Kupffer cell. (12,800x).
After internalization of two different substances, they are frequently observed together in the same smooth-walled vesicles (arrows).



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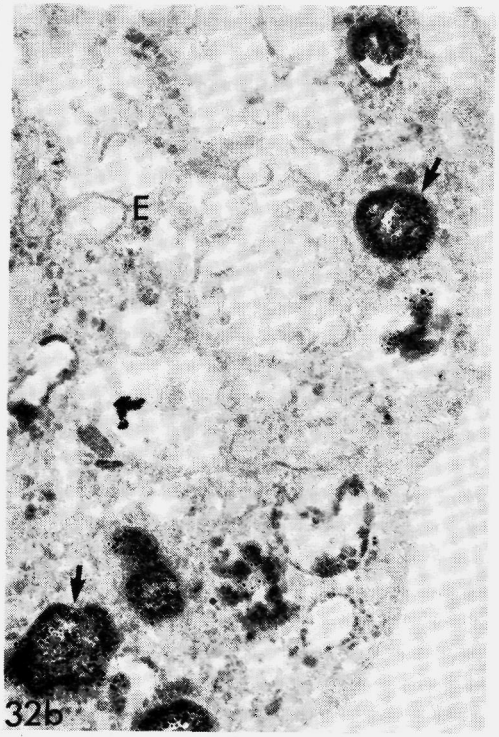

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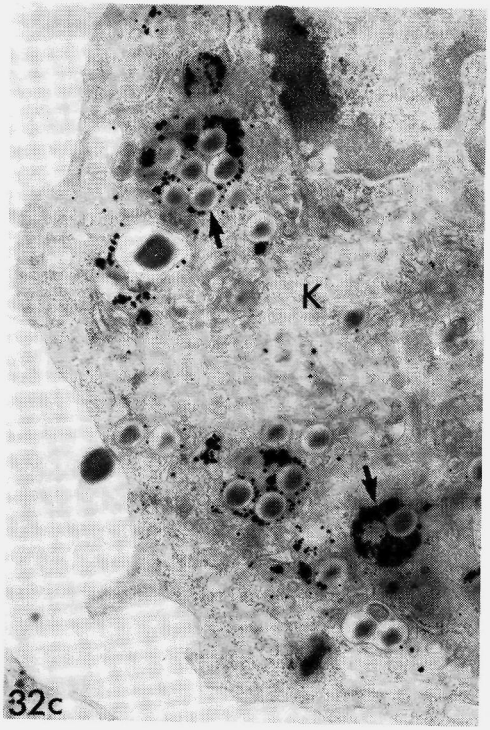

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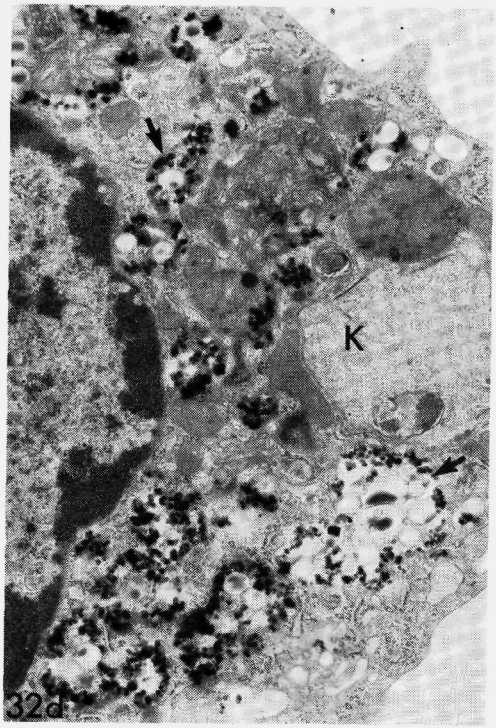

32d

cells and to prevent their clumping, BSA and FCS were generally added to the incubation media. However, BSA, FCS or homologous rat serum (not shown) appeared to inhibit endocytosis to a certain extent, since most substances were endocytosed more actively in the absence of both of these factors (Table XVII, Figs. 36 and 37). For verification and exclusion of isolation artifacts, these experiments were repeated in an in situ liver perfusion system. The results obtained were virtually the same (Figs. 36 and 37).

The effect of pronase (at 37°C) on the endocytic capacity of the cells for colloidal albumin, AGOR and AHOR (Table XXI) was discussed earlier (Section 4.3.1). The results in Table XXII and Fig. 38 show that warm pronase isolated cells also show a diminished uptake of HRP, colloidal silver and colloidal carbon, whereas endocytosis of latex beads was not influenced.

The polysaccharide mannan is thought to bind to specific mannose recognition sites present on the membrane of a variety of cells (Achord et al., 1977; Steer et al., 1979; Ullrich et al., 1979; Shepherd et al., 1981; Sung et al., 1983). When mannan was added to the incubation medium, the uptake of colloidal albumin by the cells was unchanged whereas the endocytosis of AGOR, AHOR (Table XXI) and HRP (Table XXII, Fig. 39) was greatly lessened. Other glycoproteins known to possess mannose or N-acetylglucosamine

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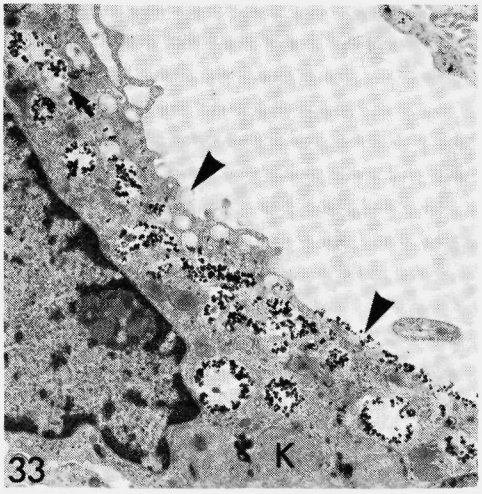
Figure 33 and 34. In situ endocytosis of latex beads (Ø 0.23 µm) after loading of the Kupffer cells in vivo.

Figure 33 Kupffer cells were loaded in vivo with colloidal carbon (see Section 3.2.2) for 5 min. The liver was then perfused in situ with latex-containing medium (0.1% solids, 37°C) for 5 min. Kupffer cells are seen to still be very active in the endocytosis of latex beads. In some smooth-walled vesicles, both colloidal carbon and latex beads are observed (arrows). Some clumps of aggregated colloidal carbon and latex beads were still attached — apparently each to specific regions — to the cell membrane (arrowheads). (7,650x).

Figure 34 Kupffer cells were loaded in vivo with latex beads (Ø 0.65 µm) (see Section 3.2.2) for 5 min. The liver was then perfused in situ as described in Fig. 33. Kupffer cells are seen to still be active in the endocytosis of the smaller beads. Both small and large beads are sometimes present in the same smooth-walled vesicles (arrows). Small as well as large beads are still attached — apparently each to specific regions — to the cell membrane (arrowheads). (7,830x).

Figure 35 Cannibalism by a Kupffer cell.
A Kupffer cell after incubation with HRP (125 µg per ml; 37°C) for 30 min. The cell seems to have ingested another complete Kupffer cell. The space between the two cells is marked by a small rim of HRP (arrows). (8,960x).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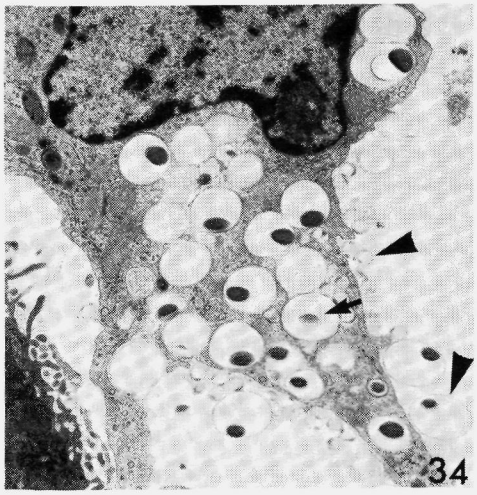
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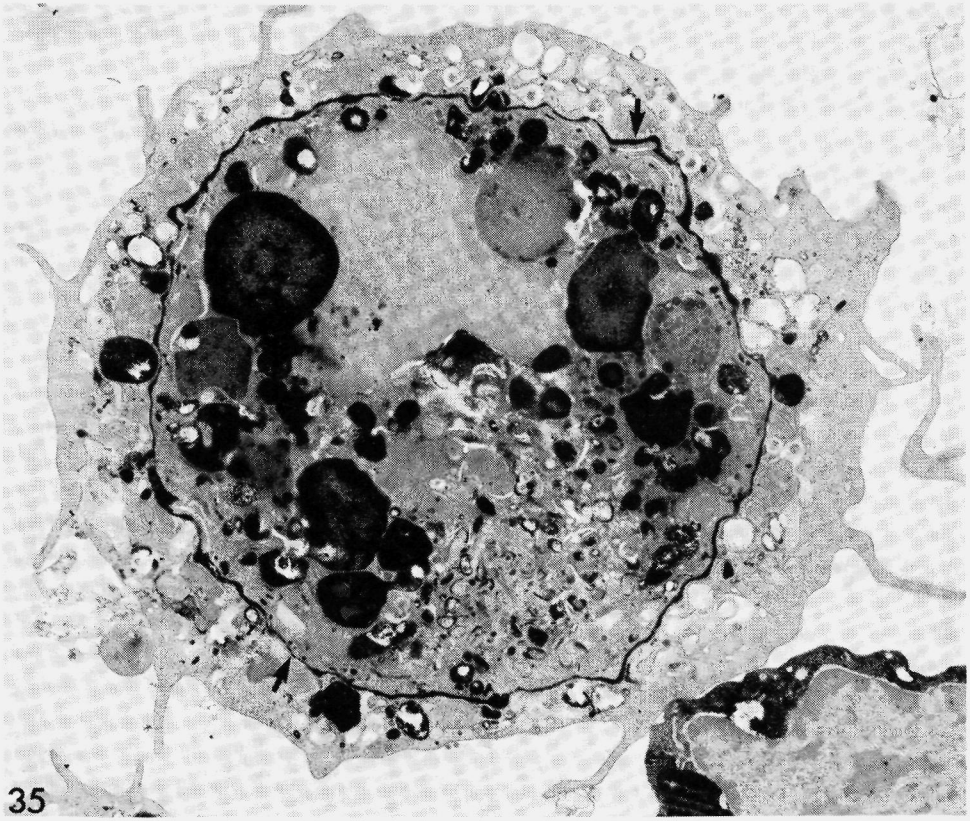
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TABLE XXI

EFFECT OF INHIBITORY COMPOUNDS ON QUANTITATIVE BIOCHEMICAL ESTIMATIONS OF ENDOCYTOSIS

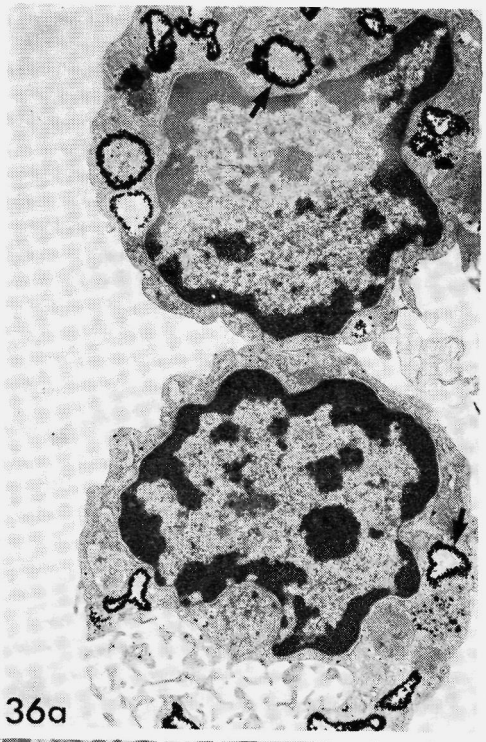
Inhibitory compound	Test substance		
	CA	AGOR	AHOR
Control	100	100	100
Pronase (0.2%)	11.9	5.9	3.3
Mannan (0.5 mg per ml)	105.8	22.0	9.9
EDTA (3.3 mg per ml)	75.8	22.4	9.7

Sinusoidal cell preparations were incubated with the test substances for 5 min as described in Section 4.2.3. Inhibitors were added at the start of the incubation period. Values are expressed as percentages of control values. Pronase refers to warm pronase isolated cells (see also Section 2.2.3). Mean values of 2 to 5 experiments. Abbreviations: CA: colloidal albumin; AGOR: agalactoorosomucoid; AHOR: ahexosaminoorosomucoid.

terminated oligosaccharide chains (like N-acetylglucosaminidase and ovalbumin) also reduced uptake of HRP; in contrast, addition of colloidal albumin (750 µg per ml) did not inhibit uptake of HRP (results not shown).

Figure 36 Endocytosis of colloidal silver (arrows) by endothelial cells in the absence of serum factors.
a) In vitro incubation (see Section 5.2.2) for 30 min. A generally higher uptake is observed when compared with incubation in presence of serum factors (see Fig. 24a). Most of the colloidal silver is present along the rim of large smooth-walled vesicles. Effective uptake is also observed in Kupffer cells (not shown). (10,300x).
b) The liver was preperfused in situ with GBSS for 5 min to remove the blood. Subsequently, it was perfused with colloidal silver (see Section 5.2.3) for 5 min. An effective uptake is observed in both endothelial and Kupffer (not shown) cells. (10,300x).

Figure 37 Endocytosis of latex beads (arrows) by endothelial cells in the absence of serum factors.
a) Latex beads of 0.23 µm diameter; in vitro incubation (see Section 5.2.2) for 30 min. A generally higher uptake is observed when compared with incubation in the presence of serum factors. (see Fig. 26c). Effective uptake is also observed in Kupffer cells (not shown). (9,690x).
b) The liver was preperfused in situ with GBSS for 5 min to remove the blood. Subsequently, it was perfused with latex beads (Ø 0.1 µm) (see Section 5.2.3) for 5 min. An effective uptake is observed in both endothelial and Kupffer (not shown) cells. (44,700x).



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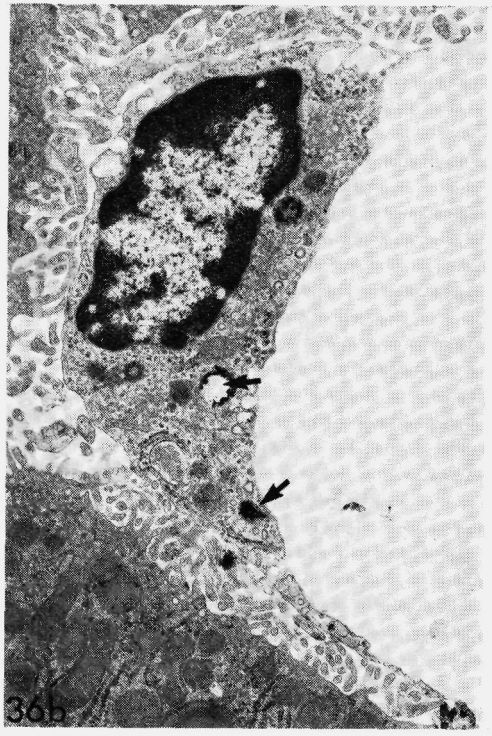

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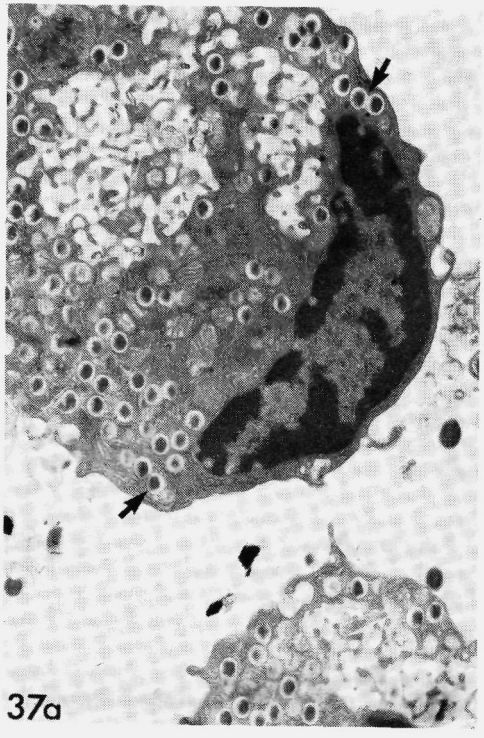

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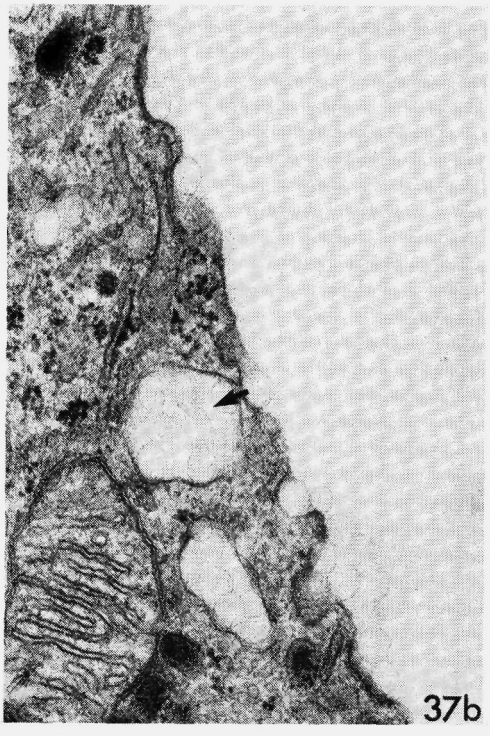

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TABLE XXII

EFFECT OF INHIBITORY COMPOUNDS ON SEMIQUANTITATIVE ULTRASTRUCTURAL ESTIMATIONS OF ENDOCYTOSIS

Inhibitory compound	Test substance			
	HRP	CS	CC	Latex*
Control	100	100	100	100
Pronase (0.2%)	0-10	0-10	0-10	100
Mannan (0.5 mg per ml)	0-10	n.d.	n.d.	n.d.
EDTA (3.3 mg per ml)	0-10	100	100	100

Sinusoidal cell preparations were incubated with the test substances for 30 min as described in Section 4.2.3. Inhibitors were added at the start of the incubation period. Values are expressed as percentages of control values. Pronase refers to warm pronase isolated cells (see also Section 2.2.3). Mean values of 2 to 5 experiments.
Abbreviations: HRP horseradish peroxidase; CS: colloidal silver; CC: colloidal carbon; n.d.: not determined. *Sizes of latex as described in Table XX.

Figure 38 Warm pronase isolated sinusoidal cells (see Section 2.2.2.1) after incubation with various test substances (see Section 5.2.2). For Kupffer (K) and endothelial (E) cells, the same results were obtained.

a) Incubation with HRP for 15 min. The amount of HRP present in the cells is negligible as compared with the cold pronase isolated cells (see Fig. 23a). The cell is still able to internalize extracellular fluid by bristle-coated micropinocytosis (arrow). In this way, small amounts of HRP can still enter the cell (arrowheads). (8,430x).

b) Incubation with colloidal silver for 30 min. The uptake is very low as compared with cold pronase isolated cells (see Fig. 24a). (8,300x).

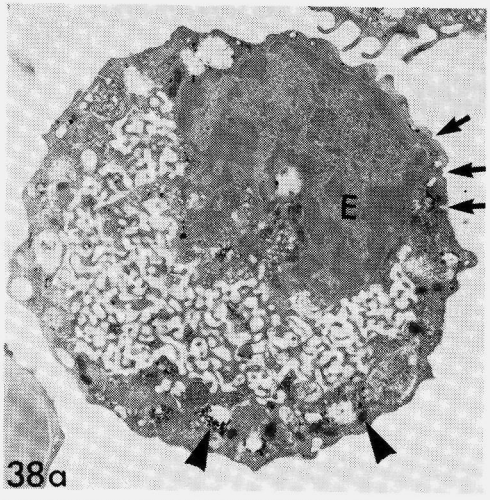
c) Incubation with colloidal carbon for 30 min. The uptake is very low as compared with cold pronase isolated cells (see Fig. 25b). (8,570x).

d) Incubation with latex beads (Ø 0.23 µm) for 30 min. Latex (arrow) is the only substance which is as actively endocytosed by warm pronase as by cold pronase (see Fig. 26c) isolated cells. (8,570x).

Figure 39 Effect of mannan on endocytosis of HRP by an isolated endothelial cell (conditions as described in Table XXII).

a) no mannan added. (9,410x).

b) 0.5 mg per ml mannan added. The uptake is strongly inhibited. The same is observed for Kupffer cells (not shown). (11,400x).



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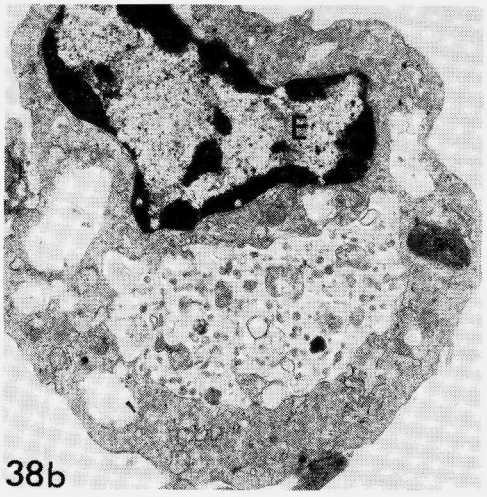

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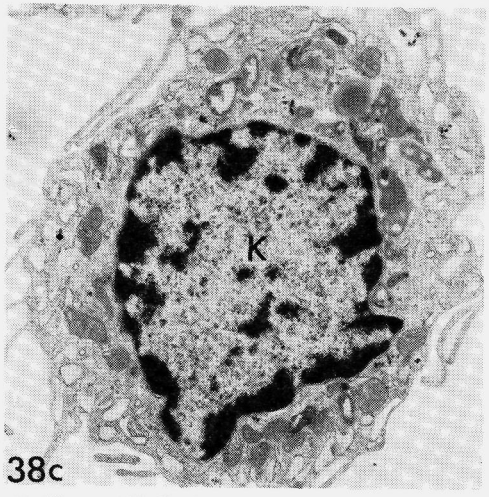

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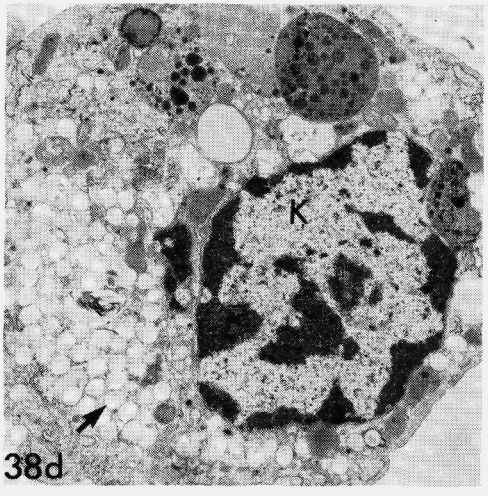

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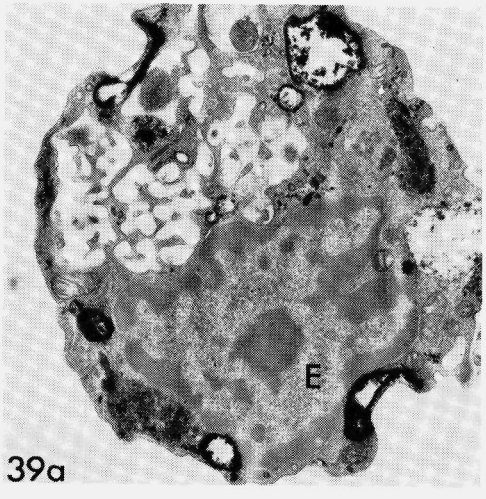

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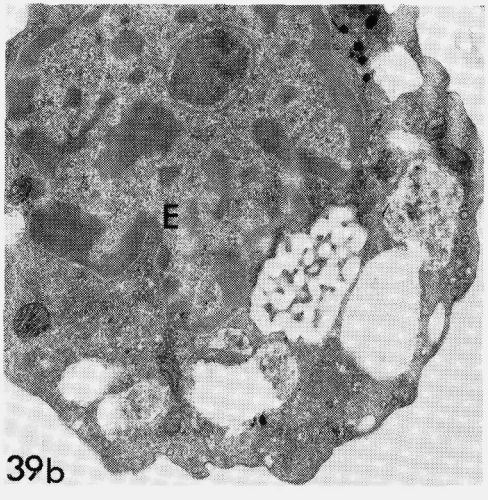


39b

The calcium dependence of the endocytic process was studied by the addition of EDTA to the incubation medium. Endocytosis of AGOR, AHOR and HRP was greatly reduced by EDTA (Tables XXI, XXII, Fig. 40), whereas it had only a moderate effect on the uptake of colloidal albumin and none on that of colloidal silver, colloidal carbon and latex (Fig.40; Tables XXI,XXII).

Table XXIII shows the effect of some metabolic and cytoskeletal inhibitors on the process of endocytosis. Antimycin A and sodium fluoride (NaF), inhibitors of mitochondrial respiration and glycolysis, respectively, both caused a decrease in the uptake of AGOR and AHOR. In the case of HRP, antimycin A had a very strong inhibitory effect on endocytosis by both Kupffer and endothelial cells (see also Fig. 41), but no effect of fluoride on the uptake of HRP could be detected. When both fluoride and antimycin A were present no further inhibition in the uptake of AGOR and AHOR occurred.

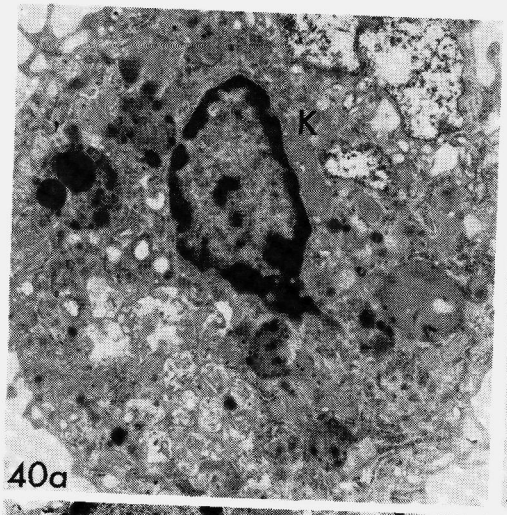
Treatment of the cells with cytochalasin B, which inhibits the action of microfilaments, resulted in a slight decrease in the uptake of AGOR and AHOR (Table XXIII). DMSO caused inhibition of the uptake of AHOR, but not of AGOR. DMSO might change some structural elements at the cell membrane necessary for the internalization step (Filosa & Fukui, 1981).

The possible involvement of microtubules in the endocytic process was investigated by incubation of the cells with colchicine. No decrease in the endocytosis of AGOR and AHOR was observed.

Figure 40 Effect of EDTA on endocytosis of various test substances by isolated sinusoidal cells. (conditions as described in Table XXII). For Kupffer (K) and endothelial (E) cells, the same results were obtained.

a) Endocytosis of HRP is greatly reduced as compared with the situation in the absence of EDTA (see Fig. 23). (8,640x).
b) EDTA has no visible effect on the uptake of colloidal silver (arrows) (compare with Fig. 24, where no EDTA was present). (10,900x).
c) Uptake of colloidal carbon (arrows) is the same in the presence or absence (Fig. 25) of EDTA. (7,430x).
d) Uptake of latex beads (Ø 0.23 µm) is also observed in the presence of EDTA (arrows) (compare with Fig. 26, where no EDTA was present). (10,500x).

Figure 41 Effect of antimycin A on endocytosis of HRP by isolated sinusoidal cells (conditions as described in Table XXIII). For Kupffer and endothelial (E) cells, the same results were obtained. When antimycin is added to the medium, endocytosis is greatly reduced (compare to the situation in which antimycin was absent in Fig. 23). HRP is present mainly in bristle-coated micropinocytic vesicles (arrows), which might not have been fully internalized. (7,370x).



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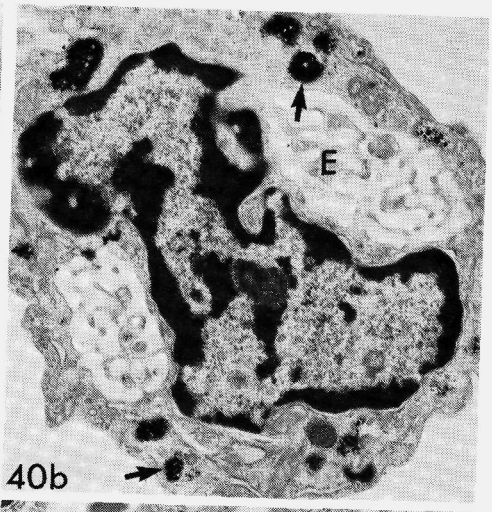

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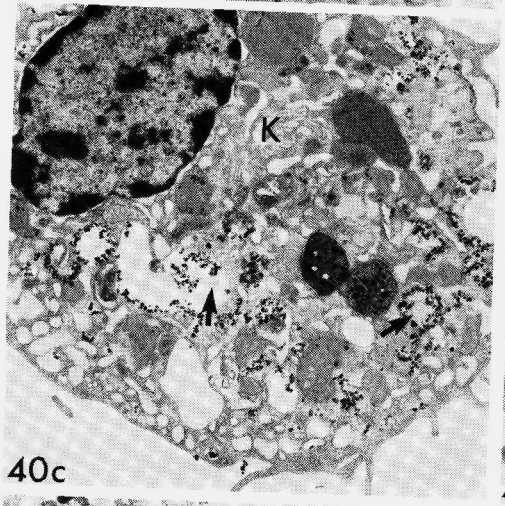

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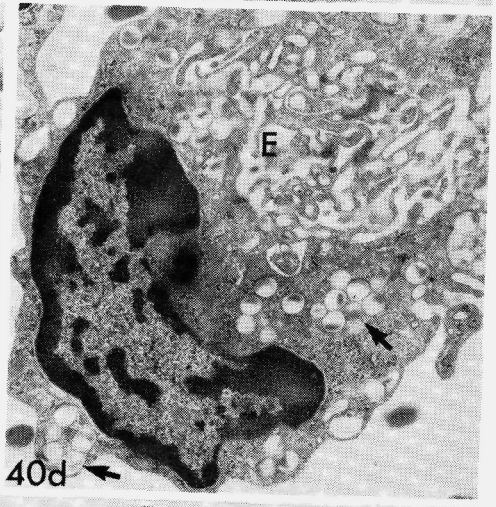

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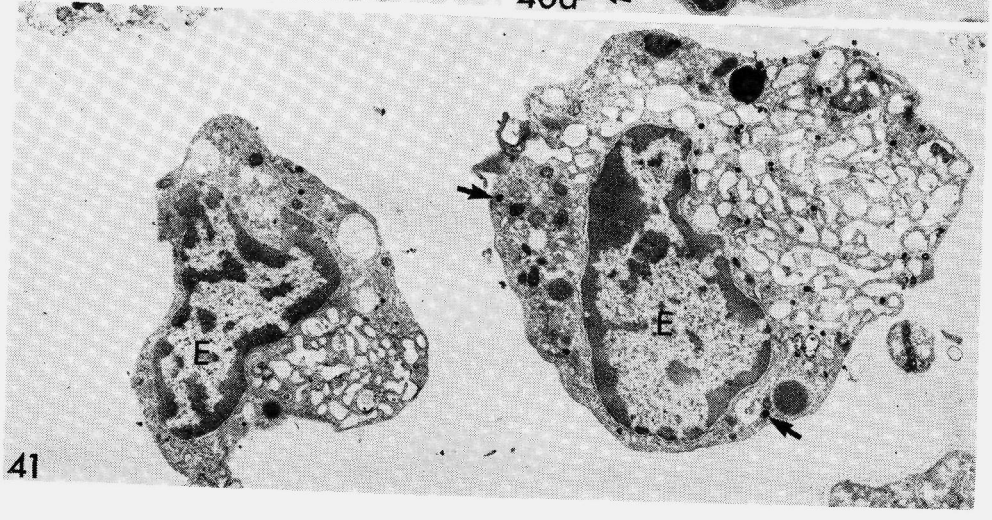

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TABLE XXIII

EFFECT OF METABOLIC AND CYTOSKELETAL INHIBITORS ON ENDOCYTOSIS

Inhibitor	AGOR	AHOR	HRP	CA*
Control	100	100	100	100
Antimycin A (0.4 mg per ml)	32.2	22.8	0-10	55.0
NaF (210 µg per ml)	77.4	76.6	100	76.0
NaF + antimycin A	23.3	31.6	0-10	29.0
Cytochalasin B (40 µg per ml)	84.5	80.8	n.d.	42.0
Colchicine (5 µg per ml)	92.3	106.3	n.d.	59.0
Chloroquine (50 µg per ml)	60.0	53.5	n.d.	n.d.
DMSO (10 µl per ml)	96.4	54.1	100	n.d.

Sinusoidal cell preparations were incubated with the test substances for 5-10 min as described in Section 4.2.3. Cells were preincubated with NaF or antimycin A for 10 min (37°C) and with cytochalasin B, colchicine or DMSO for 2 hours (4°C). For chloroquine, no preincubation was done. Antimycin was dissolved in alcohol (0.05% final concentration), NaF in sucrose (0.1% final concentration) and cytochalasin B in DMSO (1% final concentration). These solvents were also added to the control incubations. Values are expressed as percentages of control values. For abbreviations see Tables XXI and XXII. Mean values of 2 to 5 experiments. n.d.: not determined.
*values obtained with cultured Kupffer cells (Brouwer et al., 1980; 1982).

Besides an effect on the intracellular degradation of endocytosed substances, chloroquine may also influence the endocytic process itself (Attie et al., 1980). When it was added to the cells, a decrease in the uptake of AGOR and AHOR indeed occurred (Table XXIII).

As mentioned above, the effect of inhibitory substances was the same for Kupffer and endothelial cells as revealed by the ultrastructural studies. The effect of the inhibitors included in Table XXIII on the uptake of colloidal albumin has been studied in cultured Kupffer cells by Brouwer et al. (1980; 1982). For comparison, their results are included in Table XXIII. Some differences in the effect of the inhibitors on the uptake of colloidal albumin were observed as compared with the uptake of AGOR and AHOR. For colloidal albumin, there was an additive inhibitory effect when both fluoride and antimycin A were present. Moreover, cytochalasin B and colchicine resulted in a much stronger inhibition of the uptake of colloidal albumin than for AGOR and AHOR.

5.4 DISCUSSION

The different mechanisms of endocytosis in Kupffer and endothelial cells were characterized by ultrastructural examination and by use of various inhibitors or substances that influence the endocytic process. Ultrastructural studies had already indicated that endocytosis can be divided into three successive steps and that each of these steps might be mediated by a variety of mechanisms (see Chapter 1). It is questionable whether the use of inhibitors allows discrimination among the three steps or even among the different mechanisms by which these steps are performed. The monospecificity of inhibitors is not always guaranteed and one inhibitor might act on more than one mechanism and on more than one step. Furthermore, the steps involved in endocytosis are continuous and thus difficult to study separately.

By comparing the results obtained at the ultrastructural with those at the biochemical level and with some literature data, an attempt will be made here to relate the action of inhibitors to the observed mechanisms of endocytosis. Each separate step of the process will be considered successively.

5.4.1 Adsorption

All substances used in this study (except PVP) are adsorbed to the cell membrane, as can be deduced from quantitative determinations (Chapters 3 and 4) and ultrastructural investigations (Chapters 3 and 5) of the endocytic process. No visible adsorption of colloidal carbon, colloidal silver and latex beads on endothelial cells does not necessarily mean that adsorption has not occurred. Adsorption might be difficult to demonstrate if there is a rapid subsequent internalization step. In this case, only the large amount that has been internalized indicates that adsorption must have preceded the internalization. This was probably the case during the endocytosis of colloidal silver by endothelial cells, since the amount of the substance in these cells was at least as great as in Kupffer cells, to which colloidal silver does visibly adsorb. Binding of substances to the Kupffer cell membrane has been attributed to the presence of a fuzzy coat on this cell type (Wisse, 1977b). No fuzzy coat was observed in vitro. Although binding of colloidal silver, colloidal carbon and latex beads to the Kupffer cells was not pronounced in vitro (due most likely to the frequent washing of the cells), endocytosis of these substances by Kupffer cells occurred with equal efficiency in vitro as in vivo. This means that either the fuzzy coat is preserved during the cell

isolation procedure but simply cannot be demonstrated or that it is not essential for the binding.

A condition which might influence the amount of adsorbed substance is the presence of serum factors. Serum factors are thought to play an important role in the clearance of some foreign substances from the blood (see Section 1.6). Substances coated with such serum factors might be recognized by certain cell types, resulting in adsorption of the substances to the cell membrane. Some authors report that the uptake of latex or colloids is dependent on serum factors (Jenkin & Rowley, 1961; Filkins & Smith; 1965; Saba & DiLuzio, 1969; Gudewicz et al., 1980; van de Water et al., 1981). These investigators generally used substances which were specially coated with gelatin. Gelatin-coated substances are known to bind the opsonic serum protein fibronectin, which might explain their common serum dependence. Other literature data indicate that a variety of other substances, e.g., zymosan (Warr, 1980), fixed sheep red blood cells (Schneidkraut & Loegering, 1981), lysosomal enzymes (Stahl et al., 1978) and latex (uncoated; Benoliel et al., 1980) can also be bound or endocytosed in the absence of serum factors.

When fetal calf serum was present during the incubation with latex or colloids in an *in situ* perfusion or *in vitro*, endocytosis by Kupffer and endothelial cells was semiquantitatively comparable to the *in vivo* situation described in Chapter 3. Surprisingly, endocytosis of these and all of the other substances appeared to be independent of serum factors and was generally even more intensive in the absence of serum factors. The reason for this is unknown, but the same phenomenon was described for the binding of ASOR by isolated hepatocytes (Schwartz et al., 1980). Especially the uptake of colloidal silver and latex beads by endothelial cells was enhanced in the absence of serum factors. It might be that the presence of serum enhances clumping of the particles (Bloch & McCuskey, 1977). Aggregated clumps would be more difficult for endothelial than for Kupffer cells to endocytose. In accord with this suggestion, it was observed that monodispersed latex was taken up by both Kupffer and endothelial cells, whereas, with not monodispersed latex, endocytosis was limited to Kupffer cells (results not shown). Since the binding of the substances used in this study appeared to be recognized by specific binding sites on the cell membrane right away, the substances might be classified as "ligands" (see Chapter 1).

Inhibition of adsorption or endocytosis by pronase treatment of cells has been described by many authors (see also Chapter 4). In various macrophages pronase or trypsin inhibits the uptake or binding of ligands such as

erythrocytes (Schlepper-Schäfer et al., 1980), mannose-coupled BSA (Stahl et al., 1980), lysosomal enzymes (Steer et al., 1979), zymosan (Warr, 1980; Sung et al., 1983), paraffin oil (Ueda et al., 1981), gelatin-coated latex (Gudewicz et al., 1980), desialylated glycoproteins (Kolb et al., 1981), colloidal albumin and formaldehyde-treated albumin (Brouwer & Knook, 1982). It is now generally accepted that proteolytic enzymes such as pronase or trypsin destroy binding sites or receptors present on the cell membrane. Therefore, pronase incubations might be used to demonstrate the existence of binding sites or specific receptors for ligands on the cell membrane.

When isolated by pronase treatment at 37°C, Kupffer and endothelial cells were no longer able to take up any of the ligands used in this study, except latex beads, indicating that protease sensitive binding sites were necessary for the uptake of colloidal albumin, AGOR, AHOR, HRP, colloidal silver and colloidal carbon.

Binding of latex beads to Kupffer cells has been demonstrated at the ultrastructural level. Nevertheless, pronase treatment did not affect the capacity of either Kupffer or endothelial cells to endocytose this ligand. This indicates that this type of binding is not sensitive to proteolytic enzymes and that both cell types share this characteristic.

Binding of ligands that possess N-acetylglucosamine or mannose terminated polysaccharide chains by a specific recognition system has been described to occur on various macrophages (Kusiak et al., 1980; Warr, 1980; Shepherd et al., 1981; Stahl & Gordon, 1982; Nichols, 1982; Sung et al., 1983; Straus, 1983), mast cells (Straus, 1983), skin fibroblasts (Straus, 1983) and also on sinusoidal liver cells (Steer et al., 1979; Ullrich et al., 1979; Straus, 1983). Because this recognition system appeared to be specially common in macrophages, it was supposed that of the sinusoidal liver cells, the Kupffer cells were mainly responsible for the uptake of N-acetylglucosamine and mannose terminated glycoproteins. The binding specificity of this recognition sytem is often demonstrated by competition with mannan, a polysaccharide composed mainly of mannose units. The study described in this chapter showed that the uptake of AGOR (N-acetylglucosamine terminated) and AHOR and HRP (both mannose terminated) but not of colloidal albumin (no carbohydrate groups) is inhibited by mannan. In vivo and in vitro studies (Chapters 3 and 4) have demonstrated that AHOR and HRP are taken up by both Kupffer and endothelial cells. At the ultrastructural level, it was observed that mannan inhibited the uptake of HRP in both cell types. This indicates that both cell types possess the same recognition system for N-

acetylglucosamine and mannose terminated glycoproteins. The specificity of this recognition system allows the use of the term "receptor" for the binding sites (see Chapter 1). Nevertheless, the specificity of the receptor seems to be "relaxed". This is stressed by the fact that it recognizes both N-acetyl-glucosamine and mannose terminated glycoproteins, although there appeared to be some difference in the binding characteristics. Mannan, for instance, inhibited the uptake of AHOR to a much greater extent than it did that of AGOR. Some other inhibitors also had different effects on the uptake of the two substances (Tables XXI and XXIII) . The phenomenon of the relaxed specificity will be discussed in more detail in Chapter 6.

Inhibition of binding and uptake after depletion of calcium has been described for numerous ligands (Kolb et al., 1979; Shepherd et al., 1981; Ito et al., 1981; Bridges et al., 1982). There are, however, also indications that the uptake of certain ligands is independent of the presence of calcium (Fischer et al., 1980; Ose et al., 1980b). When EDTA was added to isolated Kupffer and endothelial cells, considerable inhibition in the uptake of AGOR, AHOR and HRP occurred, whereas that of colloidal albumin by cultured Kupffer cells was only slightly inhibited. Uptake of colloidal silver, colloidal carbon and latex beads in the presence of EDTA was normal. It is not known whether inhibition of endocytosis by calcium depletion is due solely to prevention of the binding step, since the involvement of calcium in the internalization and the intracellular transport has also been described (van Berkel et al., 1981a;1982b). Inhibition of the binding step might also be affected by the other inhibitors used in this study, although it seems more likely that they act on the internalization and/or the intracellular transport. These steps and the effects of the other inhibitors on endocytosis will be discussed below.

5.4.2 Internalization

At the ultrastructural level, two mechanisms of internalization have been observed in Kupffer cells: phagocytosis and bristle-coated micropinocytosis. HRP was taken up by bristle-coated micropinocytosis only, whereas particulate materials were taken up by both mechanisms. Internalization by fuzzy-coated pinocytosis or worm-like structures (Wisse, 1977b) has not been observed in Kupffer cells. Only one mechanism of internalization was observed in endothelial cells, namely, bristle-coated micropinocytosis. Strikingly, particles that were larger than a single bristle-coated pit could be taken up

by several coated pits acting together. These multiple-coated pits were also observed in Kupffer cells. We did not observe the reported internalization by macropinocytosis in endothelial cells (see Section 1.4). The results obtained demonstrate that the macromolecules AGOR, AHOR and colloidal albumin are most likely to be taken up only by bristle-coated micropinocytosis in both Kupffer and endothelial cells.

It is assumed that bristle-coated pits are specialized in the selective uptake of molecules by adsorptive pinocytosis (see Section 1.4). The substances used in this study are indeed adsorbed to the cell membrane prior to their internalization, including "unspecific" and nonphysiological substances such as colloidal carbon, colloidal silver and latex beads. Other authors have also demonstrated the involvement of coated regions at the cell membrane in the binding and uptake of nonphysiological particles (Wisse, 1972; Aggeler & Werb, 1982). A positive or negative charge present on these particles may play a role in their recognition (Bouma, 1979).

During the uptake of HRP, no HRP negative bristle-coated micropinocytic pits were formed. This indicates, that each bristle-coated pit is able to bind the same ligand.

When Kupffer and endothelial cells were incubated with various combinations of two different ligands, it appeared that, after internalization, both ligands were often present in the same vesicles, indicating that they might have been internalized in a single coated pit. Binding of more than one ligand to one coated pit has been described for other cell types (Dickson et al., 1981; Carpentier et al., 1982; Via et al., 1982). Kupffer and endothelial cells depleted of most binding sites by a 37°C pronase treatment could no longer bind the different ligands, with the exception of latex beads. Ultrastructural observations revealed that Kupffer and endothelial cells devoid of receptors for HRP were still able to perform micropinocytosis by invagination of bristle-coated pits. Since these pits were no longer able to bind HRP, the ligand could enter the cells merely by means of the fluid captured in the vesicles that were pinched off (see Fig. 38).

The findings that even in the absence of serum factors nonphysiological substances are bound and taken up by coated pits, that each pit is able to bind the same ligand, that several ligands can be internalized simultaneously by the same pit and that coated pits are also formed in the absence of adsorbed ligand indicate that bristle-coated pit formation as such is not a very specific endocytic process. Discrimination among different types of ligands appears not to be a property of the coated pit itself.

5.4.3 Intracellular transport and the effect of various inhibitors

Ultrastructural investigation of the intracellular transport of HRP in time-dependent studies revealed that several single bristle-coated vesicles fuse very rapidly after being pinched off from the cell membrane. Strikingly, the larger vesicles formed no longer possessed a coated membrane. These results indicate that the smooth-walled "macropinocytic vesicles" (see Section 1.4) may not represent structures involved in internalization as such, but are formed intracellularly by fusion of bristle-coated micropinocytic vesicles. Smooth-walled vesicles were also observed in the uptake of colloidal carbon, colloidal silver and latex beads. When two types of ligands were endocytosed by a cell, smooth-walled vesicles frequently contained both, indicating that at least a part of the intracellular pathway is identical. This does not necessarily mean that the entire intracellular route is the same. De Bruyn et al. (1975) mentioned that although colloidal carbon and ferritin are internalized by the same mechanism in endothelial cells of the bone marrow, the intracellular transport of the two ligands is not completely the same. It seems that, at least sometimes, substances can be sorted out and directed to different intracellular targets (Abrahamson & Rodewald, 1981). In many endothelial cells of the body, endocytic vesicles are mainly transported to the opposite cell membrane (Palade et al., 1978; Steinman et al., 1983). During this "transendothelial" transport, series of small vesicles that do not fuse with each other are formed. This process was not observed in the endothelial liver cells with any of the tested ligands. A very frequent destiny of endocytosed substances is the lysosome, where, if possible, the substances will be degraded (see Section 1.4). Since degradation of AGOR, AHOR and colloidal albumin in sinusoidal liver cells occurred very efficiently (see Section 2.3.4), it seems likely that these ligands are indeed directed to lysosomes. Lysosomes can be cytochemically stained for the presence of acid phosphatase. Due to the heavy density of colloidal carbon, colloidal silver and cytochemically stained HRP, it is difficult to establish whether these ligands finally enter acid phosphatase stained lysosomes. However, two forms of large smooth-walled vesicles were observed with all three ligands. Some contained only a rim of ligand bound to the membrane, whereas, in others, the ligand was scattered throughout the contents of the vesicle, which thus resembled a "dense body". The studies with HRP showed that vesicles with a rim of ligand were formed prior to the dense vesicles, indicating that the

two forms of the vesicles represent different stages in the intracellular transport.

Apart from the large smooth-walled vesicles, tubular structures containing HRP were observed to arise very soon after the internalization of the ligand. How these structures develop and what their functions are is very obscure. Tubular structures filled with endocytosed ligand have been described by many authors (De Bruyn et al., 1975; Ackerman & Wolken, 1981; Haimes et al., 1981; Nichols, 1982). Ackerman & Wolken (1981) suggest that tubulovesicles represent a special uptake mechanism. However, we never observed a connection between tubules and the outer cell membrane. Geuze et al. (1983) mention the existence of tubules in hepatocytes and suppose that their function is to transport the receptor after its dissociation from the ligand. In our case, however, the ligand was still clearly present in the tubules. Haimes et al. (1981) described tubules as being part of an extensive lysosomal compartment which forms a continuum with the endoplasmatic reticulum. De Bruyn et al. (1975) reported the existence of "transfer" tubules which contain acid phosphatase and might therefore be compared with a primary lysosome.

In this study, the influence of various inhibitors that probably act on either the internalization or the intracellular transport or both processes were investigated.

It is known that DMSO inhibits the uptake of several ligands (Benoliel et al., 1980; Filosa & Fukui, 1981), but that of some others is not inhibited (Benoliel et al., 1980). When DMSO was added to isolated Kupffer and endothelial cells, it affected the uptake of AHOR but not that of AGOR. This difference in behaviour cannot yet be explained.

Chloroquine considerably inhibited the uptake of AGOR and AHOR by the isolated Kupffer and endothelial cells. This inhibitor is known to accumulate in lysosomes, thereby raising their internal pH. In addition, it has been suggested that chloroquine also affects fusion between endocytic vesicles (Tolleshaug & Berg, 1979) and reduces the number of receptors on the cell membrane (Gonzalez-Noriega et al., 1980; Kaplan & Keogh, 1981). The latter might be the mechanism by which it inhibited the uptake of AGOR and AHOR.

Cytochalasin B is known to affect the action of microfilaments. It slightly (15-20%) inhibited the uptake of AGOR and AHOR. The uptake of colloidal albumin by cultured Kupffer cells seemed to be more dependent on the action of microfilaments (Brouwer et al., 1980). Colchicine, which dis-

sociates the labile microtubules of the cells, did not affect the uptake of AGOR and AHOR by sinusoidal cells, but that of colloidal albumin by cultured Kupffer cells was somewhat inhibited by the drug.

The uptake of AGOR, AHOR and colloidal albumin was slightly inhibited by NaF, indicating that endocytosis of these ligands is only moderately dependent on energy derived from glycolysis. Inhibition of energy supply from oxidative phosphorylation by antimycin A resulted in a stronger inhibition of the uptake of AGOR, AHOR, colloidal albumin as well as HRP. Endocytosis of colloidal albumin was inhibited at least twice as much when both NaF and antimycin A were present. This was not the case for AGOR and AHOR.

Several investigators have made attempts to discriminate between different mechanisms of internalization by the use of inhibitors. This has been especially applied to the discrimination between phagocytosis and pinocytosis with colchicine and cytochalasin B (Silverstein et al., 1977; Michl, 1980). Although it seems likely that metabolic and cytoskeletal inhibitors act primarily on the internalization and intracellular transport steps of endocytosis, several authors have reported that the binding of a substance to the cell membrane might also depend on metabolic (Ito et al., 1981; Ueda et al., 1981) or cytoskeletal (Benoliel et al., 1980; Filosa & Fukui, 1981; Ito et al., 1981) activity. The diversity of literature data given in Table XXIV illustrates that the actions of colchicine, cytochalasin B and chloroquine cannot simply be described as influencing a specific mechanism of internalization. The results presented in this chapter also indicate that inhibitors cannot be used to discriminate among the mechanisms of the different steps of endocytosis that can be vizualized ultrastructurally. When the mechanisms by which different substances are endocytosed are identical at the ultrastructural level, there may still be differences in the effects of inhibitors. For instance, all ligands used in this study were adsorbed to the cell membrane and taken up by bristle-coated micropinocytosis. Nevertheless, different effects were observed for the uptake of some ligands when pronase , EDTA, mannan, antimycin A, cytochalasin B or colchicine were added. Therefore, a specific process of endocytosis cannot be completely characterized by an ultrastructural description. However, the opposite is also true. If different mechanisms can be observed at the ultrastructural level, they might still be affected in an identical manner when inhibitors are added. This was illustrated by the fact that both bristle-coated micropinocytosis and phagocytosis of colloidal carbon and colloidal silver were inhibited by the action of pronase and not by EDTA.

The results obtained may illustrate that only a combination of ultra-

structural observations and biochemical studies offers a good approach for the characterization of certain mechanisms of endocytosis.

5.4.4 Intracellular fate of receptors

It was shown in this study, that isolated Kupffer and endothelial cells were still able to endocytose HRP by bristle-coated micropinocytic vesicles after an incubation time of 30 min with the ligand (Fig. 23e). Such a continuous uptake has also been demonstrated for various other ligands and cell types , even for several hours and in the presence of inhibitors of protein synthesis (Schwartz et al., 1982; Kolb et al., 1981). The question thus arises of how the cell is able to maintain the concentration of receptors which is necessary to mediate the continuous internalization of a ligand.

The behaviour of receptors after internalization has been studied to some extent by others and it was found that it may vary for different cell types and/or receptors. The findings and theories concerning the fate of receptors as described in those studies will be compared below with the findings of this study.

For some receptors, e.g., the insulin receptor, it has been reported that both receptor and ligand are internalized together and reach the lysosomal compartment where they are both degraded (Steinman et al., 1983). When there is an excess of ligand, this process involves a "down regulation" of receptors which might serve to prevent the cells from becoming "overfed" with a specific ligand.

If down regulation of a receptor occurs, a continuous uptake of ligand will not be possible, unless an intracellular pool which replenishes receptors at the same rate as that at which they are internalized exists. Even then, the intracellular pool would be of limited size and a continuous uptake during several hours cannot be explained this way.

There are strong indications that many receptors in various cell types are not degraded in the lysosomes but are reutilized several times. For the uptake of low density lipoproteins by fibroblasts, a continuous uptake of 6 h has been found in the presence of inhibitors of protein synthesis (Goldstein et al., 1979). This is unlikely to occur if the receptors are degraded by the cells. Stahl et al. (1980) found that when receptors for mannose glycoconjugates of alveolar macrophages were removed from the cell membrane by a 5 min incubation with trypsin, the capacity of the cells to endocytose was reduced to 30%. Assuming that a substantial number of receptors is present

TABLE XXIV

EFFECT OF VARIOUS INHIBITORS ON ENDOCYTOSIS OF SUBSTANCES BY DIFFERENT CELL TYPES

Cell type	Ligand	Cytochalasin B	Colchicine	Chloroquine	Reference
Per. μφ	IgG-RBC	+			Benoliel et al., 1980
,,	IgG-RBC	+	+		Uher et al., 1981
,,	HRP	-	+		Piasek & Thyberg, 1980
,,	HRP		+		Thyberg & Stenseth, 1981
,,	Latex	+	-	+	Kusiak et al., 1980
,,	Gel. latex	+	+		Gudewicz et al., 1980
,,	Tumor cells	+			Benoliel et al., 1980
,,	BSA-particles	+	+		Ito et al., 1981
,,	β-Hexosaminidase	+	+	+	Kusiak et al., 1980
,,	PVP	+	+		Pratten & Lloyd, 1979
,,	Colloidal gold	+	+		Pratten & Lloyd, 1979
,,	Zymosan			+	Sung et al., 1983
,,	Cat. peroxidase		+		Thyberg & Stenseth, 1981
,,	Paraffin oil	+			Ueda et al., 1981
Alveolar μφ	α_2-M-trypsin complexes	-	-		Kaplan & Nielsen, 1979
,,	α_2-M-trypsin complexes			+	Kaplan & Keogh, 1981
,,	Mannose coupled BSA			+	Tietze et al., 1980
Pulmonary μφ	colloidal gold	+	+		Valberg et al., 1982

Kupffer	Form. albumin	+			Brouwer et al., 1980;1982
,,	Colloidal albumin	+	+		Brouwer et al., 1982
,,	IgG-RBC	+	-		Munthe-Kaas, 1976
Sinusoidal	Acetyl-LDL			+	van Berkel et al., 1982a
,,	PVP		+		Ose et al., 1980a
Hepatocytes	LDL		-	-	Attie et al., 1980
,,	Lac-LDL		+	+	Attie et al., 1980
,,	Acetyl-LDL			+	van Berkel et al., 1982a
,,	Asialoglycoprotein			+	Tolleshaug & Berg, 1979
,,	PVP	-	+		Ose et al., 1980a
Fibroblasts	Glucuronidase			+	Gonzalez-Noriega et al., 1980
,,	Lysosomal enzymes			-	Willcox & Rattray, 1979
,,	Lysosomal enzymes			+	Sando et al., 1979
,,	Dextran			-	Sando et al., 1979
,,	LDL			-	Sando et al., 1979
,,	EGF			-	Sando et al., 1979
Chondrocytes	HRP	-	+		Piasek & Thyberg, 1979
Epith. carcinoma	EGF			-	McKanna et al., 1979
Clearance	ASOR, AFET	-	-		Regoeczi et al., 1978
,,	Insulin			-	Dennis & Aronson, 1981

+ = inhibition; - = no inhibition
Abbreviations: α_2-M: α_2-Macroglobulin; AFET: asialofetuin; Alveolar μφ: alveolar macrophages; BSA-: BSA-coated; Cat.: cationized; EGF: epidermal growth factor; Epith.: epitheloid; Form.: formaldehyde-treated; Gel.: gelatin-coated; Glut.: glutaraldehyde-treated; IgG: IgG-coated; Lac.: lactosylated; LDL: low density lipoprotein; Lys.: lysosomal; Per. μφ: peritoneal macrophages; RBC: red blood cells.

intracellularly, these results indicate that a considerable portion of these receptors was degraded during this short time interval. Thus, the transfer of receptors from the cell interior to the cell membrane would have to be a very rapid process. If the rate at which trypsin exhausts the number of receptors reflects the rate at which receptors are internalized and subsequently degraded by lysosomes, the cells would normally not be able to endocytose the ligand for much longer than 5 min. Since Stahl et al. (1980) demonstrated that under normal conditions the cells were able to endocytose much longer, it is reasonable to assume that no down regulation of the receptors occurred but that they could be reutilized for endocytosis. For the hepatic receptor of asialoglycoproteins, Warren & Doyle (1981) have demonstrated that its turnover is unaffected by the presence of ligand. The half-life of this receptor is about 20 h, which is several times longer than that of the ligand. This indicates that the receptor somehow manages to escape from lysosomal degradation.

The escape of receptors from lysosomal degradation prompted several investigators to speculate on the mechanism by which this may be accomplished. For cultured fibroblasts, Willingham et al. (1981c) postulated that the functional receptors for α_2-Macroglobulin are present mainly on the membrane and not in the interior of the cell. During endocytosis of the ligand, cointernalization of the receptor is somehow avoided and the receptor is very soon able to bind the next ligand molecule. Since the receptor remains at the cell surface, it automatically escapes lysosomal degradation. This mechanism, however, appears not to be a general feature of all cell types. Stahl et al. (1980) incubated alveolar macrophages for 30 min with pronase or trypsin at 4°C, a temperature at which the proteolytic enzymes were still able to degrade receptors on the membrane but at which internalization is strongly inhibited. After warming up to 37°C, the endocytic capacity of the cells was only slightly reduced as compared with cells which had not been incubated with proteolytic enzymes. These results indicate that, during the incubation with proteolytic enzymes at 4°C, the majority of the receptors was present inside the cells and not at the cell membrane, where they would have been degraded. Apparently, the transport of receptors was inhibited at 4°C, whereas, after rewarming, receptors immediately started to manifest themselves again at the cell surface.

If a receptor is indeed cointernalized with its ligand, the question of how it manages to escape from lysosomal degradation still remains unanswered. Blumenthal et al. (1980) mention the possibility of translocation of

the asialoglycoprotein receptor on parenchymal cells from one side of the cell membrane to the other. They suggested that receptors may escape lysosomal degradation by moving to the outer membrane of the lysosome and subsequently recycle back to the cell membrane. A more common theory is based on the assumption that receptor and ligand are separated before they reach the lysosomal compartment. The ligand is degraded in the lysosomes, whereas the receptor is spared from this fate and recycles back to the cell surface.

The recycling of a receptor should not be taken too literally. It is possible that the intracellular route of receptors involves a long and complex cycle. In this case, the number of receptors inside the cell may greatly exceed the number present at the cell membrane, which would give the impression of an intracellular pool of receptors, as was found in the study of Stahl et al. (see above).

Although the behaviour of receptors was not explicitly examined in this study, two findings, i.e., the complete abolishment of virtually all types of receptors by a 60 min warm pronase treatment and the preservation of the endocytic capacity of the cells treated by pronase at 10°C, may allow some conclusions. Comparable to the situation in alveolar macrophages (Stahl et al., 1980, see above) and in contrast to the findings of Willingham et al. (1981c, see above), the majority of receptors must have been present inside the sinusoidal cells and must have remained there during the pronase treatment at 10°C. At 37°C, however, all receptors must have appeared at the cell membrane to become sensitive to pronase. In this way, the supply of intracellular receptors completely disappeared within 60 min. The cells appear not to be able to restore this supply within a comparably short time, since at least a 24 h culture period is needed to restore their endocytic capacity (Brouwer & Knook, 1977). These findings make it very unlikely that receptors are degraded by lysosomes under normal circumstances. In that case, the cells would depend solely on a pool of receptors to replenish the internalized ones. To explain the findings of this study, it is more likely to assume that the intracellular supply of receptors represents part of a receptor recycling system. This would apply to all binding sites or receptors investigated here, with the possible exception for those binding latex beads.

Our findings and those of Stahl et al. show that the complete degradation of all receptors for a specific ligand by extracellular proteolytic enzymes at 37°C occurred in the absence of the ligand. This indicates that receptor recycling is a ligand independent process. Although it was expected that cells treated by pronase at 10°C would possess no receptors at their mem-

branes before warming up, they appeared to be able to directly bind a certain amount of colloidal albumin, AGOR, AHOR (Table XVI) and HRP (Fig. 28f). This phenomenon might be explained by a very slow receptor recycling, which has also been described to occur even at 4°C (Aulinkas et al., 1982). Cells isolated by the 10 min cold pronase treatment would have the opportunity to recycle some of their receptors during the subsequent isolation procedure which is performed at 4°C and takes about 90 min (see Section 2.2.2).

Techniques have now been developed to simultaneously demonstrate the receptor and the ligand. Geuze et al. (1983) have described a structure called CURL (Compartment Uncoupling Receptor Ligand) in which separation of the ligand and receptor seems to occur. This separation might be induced by the acid pH which the vesicles acquire shortly after their internalization (Tycko & Maxfield, 1982). Agents such as chloroquine and monensin which prevent acidification of the vesicle might thus affect both the separation of receptor and ligand (which automatically inhibits the recycling of unoccupied receptors, Tietze et al., 1980; Basu et al., 1981) and the fusion of the incoming vesicle with the organelle which has to transport either receptor or ligand (see also Praaning-van Dalen et al., 1982).

In summary, experiments described in this chapter have shown that Kupffer and endothelial cells both have the capacity to internalize different ligands by adsorptive endocytosis. At the ultrastructural level, the degree of adsorption for some substances appeared to be different for each cell type, although an apparent efficient adsorption might be the result of a slow internalization step. Only two mechanisms of internalization were observed: bristle-coated micropinocytosis (Kupffer and endothelial cells) and phagocytosis (Kupffer cells). A discrimination between these two mechanisms is possible at the ultrastructural level, but appears to be difficult by biochemical inhibition studies. Bristle-coated micropinocytosis was also performed by receptor depleted cells. One bristle-coated vesicle is probably able to internalize at least two types of ligands at the same time. Both cell types internalized HRP via specific receptors for mannose. The intracellular transport of HRP was very rapid and included the formation of a wide variety of different vesicles.

Although receptors as such were not visualized, the results presented indicate that receptors and ligands are separated before the ligand is delivered to the lysosomes. The receptors appear to escape from lysosomal degradation and recycle back to the cell membrane.

CHAPTER 6

GENERAL DISCUSSION

The aim of the study described in this thesis was to obtain more information on the capacity of Kupffer and endothelial liver cells to endocytose various substances presumed to be cleared by the RES, the mechanisms by which this occurs and the role of the cells in the clearance function of the RES. This was investigated by a combination of studies at the plasma, organ and cellular levels.

6.1 PLASMA CLEARANCE OF TEST SUBSTANCES

There are at least six important factors which might influence the rate at which a specific intravenously injected RES test substance is removed from the blood (see also Section 1.6):

1) the rate of blood flow, which determines the rate at which the test substance is delivered to an organ and its cells;
2) the physiological composition of the blood;
3) the size of the population of RES cells;
4) the extent of adherence of a substance to the cellular surface;
5) the injected dose of the test substance, which determines whether a saturation effect of the clearance occurs;
6) the intrinsic clearance capacity of the cells belonging to the RES.

The relative significance of each of these factors with regard to its actual influence on the clearance rate will also depend on the dose and nature (e.g., the size, surface charge and the property to aggregate in the blood) of the test substance.

After intravenous injection of several of the test substances used in this study, it appeared that there were indeed differences in the clearance rates (see Section 3.3.1). Heparin and PVP were cleared in a relatively slow, nonexponential way. However, at the concentrations used, ASOR, AGOR, AHOR and colloidal albumin were cleared in an exponential way, in-

dicating a one-compartment clearance system, which appeared to be the liver for all four substances. From the slope of the curves and the half lives of the substances in the plasma, it could be deduced that they all were cleared at high rates, with slight individual variations.

An exponential clearance rate has generally been considered to be a specific characteristic of the colloid clearance by the RES (see Section 1.6). However, the results in this study have shown that some noncolloidal substances (e.g., ASOR, AGOR and AHOR) are also cleared at an exponential rate. In addition, some other substances which are supposed to be taken up by the RES are not cleared in an exponential way.

The six factors mentioned which might generally determine differences in clearance rates will now be discussed in relation to the results obtained in this study. The intrinsic clearance capacity mentioned as factor 6 will be discussed under Section 6.3.

1) It was shown that the rate of blood flow through the liver indeed determines the rate at which a substance is cleared from the plasma (see section 3.3.2). This situation can occur only if the concentration of a substance is so low that almost no substance can pass through the liver without being cleared. It has been suggested that such an effective clearance is a specific characteristic of the "colloid clearance test" because only the cells of the RES are assumed to be capable of removing all substances at one passage (see Section 1.6). This study has shown, however, that this clearance characteristic is not limited to colloids, since the clearance of noncolloidal substances such as ASOR can also reflect the rate of blood flow. Moreover, the study with ASOR has shown that a liver cell population which is assumed not to belong to the RES, the parenchymal cells, is also able to clear almost all of the substance at one passage (see Section 3.3.2).

The intrinsic properties of a substance might induce changes in the rate of blood flow. Aggregation of a substance in the blood, as was observed for colloidal carbon, might reduce the rate of blood flow through the liver.

2) Factors which define the physiological composition of the blood can also influence the clearance rate of an injected substance. These factors include the concentration of serum components, i.e., opsonins, by which some substances have to be covered before cellular recognition can occur, the Ca^{++} concentration, the presence of substances which may compete with test substance in the clearance and the pH of the blood. The uptake of none of the test substances appeared to be dependent on serum factors (see section 5.3.2). This implies that a reduction in the clearance rate as a result of

depletion of serum factors as suggested by Saba & DiLuzio (1969) will not occur for the substances used in our study. The clearance of AGOR, AHOR, HRP and colloidal albumin might, however, be influenced by the Ca^{++} concentration of the blood, since in vitro studies have shown that the uptake of these substances is Ca^{++} dependent (see Section 5.3.2). In addition, the removal of AGOR, AHOR and HRP might be influenced by blood borne glycoproteins that possess oligosaccharide chains with terminal N-acetylglucosamine or mannose groups. Such glycoproteins, which might be present in the blood during injury (e.g., lysosomal enzymes) or infection (e.g., bacterial cell walls), will compete with the clearance of HRP, AGOR and AHOR, since they are all recognized by the same receptors on Kupffer and endothelial cells (see Sections 3.4 and 5.3.2). Finally, the pH of the blood might influence the clearance rate of a substance. The affinity of the cell membrane for specific substances is reported to be influenced by the pH (Fischer et al., 1980; Aulinkas et al., 1982). Apart from this effect, an increased blood pH may also affect the pH of endocytic vesicles, which may in turn affect the endocytic capacity by inhibiting the reappearance of receptors on the cell membrane (Tolleshaug & Berg, 1979). It was demonstrated here that an increase in the pH by means of chloroquine reduced the endocytic capacity of the sinusoidal cells (see Table XXIII).

3) The rate at which a substance is cleared from the blood may depend on the size of the population of cells which contribute to the clearance. In this study, the general assumption that the major cell population contributing to the clearance of RES test substances is situated in the liver was confirmed for most substances; an exception was heparin. It is generally assumed that the Kupffer cell population makes the major contribution to the clearance of injected RES test substances (see Section 1.6). As shown in Chapters 3 and 4, the clearance of colloidal carbon and large latex particles was indeed done primarily by the Kupffer cells. However, it was demonstrated in those chapters that endothelial liver cells are able to clear several other RES test substances, often even more effectively than Kupffer cells.

The cell type(s) and the number of cells of a certain type which are involved in the clearance can depend on the nature as well as the concentration of substance injected. At least three reasons can be given for fluctuations under the influence of the amount administered.

a) Firstly, the dose might determine to which zone of the liver the substance is delivered. At a relatively small dose, only the cells of zone 1 of the liver acinus (see Section 1.1) would be in contact with the substance,

whereas at a higher dose also the cells in zones 2 and 3 might have the chance to take up some substance. In vivo, indeed some Kupffer cells seemed to be more active in the clearance of colloids and latex than were others (see Section 3.3). However, this was also observed in vitro (see Section 4.3), indicating that it is probably not due to the different location of the cells in the acinus but to a true heterogeneity within the Kupffer cell population. This would agree with the results of Sleyster & Knook (1982), who found that, during an in situ perfusion of the liver, the clearance by Kupffer cells in zone 1 was higher than in the other zones of the acinus, independent of the direction in which the test substance was flushed through the liver.

b) Secondly, the magnitude of the administered dose might determine which type of cell contributes to the clearance. At a relatively high dose, more cell types might be involved than at a lower one, as has been suggested for colloidal carbon, the uptake of which by endothelial cells was increased at a high dose (Patek et al., 1967). This implies that such high doses of colloidal carbon cannot be used as a marker for the clearance function of Kupffer cells. In the experiments described here, an injection of a relatively high (7 mg per 100 g rat) but not yet blockading (Donald & Tennet, 1975) dose of colloidal carbon indeed did not result in demonstrable inhibition in the capacity of Kupffer cells to endocytose latex beads of 0.23 µm diameter. However, the uptake of latex beads by endothelial cells was increased under these conditions (see Section 5.3.1). The same phenomenon was observed when large latex beads (0.65 µm) instead of colloidal carbon were first injected. A slightly diminished uptake of latex beads by Kupffer cells resulting from the preinjected substance still occupying the surface of the cells might have increased the chance that latex beads also reached the endothelial cells.

c) Thirdly, the physiological condition of the animal might determine the size of the cell population available for the clearance. Several factors which are normally present in the blood such as hormones and bacterial products are reported to influence both the RES clearance rate and the size of the population of cells contributing to it (Altura, 1980).

4) The adherence of a test substance to the cell membrane as such is sufficient to determine its clearance without internalization being necessary. Therefore, it is not possible to determine the actual rate of internalization in clearance studies. This may be especially important in the case of a high binding capacity combined with a low rate of uptake.

5) The administered dose of a substance might not only determine which population of cells contributes to the clearance but might also influence the

clearance in other ways. The clearance of a very low dose of a rapidly cleared ligand will merely reflect the rate of liver blood flow. However, as shown by Benacerraf et al. (1957a) for colloidal albumin, the clearance rate will decrease when very high doses of substances are injected. In the present study, it was also shown that a higher dose of colloidal albumin was cleared at a slower rate than was a lower dose. Since the uptake of colloidal albumin was not dependent on opsonins (see Table XVII), a decrease in clearance rate at higher doses is probably due to a partial saturation of binding and subsequent uptake by the cells. Such a saturation effect indeed occurred when the cells were exposed to various concentrations of colloidal albumin in vitro. This was also observed for the uptake of AGOR and AHOR. At saturating concentrations, the adsorption step of the endocytic process is probably the rate limiting one. For substances like PVP that are endocytosed without prior adsorption to the cells, no saturation effect is found (Munniksma et al., 1980).

6.2 CONTRIBUTION OF VARIOUS ORGANS TO THE CLEARANCE OF TEST SUBSTANCES

It has been supposed that 85-90% of the RES clearance function is performed by the liver (see Section 1.6). This would imply that, irrespective of the rate of clearance, 85-90% of the fraction of a RES test substance removed from the blood should be recovered in the liver at any given time, provided that no degradation of the substance has occurred. In Table XII, it was shown that such a situation was found only for the low dose of colloidal albumin employed and for ASOR, which is taken up mainly by the parenchymal cells. For AGOR, AHOR and the high dose of colloidal albumin, not more than 55-72% of the amount cleared from the plasma was found in the liver. For PVP and heparin, these percentages were only 4% and 13%, respectively. These results show that a high clearance by the liver per se cannot be regarded as a specific characteristic of the RES, since an 85% recovery in the liver might also be found for substances like ASOR which are not cleared by cells of the RES. Furthermore, the assumption that 85% of a RES test substance should be recovered in the liver is not valid for all test substances. Nevertheless, our study showed that the contribution of other organs to the clearance of the test substances was always much lower than that of the liver, with the exception of heparin, which was also cleared to a considerable

extent by the kidneys (Table XI). It is possible that other organs have the potential capacity to clear colloidal substances, but do not get the opportunity due to the effective clearance by the liver. In that case, it would be expected that incomplete clearance by the liver would result in increased clearance by the other organs. However, this was not found when colloidal albumin was used as a test substance (see Table XI). Thus, these organs appeared not to be able to take over the clearance function of the liver.

6.3 CONTRIBUTION OF KUPFFER AND ENDOTHELIAL CELLS TO THE CLEARANCE OF TEST SUBSTANCES

The clearance of RES test substances by the liver is often assumed to be carried out by the Kupffer cells exclusively (see Section 1.6). This is based mainly on plasma clearance studies or on the localization of the test substances in the liver by light microscopy or radioactive liver scan. No exact information on an exclusive localization in Kupffer cells can be obtained by these means. Literature reports have indicated a possible contribution of also endothelial cells to the clearance of RES test substances (see Section 1.6). The contribution of Kupffer and endothelial cells to the clearance of RES test substances can most accurately be studied if the cells can be isolated and purified after they have taken up the test substances in vivo. The most suitable isolation method for performing such studies is that of cold pronase (see Section 2.3.4). This isolation method also appeared to be suitable for the study of the endocytic capacity of both cell types in vitro.

As was shown in Chapters 3 and 4, endothelial cells were indeed able to take up all the RES test substances investigated. Since the endothelial cells are 2-3 times more abundant than Kupffer cells in the rat liver, their contribution to the clearance of heparin, AGOR, AHOR, colloidal albumin, colloidal silver and HRP can be expected to be at least as high as that of the Kupffer cells. Previous findings have indicated that parenchymal cells might also contribute to the clearance of heparin, PVP and colloidal albumin (Praaning-van Dalen et al., 1981). Thus, only certain RES test substances are cleared by Kupffer cells exclusively (e.g., large latex beads and colloidal carbon when given in a low dose) and others are also cleared by endothelial or even parenchymal cells. If one does not wish to consider other cell types of the liver than Kupffer cells as belonging to the RES, only substances such as large latex beads and colloidal carbon should be chosen to determine the

clearance function of these cells. Whether other substances not tested by us are usable for this purpose can be established only if the exact localization of a substance in the liver following its administration is ascertained.

6.4 MECHANISMS BY WHICH KUPFFER AND ENDOTHELIAL CELLS ENDOCYTOSE RES TEST SUBSTANCES

The intrinsic cellular endocytic capacity is one of the most important factors that determines the plasma clearance rate. The different rates at which various substances are cleared suggest the existence of different cellular mechanisms of uptake. It has been assumed that colloidal or particulate RES test substances are taken up solely by the mechanism of phagocytosis. Since colloids such as colloidal albumin, colloidal silver and colloidal carbon were also taken up by endothelial cells, which are not able to phagocytose, this assumption cannot be correct. To obtain more detailed informations on the mechanisms of endocytosis, the process was studied not only in vivo and in a perfused liver system but also in vitro by the use of freshly isolated and purified cells.

As described in Section 1.4, the process of endocytosis can be divided into three steps, i.e., the events at the cell membrane, internalization and the intracellular transport.

6.4.1 Events at the cell membrane

The results in this study have demonstrated that all substances except PVP are taken up by adsorptive endocytosis. Adsorption was demonstrated by either the effective rate of endocytosis, saturation kinetics or ultrastructural visualization (Chapters 2, 4 and 5). At the ultrastructural level, binding of a substance at 37°C was not always visible, which might have been due to the rapid subsequent internalization step (see Section 5.3.1). Only if the internalization is a slow, rate limiting step binding at 37°C might be visible, but even then only when the binding is stable. In contrast to the endothelial cells, binding to the membrane of Kupffer cells was observed during the uptake of colloidal silver, colloidal carbon and latex beads.

Adsorption of a substance to a cell occurs if the membrane contains certain sites that are able to bind the substance (ligand). The results in Chapter 5 indicate that there probably are at least three different types of bin-

ding sites on sinusoidal cells, i.e., for latex beads, for colloidal silver, carbon or albumin, and for AGOR, AHOR or HRP. The number of binding sites as well as their affinity for the ligand will determine the amount of a substance that becomes adsorbed to the cell membrane. This will also determine the amount of substance that can be subsequently internalized by the cells.

The binding of a ligand (L) to a binding site (B) occurs at a specific rate (k_1). The ligand may dissociate from the binding site again at a specific rate (k_2). The rates of binding and dissociation determine the affinity of the binding. Bound ligands can be internalized in an irreversible step at a specific rate (k_3). The overall process can be expressed most simply as:

$$L + B \underset{k_2}{\overset{k_1}{\rightleftharpoons}} L\text{-}B \xrightarrow{k_3} [L\text{-}B]$$

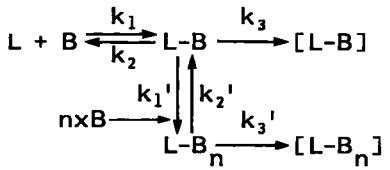
It is difficult to assess the actual number of binding sites present on the cell membrane if the rate of dissociation is unknown. At the physiological temperature of 37°C, the rate of dissociation might not be measurable due to a rapid internalization of the ligand/binding site complexes. Therefore, the number of binding sites (and the dissociation rate) is often assessed at a temperature of 4°C, at which no internalization occurs. As will be discussed later, this method might not always be accurate.

If related types of ligands inhibit the binding of each other, the binding site has a certain specificity and may be called a receptor. The results described in this thesis demonstrated receptor mediated binding and uptake by Kupffer and endothelial cells for ligands which possessed mannose or N-acetylglucosamine terminated oligosaccharide chains (Section 5.3). Mere binding of a ligand to a receptor does not necessarily directly induce its internalization. For several cell types, including Kupffer cells, it has been reported that receptor bound ligands are diffusely distributed over the cell membrane at 4°C and that at 37°C the receptor/ligand complexes shift over the membrane to cluster together and form patches or even caps at the location where the internalization step is to take place (McKanna et al., 1979; Kolb-Bachofen, 1981; Salisbury et al., 1981; Dickson et al., 1982b; Zeitlin & Hubbard, 1982). Other authors report that binding at 4°C occurs exclusively in coated pits (Willingham et al., 1981a; Wall & Hubbard, 1981; Kolb-Bachofen et al., 1982). It is not yet clear whether these different findings are due to varied experimental approaches or whether some receptors indeed never leave the coated pits. Kolb-Bachofen (1981), for instance, found that when hepatocytes were incubated with galactose terminated glycoproteins at

4°C for 10 min, the ligand was diffusely distributed over the cell membrane. After prolonged incubation at 4°C, however, the ligand was also found in coated pits. Wall & Hubbard (1981), who performed the same experiment during an incubation period of 30 min, found the ligand in coated pits only. Thus, movement of receptor/ligand complexes into clusters and coated pits might also occur at 4°C, although at a much slower rate than at 37°C. In some cells, the clustering of receptor/ligand complexes into coated pits is reported to depend on the enzymatic action of transglutaminase, the process being inhibited by dansylcadaverine and other inhibitors of this enzyme (Davies et al., 1980; Dickson et al., 1982a).

Since the location of receptors is demonstrated by the binding of a ligand in most of the experiments, there are still differences of opinion as to whether the clustering and internalization of some receptors might also occur without added ligand. One might assume that the binding of a ligand to a receptor triggers the action of transglutaminase or other enzymes necessary to start the proces of receptor clustering and movement into coated pits.

The rate of clustering of the receptor/ligand complexes might depend on the spreading of the specific receptors over the membrane and on the composition of the membrane of a certain cell type. If one assumes that clustering and internalization of a receptor is a continuous, ligand independent process, the ligand itself will not be able to induce its own internalization. However, whether receptor clustering is a ligand dependent process or not, the clustering of receptors might function to increase the affinity of binding. This might happen if a ligand is able to bind to more than one receptor, resulting in an increase in the binding valency if the receptors are clustered. In this case, the formula given above would be extended to:



This formula clearly shows that the chance of ultimate dissociation, expressed by k_3, is significantly reduced if multiple ligand/receptor interaction occurs. This hypothesis would also explain the results of Dickson et al. (1982a), who found two types of binding sites on fibroblasts for α_2-macroglobulin, i.e., low affinity sites distributed over the cell membrane (single ligand/receptor interaction, thus a high dissociation rate) and high affinity sites present in coated pits (multiple ligand/receptor interaction, thus a low

dissociation rate). It would also explain why mannose terminated glycoproteins must possess a critical number of mannose residues (probably 5 or 6; Stahl & Lee, 1982) to be endocytosed by sinusoidal cells (necessary for multiple ligand/receptor interaction), whereas the nonendocytosible simple sugars (multiple ligand/receptor interaction not possible; high dissociation rate), when added at a high concentration, are able to inhibit binding of the glycoproteins (Summerfield et al., 1982). Finally, a multiple ligand/receptor interaction combined with the spacing of receptors on the cell membrane versus spacing of recognition groups on the ligand might explain the results of Schlepper-Schäfer et al. (1982), who found that asialoglycoproteins are not endocytosed by Kupffer cells, whereas galactose-coated particles of 5 nm and larger are recognized by a galactose-specific receptor and endocytosed. Probably due to the spacing of the receptors, only these larger particles are able to induce a multiple ligand/receptor interaction, resulting in a stabile complex with a low dissociation rate. The importance of the spacing of recognition groups on a ligand versus receptor spacing has also been described by Baenziger & Fiete (1980). The findings described above indicate that not only the density of receptors and the affinity of a single ligand/receptor interaction but also the spacing of receptors and recognition groups in relation to one another can determine the amount of endocytosed ligand.

Although the spacing of the receptors for mannose or N-acetylglucosamine and their distribution over the cell membrane has not yet been studied, some indications of the process can be given from the results described in this thesis. A receptor/ligand binding in and outside coated pits could be demonstrated in endothelial cells when the liver was perfused with HRP for 45 seconds at a temperature of 4°C (see Section 5.3). No binding outside coated pits was observed on Kupffer cells. This might indicate that the receptors on the Kupffer cell membrane are present only in the coated pits. However, it might also have been due to a lower binding affinity or to such a diffuse distribution of the receptors that the binding of HRP was not demonstrable by the cytochemical staining procedure used. There are more indications that the Kupffer cells have a lower binding affinity than the endothelial cells. HRP added in low concentrations or present during a very short time interval is endocytosed at 37°C by isolated endothelial cells, but very little by Kupffer cells (see Sections 4.3 and 5.3). Moreover, the amount of HRP, AHOR and AGOR endocytosed by endothelial cells was greater than found for the Kupffer cells in vivo as well as in vitro (see Sections 3.3 and 4.3). The capacity of Kupffer cells to endocytose AGOR in vivo and in

vitro is much smaller than that for AHOR, whereas endothelial cells have the same endocytic capacity for both substances under both experimental conditions (see Sections 3.3 and 4.3). These results indicate that Kupffer cells somehow handled AGOR differently than they did AHOR, while endothelial cells do not discriminate between these two ligands.

The phenomenon of one receptor being able to recognize two different groups was called relaxed specificity by Achord (1977). Such specificity for the receptor for asialoglycoproteins has been described for hepatocytes (Baenziger & Maynard, 1980). The relaxed specificity of the receptor for mannose or N-acetylglucosamine present on sinusoidal cells was studied in detail by Maynard & Baenziger (1981), who described the structure which a ligand should minimally possess to enable binding and endocytosis. Their study, however, does not give a complete explanation of the nature of the relaxed specificity. Firstly, the binding of L-fucose, which is said to bind to the mannose receptor of alveolar (Shepherd et al., 1981), peritoneal and bone marrow macrophages (Stahl & Gordon, 1982; Straus, 1983), was not studied. Secondly, no distinction was made between the binding on endothelial and Kupffer cells. Thirdly, they did not consider the possibility of a multiple ligand/receptor interaction which, as will be discussed below, might influence the expression of relaxed specificity.

In spite of the apparent differences in the binding capacity of the receptors of Kupffer and of endothelial cells, there are indications that the recognition system is basically the same. For instance, the uptake of HRP and AHOR, which were both taken up by Kupffer as well as endothelial cells in vivo and in vitro (see Sections 3.3 and 4.3), was almost completely inhibited by mannan in both cell types (see Section 5.3). The isolation of only one type of mannose binding protein from isolated sinusoidal cells by Mori et al. (1983) indicates that the receptor on both Kupffer and endothelial cells has the same structure.

Thus, the differences between Kupffer and endothelial cells with respect to their interaction with the ligands cannot be explained by a difference in the receptor as such. One can then only speculate on how these differences are accomplished. A possible explanation which is compatible with all of the results found in this study is exemplified in Fig. 42. In this model, it is assumed that there is one type of receptor which recognizes AGOR, AHOR and HRP and which is expressed on both Kupffer and endothelial cells. It is further assumed that multiple ligand/receptor interaction occurs and even is necessary for achieving stable binding and endocytosis. Receptors are ini-

tially distributed over the cell membrane at relatively low density and finally cluster into coated pits. In Kupffer cells, a wider local distribution of receptors close to the site of internalization might result in a receptor/ligand interaction of lower affinity than occurs in endothelial cells. This model would explain why endothelial cells internalized the ligands more effectively than did the Kupffer cells (without a difference in the number of receptors being necessary) and also why endothelial, but not Kupffer cells, endocytose AGOR as effectively as they do AHOR (see Fig. 42). The model also shows that it might be difficult to determine the number of receptors on a cell type if the extent of multiple/ligand receptor interaction is not known. Moreover, at 4°C, the temperature at which the number of receptors is usually determined, the rate of receptor clustering is slower than at 37°C, resulting in different affinities if multiple ligand/receptor interaction occurs.

The model described above might also be valid for other receptors and binding sites present on Kupffer and endothelial cells and might govern the rate of internalization of particulate and colloidal RES test substances.

6.4.2 Mechanisms of internalization

One of the most important features of the cells belonging to the RES is their capacity to internalize colloidal and particulate materials by the mechanism of phagocytosis (see Section 1.6). The finding that endothelial cells, which are not able to phagocytose, also contribute to the uptake of colloids and latex beads (see Sections 3.3 and 4.3) indicated that other mechanisms of internalization must also be involved. Ultrastructural studies showed that endothelial cells internalized all substances used in this study by bristle-coated micropinocytosis (see Section 5.3). Substances larger than the diameter of one bristle-coated pit were internalized by several bristle-coated pits acting together. Although it is supposed that Kupffer cells internalize colloids and latex beads by phagocytosis, it was striking to observe that the cells internalized these substances by bristle-coated micropinocytosis or multiple bristle-coated micropinocytosis as well. The mechanism of phagocytosis was employed mainly when the substances were aggregated into clumps (see Section 5.3).

It is generally assumed that bristle-coated pits are specialized for internalizing substances by adsorptive pinocytosis (see Section 1.4). The formation of bristle-coated pits is frequently observed in vivo, especially in endothelial cells (see Section 1.3). It has not yet been established whether

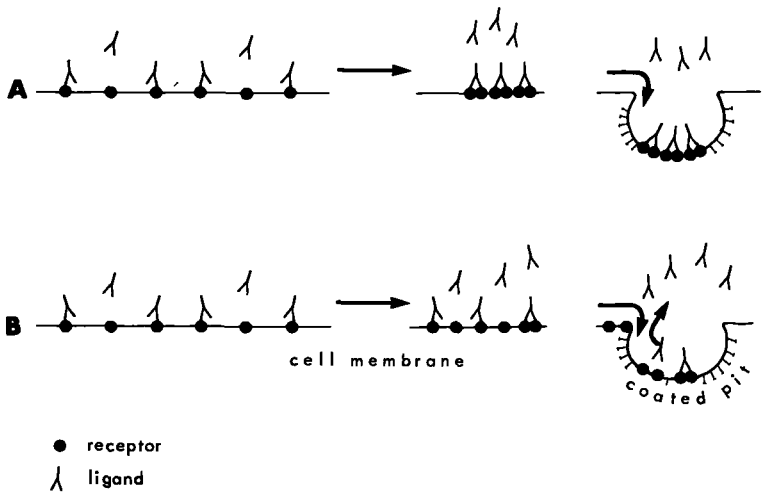


Figure 42 Possible explanation for differences in affinity and rate of binding of N-acetylglucosamine or mannose terminated glycoproteins to the same type of receptor on Kupffer and endothelial cells.
Receptors are initially distributed over the cell membrane before clustering occurs. A certain amount of ligand bound to the receptors may again dissociate. The chance of dissociation is greatest as long as there is only one binding event between receptor and ligand. In the case of multiple ligand/receptor interaction, which only occurs at sufficient local density of receptors (patching), the chance of dissociation will become considerably less. Thus, if in case A the patching and clustering is more rapid, the risk of dissociation during binding and internalization will be less than in case B. If the affinity of the receptor for one glycoprotein is less than that for another (i.e., AGOR versus AHOR), the patching in situation A might be so effective that the lower affinity is masked by multiple receptor/ligand interactions, whereas in case B the lower affinity would lead to increased dissociation. A prerequisite for this model is multivalency of the ligand. However, multivalency of the receptor is also possible. The rate of receptor clustering can depend on various factors, such as the speed at which the receptor is able to move over the cell membrane, the distance over which the receptor has to travel before reaching the pit (which in turn depends on the number of receptors versus the number of coated pits) and the speed at which a pit internalizes while receptor clustering is still taking place.

bristle-coated micropinocytosis also takes place in the absence of a receptor bound ligand. However, it has been demonstrated in this study that isolated sinusoidal cells treated with pronase at 37°C were still able to pinch off bristle-coated pits in the absence of added ligand (see Section 5.3). Since the cells were probably depleted of the majority of their receptors, this result indicates that the mechanism of bristle-coated micropinocytosis is a continuous and regular process which is receptor and ligand independent. Since

it is the only mechanism of internalization observed in endothelial cells, it seems to serve not only for adsorptive but also fluid-phase pinocytosis. Based on a diameter of 0.1 µm-0.15 µm (see Section 1.4) for bristle-coated pits (corresponding to a volume of 0.52 to 1.8×10^{-12} µl) and a rate of fluid-phase pinocytosis of 3.61×10^{-10} µl per min per endothelial cell (see Section 3.3.4), it can be calculated that one cell will internalize at least 200-690 bristle-coated micropinocytic vesicles per minute. The results given in Section 3.3.4 have shown that the rate of adsorptive endocytosis can be 5000 times higher than that of fluid-phase endotytosis. The great difference might be explained in part by induction of additional vesicle formation. However, a very highly increased rate of vesicle formation is not observed and might not be necessary if the efficiency of receptor clustering is so high that all coated pits present are almost immediately filled with a high concentration of bound ligand. Several reports mention that the amount of fluid-phase endocytosis before and after the addition of a ligand does not change (Besterman et al., 1982; Goud et al., 1981; Steinman et al., 1983), indicating that no induction of additional vesicle formation occurs upon adsorption of a ligand. Moreover, when sinusoidal cells were incubated with HRP at 37°C for only 15 sec, all coated pits present on the cell membrane were already filled with the ligand (see Section 5.3), indicating that a very efficient clustering of receptors had indeed occurred. Since receptor recycling appears to occur continuously, even in the absence of ligand (see Section 5.4.4), the clustering of receptors in the pits might be a ligand independent process. This does not exclude, however, the possibility that an increase in the rate of receptor clustering and recycling occurs upon addition of a ligand.

Unlike the internalization by bristle-coated pits, phagocytosis has never been observed to occur without adsorbed ligand and therefore seems to require previous binding of a substance to the cell membrane. The process of phagocytosis, by which pseudopodia must form and engulf a particle, is accomplished at a slower rate than is bristle-coated micropinocytosis. For complete internalization of a yeast cell by phagocytosis, a time period of about 4 min is required (Barelds et al., 1982), whereas bristle-coated micropinocytosis occurs within 15 seconds (see Section 5.3). This would imply that substances which are phagocytosed remain for a relatively longer time at the cell membrane than do those which are internalized by bristle-coated micropinocytosis. Indeed, it was shown at the ultrastructural level that at 37°C colloidal and particulate substances which are taken up by, among others, phagocytosis are found to be adsorbed to the membrane of Kupffer cells, in contrast

to HRP, which is internalized by bristle-coated micropinocytosis exclusively. In agreement with this, endothelial cells, which internalize substances by bristle-coated micropinocytosis only, showed no adsorption of substances on the cell membrane at 37°C; they were found only in the coated pits. These observations imply that large particles which are merely phagocytosed are internalized more slowly than are substances which are also internalized by bristle-coated micropinocytosis. Support for this assumption comes from the observation that, when large latex particles (0.65 µm) were injected and the liver was perfused 5 min later with smaller latex particles (0.23 µm), several Kupffer cells which had already internalized the small particles but were still in the process of phagocytosing the larger ones, were observed. Therefore, the physical size of an injected substance might determine the rate at which it is endocytosed. A higher rate of clearance of smaller particles has also been described for colloidal carbon as compared with foreign red blood cells (Halpern et al., 1957). It is of importance therefore to always check for the mechanism of internalization before drawing conclusions upon the clearance rate of an injected RES test substance.

The internalization step is completed by the formation of an intracellular phagocytic or bristle-coated micropinocytic vesicle. Although it is generally believed that bristle-coated pits are internalized by the cell and form coated vesicles, it is difficult to judge from ultrathin sections whether actual internalization occurs. Willingham et al. (1981b) claimed that during endocytosis of α_2-macroglobulin by fibroblasts, coated pits invaginate and form a long neck but never completely pinch off. Fan et al. (1982), however, showed on serial sections that during the uptake of insulin and ferritin by adipocytes about 50% of the coated vesicles are really closed and have no connection with the outer membrane. Since the long neck described by Willingham was never observed in this study, we tend to believe that at least a portion of the vesicles is actually closed.

6.4.3 Intracellular transport of endocytosed substances

After internalization by bristle-coated micropinocytosis or phagocytosis, all substances were eventually found in large smooth-walled vesicles (≥ 0.7 µm) (see Section 5.3). In Kupffer cells, the substances were sometimes also located in fuzzy-coated vesicles. During incubation with HRP, it was observed in both cell types that the large smooth-walled vesicles were apparently derived from bristle-coated vesicles which had quickly fused with each other

(see Section 5.3).

There are several other speculations about the development of these smooth-walled vesicles. Willingham et al. (1981b) suggested that, in fibroblasts, the vesicles arise by a bulge from the long neck which forms the channel between a coated pit and the outer membrane. These large vesicles were designated as receptosomes. The large vesicles have also been considered to be too large to arise from merely fusion of smaller ones. It was suggested that preexisting smooth-walled structures (e.g., lysosomes) fuse with one or two coated vesicles (Praaning-van Dalen et al., 1982).

It can be calculated how many vesicles with a diameter of about 0.1 µm must fuse to give rise to a vesicle with a diameter of about 0.7 µm. To obtain the amount of membrane present around a vesicle of 0.7 µm, 49 vesicles of 0.1 µm are necessary. However, to reach the volume of a 0.7 µm vesicle, fusion of 343 vesicles of 0.1 µm is required, which would give rise to a great excess of membrane (of 294 vesicles). This large volume, however, might also be reached merely by diffusion. It is not unlikely that fusion of 49 vesicles and the diffusion necessary to fill up the volume might both occur within a fraction of a minute.

No structures resembling the receptosomes described by Willingham, (i.e., vesicles of about 0.4 µm, with a broad rim along part of their membrane and with a smaller vesicle inside) were ever observed in Kupffer or endothelial cells. Therefore, it seems likely that the large smooth-walled vesicles present in these cells arise from fusion and not from a bulge that pinches off from a channel.

It is still unclear how a fusion event might be regulated. There are several indications for an induction of the fusion by a decrease in the pH. Firstly, the fusion of some viruses with plasma membranes (Marsh et al., 1982) are pH dependent. Secondly, endocytic vesicles become acidic prior to fusion with the (also acidic) lysosomes (Tycko & Maxfield, 1982). Thirdly, it has been suggested that agents which increase the internal pH of the vesicles such as chloroquine and ammonia inhibit the fusion of endocytic vesicles with lysosomes (Tolleshaug & Berg, 1979; Gordon et al., 1980). Finally, the involvement of a proton gradient in the transfer of a ligand from a coated pit to a large smooth-walled vesicle is indicated by the inhibition of this process by monensin, a monovalent ionophore which disrupts proton gradients (Dickson et al., 1982b). Fusion might probably also depend on the action of the Ca^{++} binding protein calmodulin, since various inhibitors of calmodulin prevent this event (van Berkel et al., 1982b). Fusion seems to be

independent of the type of ligand present in the vesicles, since smooth-walled vesicles filled with various types of ligands were observed in this study (see Section 5.3).

The coat of bristle-coated pits and vesicles consists of a molecule called clathrin. Several molecules of this protein are able to form a basket-like network of pentagons and hexagons. During invagination of the coated membrane, a rearrangement of this network in which hexagonal facets are somehow exchanged for pentagons that allow the network to close, occurs (Ungewickell & Branton, 1982). Clathrin might be needed for the vesicle formation and/or the stabilization of the membrane curvature as well as for further intracellular transport of the vesicles and for fusion events. The mechanism by which bristle-coated vesicles loose their coats after fusion is still unknown. Patzer et al. (1982) reported that clathrin can be released in a soluble form when isolated coated vesicles are incubated with ATP and a cytosol fraction. Dissociated clathrin looses its basket-like appearance and thus might not be recognized at the ultrastructural level. Thus, dissociated clathrin might be present all over the cell, ready to reassociate at the site of internalization.

Smooth-walled vesicles which had lost their clathrin coat showed a rim of ligand attached to their membranes or a distribution of ligand throughout the contents. These two phenomena appeared to represent subsequent steps in the internalization process (see Section 5.3). The ultimate fate of the ligands could not be established by ultrastructural investigation, but it is likely that they all accumulate in dense bodies or lysosomes. Apart from the large smooth-walled and bristle-coated vesicles, tubular vesicles were observed during the intracellular transport of HRP. Fusion between these tubules and larger vesicles seemed to occur. HRP is most likely taken up by Kupffer and endothelial cells by the mannose/N-acetylglucosamine recognition system (see Section 5.3). On basis of the observations on the uptake of HRP, a possible mechanism of internalization and transport of mannose or N-acetylglucosamine terminated glycoproteins in Kupffer and endothelial cells is as depicted in Fig. 43.

Since only ligands were demonstrated in this study, the fate of receptors is not known. The results given in Chapter 5 indicate that at least some types of receptors in sinusoidal cells are dissociated from their specific ligands before the ligand reaches the lysosomal compartment and that the receptors recycle back to the membrane. If endothelial cells are indeed able to internalize up to 700 vesicles per min, not only the receptors but also the internalized membrane will have to recycle back. Nothing is yet known about

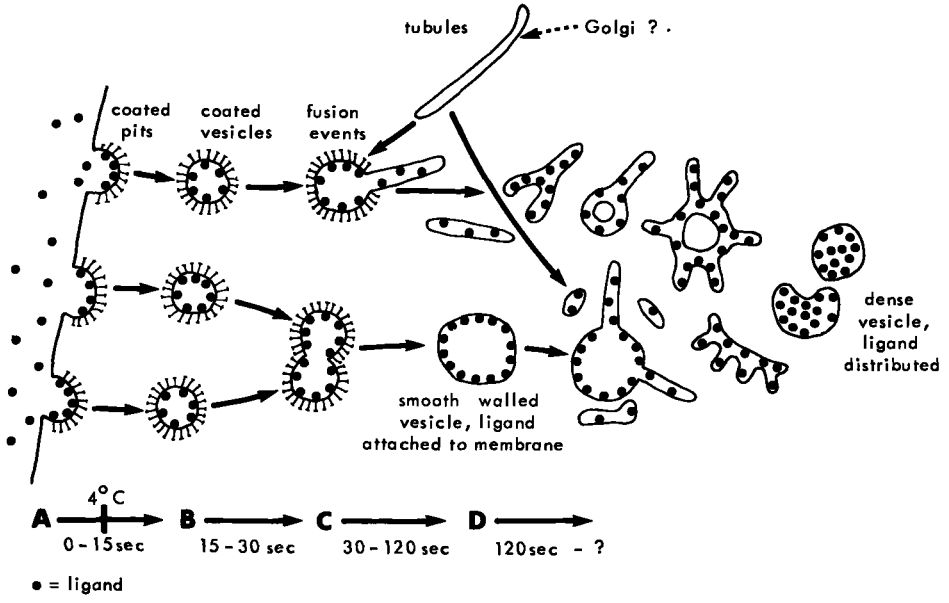


Figure 43 Proposed mechanism by which N-acetylglucosamine or mannose terminated glycoproteins might be internalized and transported by Kupffer and endothelial cells.

the mechanisms by which this is accomplished. It would be interesting to know what factors determine the fates of a ligand, a receptor and a membrane. Solving this problem might open up the possibility to manipulate the intracellular fate of certain endocytosed ligands, e.g., drugs. Moreover, it might give some insight into the mechanisms of RES blockade.

6.4.4 Possible mechanisms of RES blockade

RES blockade or depression, generally induced by intravenous administration of colloids, is still used as an experimental procedure for the study of the pathophysiology of the RES. Especially colloidal carbon is employed to induce RES blockade. Some theories which attempt to explain the mechanisms of RES blockade were described in Chapter 1.

In principle, all factors which influence the clearance rate of an injected substance (see Section 6.1) might be involved in the depression of the clearance during RES blockade. The results of our study allow the elimination of some of these factors.

Aggregation of colloids in the blood when given at a high dose might

decrease the hepatic blood flow and result in a decrease in the rate of clearance of a subsequently injected RES test substance. In the experiments of this study, the observed aggregation of colloidal carbon and to a small extent colloidal silver did not result in a complete blockade in the clearance by the liver cells.

The clearance of none of the substances was dependent on serum factors; thus, depletion of opsonins is unlikely to cause a decrease in the clearance of these substances.

A clearance decrease by saturation of the binding to the cell membrane might also occur (Norman, 1973). When a relatively high concentration of colloidal carbon was injected and the liver was perfused 5 min later with a latex containing medium, the endothelial cells ingested more latex than normal (see Section 5.3). This suggests that more latex beads were available for endothelial cells and thus a lesser (although not measurable at the ultrastructural level) amount had been taken up by Kupffer cells, which still showed adsorbed clumps of colloidal carbon at their cell membranes. Since the Kupffer cells were still quite active in the uptake of latex beads, such a temporary occupation of the cell membrane does not result in a complete RES blockade.

Kolb et al. (1981) found that colloidal carbon affects the recycling of receptors for galactose in Kupffer cells and thus inhibits the uptake of another ligand. No such effect on the physiological state of the cells which affected their intrinsic endocytic capacity was observed in the present study. In vitro experiments showed that different substances could be internalized along with colloidal carbon; thus, no inhibition of the internalization of each other seemed to occur (see Section 5.3).

6.5 IS THERE A POSSIBLE DEFINITION FOR THE RES?

It was proposed in Chapter 1 that the clearance of several RES test substances is not done by macrophages only and that apart from phagocytosis other mechanisms of internalization are also involved in the clearance. The results discussed in this thesis proved that the assumptions were correct. In addition to Kupffer cells, endothelial cells of the liver also contributed significantly to the clearance of colloidal substances and, in addition to phagocytosis, bristle-coated micropinocytosis is involved in the clearance. Therefore, the RES colloid clearance test is not an expedient to determine the phagocytic capacity of the macrophages (especially those of the liver)

lining the blood stream.

Moreover, the clearance of colloidal substances is not always an accurate reflection of the intrinsic endocytic capacity of the cells. At low doses, the clearance reflects only the blood flow and at high doses, the adsorption of colloids to the cell membrane will be the rate limiting step in the clearance instead of the internalization. Since the extent of adsorption will depend on the nature of the colloid, different colloids might be cleared at different rates, which only partly represent the actual endocytic capacity of the cell.

The results described in this thesis might form a basis for a revised definition of the RES. The definition of it as a population of cells which is able to remove colloidal, particulate or other substances generally used for a "RES" test proved to be impracticable. It was shown in this thesis that the cell population contributing to the clearance of different RES test substances is not a definite one, since Kupffer as well as endothelial and even sometimes parenchymal cells may be involved. In addition, different uptake mechanisms may be involved in the clearance of distinct substances and this will lead to great variations in clearance characteristics.

It is also impractical to regard the RES as being the population of all macrophages in the body designated as MPS (see Section 1.6). Since according to this definition those macrophages are also included which do not line the blood stream, only part of the system will be involved in the clearance of injected test substances. Therefore, it is unjustified to test the function of this system by its clearance capacity. This, of course, especially holds if the test substance used is also cleared by cells other than macrophages.

Probably, the best solution is to limit the definition of the RES to the definite population of macrophages lining the blood stream which is active in the clearance of substances by one specific mechanism, i.e., phagocytosis. In that case, it will be possible to test the clearance function of the RES if the correct test substance is used, e.g., large latex beads or erythrocytes, although one should always be aware of the general shortcomings of clearance studies. According to this definition, all results reported in the literature on the clearance of colloidal or other particulate materials which might be taken up by cells other than macrophages and not by phagocytosis cannot be regarded as reflecting the RES function. Although endothelial liver cells would not be included in this definition of the RES, these cells form a separate cell population which proves to have an important physiological function in the clearance of a variety of substances such as glycoproteins (e.g., lysosomal enzymes), mucopolysaccharides and colloids.

SUMMARY

The mammalian liver makes an important contribution to the clearance of potentially toxic endogenous and exogenous substances from the blood. However, there are striking discrepancies in the rate at which the liver is able to clear substances of different nature. This indicates the involvement of various factors which control the rate of clearance of distinct substances. These factors include the size of the population of liver cells involved in the clearance and the mechanims by which these cells are able to recognize and internalize a specific substance.

Certain substances, e.g., colloids, are cleared by the liver in a very specific way. After intravenous injection, colloids are removed from the bloodstream in an exponential way, which indicates a one-compartment clearance system. The liver is able to clear most colloids at such a high rate that nearly all of the substance is removed from the blood during its first passage through the organ. Therefore, the rate of clearance will primarily depend on the rate of blood flow through the liver.

Light microscopic or autoradiographic studies on the localization of colloids within the liver have prompted investigators to believe that the substances are cleared exclusively by a specific type of liver cell, the Kupffer cell. Kupffer cells are macrophages which are able to internalize (i.e., endocytose) certain substances by a specific mechanism: phagocytosis. Characterization of the phagocytic process requires ultrastructural studies. Nevertheless, it was concluded that Kupffer cells clear colloids by phagocytosis on the basis of light microscopic observations only.

The population of all macrophages in the body lining the blood stream and principally able to clear colloids by phagocytosis was designated as the reticuloendothelial system (RES). Since most injected colloids are primarily cleared by the liver, the Kupffer cells are thought to represent 85-90% of the RES. Although other denominations and more extensive definitions of the RES also exist, the means for testing the functional capacity of the system are basically the same. The most frequently used is the so-called colloid clearance test based on the determination of the rate of clearance of injected colloids from the plasma.

During the passage of time, other substances such as denatured proteins and immune complexes have been reported to also be cleared by the RES. Ultrastructural studies on the localization of substances which are supposed to be cleared by the RES (a.o., some colloids) revealed that some of them are also cleared by the endothelial cells of the liver. Since these cells appeared not to be able to phagocytose, these substances must have entered the cells by another mechanism. As a result of this discovery, not only the colloid clearance test but also the RES clearance characteristics in general and thereby the complete definition of the RES became questionable.

To achieve a better understanding of the mechanisms and cell types involved in the clearance of various substances reported to be removed by the RES, the study described here involved the determination of the rates at which test substances injected into rats were removed from the blood and the assessment of the capacity of the rat liver and other organs to clear these substances. Moreover, the involvement of Kupffer and endothelial cells in the clearance was studied both in vivo and in vitro (i.e., after isolation and purification of the cells). The in vivo and in vitro studies were performed quantitatively (by the use of radioactively labeled substances) as well as semi-quantitatively (by localization of substances at the ultrastructural level). The mechanisms by which the cells endocytosed were also studied quantitati-

vely and ultrastructurally, amongst others, by the use of certain inhibitors of cellular processes. These studies, which were mainly performed in vitro, were supplemented in some cases by studies using an in situ perfused liver system.

To accurately determine the amount of substance endocytosed in vivo, Kupffer and endothelial cells were isolated and purified. The most suitable isolation method had to be developed for in vivo as well as in vitro investigations. An isolation procedure based on the digestion of the liver by the proteolytic enzyme pronase at a temperature of 10°C appeared to be more applicable for both experimental approaches than other isolation methods. Because of the low temperature, no intracellular digestion of in vivo endocytosed substances occurred during isolation. Moreover, redistribution of substances released from damaged cells to viable ones during the isolation procedure was inhibited. For in vitro studies, the cells obtained by this isolation method appeared to be intact, easy to identify and still in the possession of the capacity to endocytose substances which are also endocytosed in vivo.

The substances used in this study were: 1) polyvinylpyrrolidone (PVP), a synthetic polymer which can be considered as a suitable marker for a specific mechanism of endocytosis, i.e., nonadsorptive fluid-phase endocytosis; 2) heparin, a mucopolysaccharide which is a normal constituent of the blood; 3) orosomucoid, a plasma glycoprotein which had been modified in 3 ways: asialoorosomucoid (ASOR) with galactose terminated oligosaccharide chains, agalactoorosomucoid (AGOR) and ahexosaminoorosomucoid (AHOR) with N-acetylglucosamine and mannose terminated oligosaccharide chains, respectively; 4) horseradish peroxidase, an exogenous glycoprotein which also possesses mannose terminated oligosaccharide chains; and 5) several colloids, including heat denatured colloidal albumin, colloidal silver and colloidal carbon. Finally, as a particulate test substance, latex beads of different sizes were used.

After injection of the radioactively labelled substances ASOR, AGOR, AHOR and colloidal albumin, they were all cleared in a rapid exponential way, with half lives in the blood of about 2 min. Heparin and PVP were cleared in a more slow, nonexponential way, exhibiting half lives of about 20 min and 18 h, respectively. After an established period, the primary location of all these substances in the body proved to be the liver, except for heparin which was also found in the kidneys. When compared with that of the marker for fluid-phase endocytosis PVP, the rates of clearance for all other substances by the liver was much higher, indicating that these substances were cleared in a concentrating, adsorptive way. The rate at which the liver was able to take up ASOR and a low dose of colloidal albumin was so high that the clearance rate simply reflected the rate of blood flow through the liver. The difference between this high rate and the rate at which a soluble substance (e.g., PVP) passively enters the liver cells reflects the difference between the maximal rate and the minimal rate at which a substance can possibly be cleared by the liver. These rates appeared to differ by a factor of at least 5000.

Isolation of Kupffer and endothelial cells (i.e., sinusoidal cell preparations) revealed that most of the radioactively labeled substances tested were primarily cleared by these cell types. ASOR and PVP were most probably cleared mainly by the parenchymal cells of the liver. The capacity of Kupffer cells to endocytose PVP, colloidal carbon and latex beads in vivo was higher than that of the endothelial cells. For colloidal albumin, AHOR and colloidal silver, the endocytic capacity was about the same for both cell types, whereas the capacity of endothelial cells to endocytose heparin, AGOR, and HRP in vivo was higher than that of the Kupffer cells. Since

there are 2 to 3 times more endothelial cells present in the rat liver than Kupffer cells, the contribution of endothelial cells to the clearance of the latter substances as well as colloidal albumin and colloidal silver was higher than that of Kupffer cells.

The observed differences in the endocytic capacity between Kupffer and endothelial cells in vivo were largely confirmed by experiments in vitro. However, in contrast to the in vivo situation, endothelial cells were able to endocytose latex beads of up to a diameter of 0.65 µm in vitro.

In vitro, the specific characteristics of the endocytic processes in sinuoidal cells could be studied by manipulating the incubation conditions and by the addition of inhibitors of specific cellular processes. A strong reduction in the uptake of all substances tested was observed when incubations were performed at 4°C. The uptake of AGOR, AHOR and HRP was highly dependent on the presence of calcium, whereas that of colloidal albumin was only slightly so. The uptake of all other substances was Ca^{++} independent. The addition of an inhibitor of mitochondrial respiration caused a strong decrease in the uptake of AGOR, AHOR, HRP and colloidal albumin, whereas the inhibition of glycolysis caused only a moderate reduction in the uptake of these substances. Treatment of the cells with an inhibitor of the action of microfilaments resulted in a slight decrease in the uptake of AGOR and AHOR, while the uptake of colloidal albumin was more strongly inhibited. The addition of an inhibitor of the action of microtubules did not result in a decrease in the uptake of AGOR or AHOR, whereas the uptake of colloidal albumin was clearly reduced. An increase in the intravesicular pH in the cells caused by the addition of chloroquine reduced the uptake of AGOR and AHOR to about 50%.

When incubated with various substance concentrations, the uptake of AGOR, AHOR and colloidal albumin appeared to obey Michaelis-Menten kinetics, indicating that the substances are adsorbed to the cell membrane prior to internalization. It was observed at the ultrastructural level that adsorption also occurred during the uptake of HRP, colloidal silver, colloidal carbon and latex beads.

These results indicate that binding sites or receptors which are able to recognize the substances are present on the cell membranes of both Kupffer and endothelial cells. When the cells were pretreated with pronase at 37°C, endocytosis of all substances except latex beads was completely inhibited, indicating that most of these binding sites or receptors had been degraded. The recognition of the substances by the cells was apparently not mediated by certain serum factors, since endocytosis of all substances tested was at least as efficient in the absence of these factors. The endocytosis of AGOR, AHOR and HRP was strongly inhibited by the addition of mannan, a polysaccharide composed of mannose units. These results indicate that the uptake of AGOR, AHOR and HRP was mediated by a receptor that is able to specifically recognize mannose units.

Based on the observations that a 37°C pronase treatment resulted in the complete degradation of receptors whereas a 10°C pronase treatment did not, it was concluded that only a portion of the total number of receptors is present on the cell membrane and that the intracellular supply of receptors normally forms part of a receptor recycling system which is inhibited at a temperature of 10°C.

The mechanisms by which the various test substances were endocytosed by both Kupffer and endothelial cells were studied in more detail at the ultrastructural level. For the uptake of the substances by endothelial cells, only one mechanism was discovered, namely, the so-called bristle-coated micropinocytosis. During this method of uptake, an invagination of the cell of 0.1-0.15 µm in diameter resulted in the formation of a vesicle of the same

diameter. These vesicles contained a bristle-like coat. During internalization of latex beads larger than 0.15 µm, several invaginations were formed next to each other. Bristle-coated vesicles appeared to fuse very rapidly with each other to form larger smooth-walled vesicles. For Kupffer cells, two mechanisms of endocytosis were revealed during the uptake of the test substances. All substances except latex beads ≥ 0.29 µm were taken up by bristle-coated micropinocytosis. The larger beads were taken up by phagocytosis. The latter mechanism was also observed during the uptake of aggregated clumps of smaller latex beads, colloidal silver and colloidal carbon.

In conclusion, the clearance kinetics of a substance supposed to be taken up by the RES do not indicate by which mechanism and cell type the substance is taken up. Depending on a variety of factors, which are completely independent of the intrinsinc endocytic capacity of the cells under study, different clearance kinetics may be found for different substances. Colloids are not as is often assumed cleared exclusively by Kupffer cells and by the mechanism of phagocytosis. Endothelial cells appeared to be active in endocytosing all substances tested. Discrimination between phagocytosis and other endocytic mechanisms cannot be accomplished by clearance studies nor by determination of the endocytic capacity under the influence of various inhibitors of cellular processes.

If it is the aim to test for clearance by Kupffer cells and by phagocytosis exclusively, the correct test substance should be used, e.g., large latex beads or erythrocytes. However, one should always be aware of the general shortcomings of clearance studies.

The definition of the RES as a population of cells which is able to remove colloidal, particulate or other substances as generally used for a "RES" test appeared to be impracticable. In this thesis, it was therefore suggested the definition of the RES be limited to the definite population of macrophages which line the bloodstream and which are active in the clearance of substances by one specific mechanism, i.e., phagocytosis. Although endothelial liver cells would not be included in this definition, these cells constitute a separate cell population which plays an important role in the clearance of a variety of blood borne substances.

SAMENVATTING

De lever vervult een belangrijke taak in de verwijdering of klaring van in het bloed aanwezige stoffen met een mogelijke schadelijke werking. De snelheid van deze klaring verschilt echter van stof tot stof. Dit wijst erop dat verschillende factoren de klaringssnelheid bepalen. Hierbij kan gedacht worden aan de grootte van de bij de klaring betrokken levercelpopulatie en aan het mechanisme waarmee de levercellen verschillende stoffen herkennen en opnemen.

Bepaalde stoffen, waaronder colloïdale deeltjes, worden op zeer specifieke wijze door de lever geklaard. De verdwijningscurve van intraveneus geïnjecteerde colloiden vertoont een exponentiëel verloop. Dit duidt erop dat de klaring door één compartiment (orgaan) wordt verzorgd. De lever kan een lage concentratie van colloïden meestal zo effectief klaren, dat het bloed al na de eerste passage door de lever vrijwel geheel is ontdaan van colloïd. In dit geval wordt de klaringssnelheid voornamelijk bepaald door de snelheid waarmee het bloed door de lever stroomt.

Op grond van lichtmicroscopische en autoradiografische waarnemeningen is lang gesteld dat colloïden uitsluitend worden opgenomen (geëndocyteerd) door één type levercel, de Kupffercel. Kupffercellen zijn macrofagen en kunnen stoffen endocyteren mede met behulp van een specifiek mechanisme, namelijk fagocytose. Het onderscheid tussen fagocytose en andere endocytoseprocessen kan alleen goed worden gemaakt op basis van ultrastructureel onderzoek. Desalniettemin meende men aan de hand van slechts lichtmicroscopische waarneming te kunnen stellen dat colloïden uitsluitend door Kupffercellen en alleen via fagocytose worden opgenomen.

De populatie van alle macrofagen die aan de bloedbaan grenzen en die in principe in staat zouden zijn om geïnjecteerde colloïden via fagocytose te klaren, heeft men het reticuloendotheliale systeem (RES) genoemd. Daar de meeste geïnjecteerde colloïden door de lever verwijderd worden, wordt vaak aangenomen dat het RES voor 85-90% door de Kupffercellen vertegenwoordigd wordt. Hoewel aan het RES ook andere benamingen en meer uitgebreide definities zijn toegekend, komen de methoden die gebruikt worden om de functionele capaciteit van het systeem te testen sterk overeen. De meest gebruikelijke methode is de zogenaamde colloïdklaringstest, waarbij de snelheid wordt bepaald waarmee een geïnjecteerd colloïd uit het bloed wordt verwijderd.

In de loop der tijd werd gemeld dat ook andere stoffen, zoals immuuncomplexen en gedenatureerde eiwitten, door het RES worden geklaard. Toen eenmaal de exacte lokalisatie van geklaarde stoffen via ultrastructureel onderzoek mogelijk was geworden, bleek dat diverse stoffen (o.a. bepaalde colloïden), waarvan men aannam dat ze door het RES en dus door de Kupffercellen werden geklaard, ook door een ander type levercel, de endotheelcel, werden opgenomen. Aangezien deze cellen niet in staat zijn te fagocyteren, moeten deze stoffen via een ander mechanisme zijn geëndocyteerd. Deze ontdekking leidde ertoe dat niet alleen de colloïdklaringstest, maar ook de klaringskarakteristiek van het RES in het algemeen en hierdoor de totale definitie van het RES op losse schroeven kwamen te staan.

De hier beschreven studie werd ondernomen voor een beter begrip van de mechanismen en celtypen die betrokken zijn bij de opname van stoffen, die door het RES zouden worden geklaard. De snelheid werd gemeten waarmee geïnjecteerde testsubstanties uit het bloed van de rat worden verwijderd en de capaciteit werd bepaald waarmee de lever en andere organen in staat zijn deze stoffen op te nemen. Daarnaast werd de rol van Kupffer- en endo-

theelcellen bij het opnameproces zowel in vivo als in vitro onderzocht. De opname werd kwantitatief bestudeerd m.b.v. radioactief gemerkte stoffen en kwalitatief via ultrastructureel onderzoek. De mechanismen waarmee de cellen de stoffen endocyteerden werden eveneens kwantitatief en ultrastructureel bestudeerd, o.a., met behulp van remmers van bepaalde cellulaire processen. Deze laatste studies werden voornamelijk in vitro verricht en aangevuld met experimenten waarbij levers in situ werden doorstroomd met een vloeistof die de testsubstanties bevatte.

Om de hoeveelheid in vivo door de cellen opgenomen testsubstantie nauwkeurig te kunnen kwantificeren, werden de Kupffer- en endotheelcellen na het klaringsproces uit de lever geïsoleerd en vervolgens gezuiverd. Voor een goede kwantificering van de hoeveelheid in vivo geëndocyteerde stof is van belang dat tijdens de isolatieprocedure geen afbraak en daarom verlies uit de cellen plaatsvindt. Hiertoe moest een geschikte isolatiemethode worden ontwikkeld. Verschillende methoden werden onderzocht. Er trad geen afbraak van de geëndocyteerde stof op wanneer de lever met behulp van het eiwitsplitsende enzym pronase bij een temperatuur van 10°C werd verteerd. Daarnaast is het voor de kwantificering van belang dat de in vivo geëndocyteerde stoffen die tijdens de isolatieprocedure vrijkomen uit beschadigde cellen niet opnieuw worden opgenomen door de intacte cellen. Ook dit proces werd door de isolatie bij lage temperatuur voorkomen. De cellen die m.b.v. deze isolatiemethode werden verkregen waren intact, goed te identificeren en in staat stoffen te endocyteren die ook in vivo worden opgenomen. De gebruikte isolatiemethode was daarom ook geschikt voor in vitro endocytose studies.

De gebruikte testsubstanties waren: 1) polyvinylpyrrolidon (PVP), een synthetisch polymeer dat een goede indicator is voor een specifiek endocytosemechanisme, de vloeistoffase endocytose, waarbij adsorptie van de stof aan de celmembraan geen rol speelt; 2) het mucopolysaccharide heparine, een normaal bestanddeel van het bloed; 3) orosomucoide, een glycoproteïne uit het bloed dat op drie verschillende manieren gemodificeerd was: asialoorosomucoide (ASOR) dat op galactose eindigende suikerketens bezit, agalactoorosomucoide (AGOR) en ahexosaminoorosomucoide (AHOR), die respectievelijk op N-acetylglucosamine en mannose eindigende suikerketens bezitten; 4) horseradish peroxidase (HRP), een lichaamsvreemd glycoproteïne dat eveneens op mannose eindigende suikerketens bezit; 5) een aantal colloïden: hitte gedenatureerd colloïdaal albumine, colloïdaal zilver en colloïdaal koolstof; en 6) latex bolletjes met verschillende diameters.

De radioactief gemerkte stoffen ASOR, AGOR, AHOR en colloïdaal albumine werden na intraveneuze injectie op exponentiële wijze uit de bloedbaan verwijderd. De halfwaardetijd van deze stoffen bedroeg ongeveer 2 minuten. De klaring van heparine en PVP verliep aanzienlijk langzamer en niet exponentiëel. De halfwaardetijd van deze stoffen was respectievelijk 20 minuten en 18 uur. Alle stoffen bleken voornamelijk door de lever te zijn opgenomen. Een uitzondering vormde heparine, dat ook door de nieren werd geklaard. Alle stoffen werden veel sneller door de lever opgenomen dan de indicator voor vloeistoffase endocytose, PVP, hetgeen erop wijst dat deze stoffen via adsorptie, geconcentreerd, door de cellen werden geklaard. De lever was in staat om met name ASOR en colloïdaal albumine in lage doses zo efficiënt uit de bloedbaan te verwijderen, dat de klaringssnelheid van deze stoffen volledig door de bloeddoorstromingssnelheid van de lever werd bepaald. Het verschil tussen deze hoge klaringssnelheid en die van PVP weerspiegelt het verschil tussen de maximale en minimale snelheid waarmee een willekeurige oplosbare stof door de lever geëndocyteerd kan worden. Dit verschil bleek tenminste een factor 5000 te bedragen.

Na isolatie van Kupffer- en endotheelcellen kon worden vastgesteld dat de meeste testsubstanties voornamelijk door deze sinusoïdale cellen werden

geklaard. Alleen ASOR en PVP werden hoofdzakelijk door de parenchymcellen van de lever geklaard. De capaciteit van de Kupffercellen om PVP, colloïdaal koolstof en latex bolletjes in vivo te endocyteren was groter dan die van de endotheelcellen. Colloïdaal albumine, colloïdaal zilver en AHOR werden door Kupffer- en endotheelcellen in ongeveer gelijke mate opgenomen, terwijl de capaciteit van de endotheelcellen om in vivo heparine, AGOR en HRP te endocyteren groter was dan die van de Kupffercellen. Aangezien de rattelever 2 tot 3 maal meer endotheelcellen bezit dan Kupffercellen, is het aandeel van alle endotheelcellen in de klaring van zowel de laatstgenoemde stoffen als van colloïdaal albumine, colloïdaal zilver en AHOR groter dan van de Kupffer cellen.

De verschillen in de capaciteit waarmee endotheel- en Kupffercellen de testsubstanties in vivo endocyteerden, werden in essentie ook in vitro gevonden. De endotheelcellen in vitro waren echter, in tegenstelling tot in vivo, wel in staat om latex bolletjes met een diameter tot en met 0,65 µm te endocyteren.

Door de incubatiecondities te variëren en remmers van bepaalde cellulaire processen toe te voegen was het mogelijk om in vitro specifieke karakteristieken van het endocytoseproces in de sinusoïdale cellen te bestuderen. Wanneer de incubaties werden verricht bij 4°C in plaats van 37°C, bleek er een sterke reductie in de opname van alle geteste substanties op te treden. De opname van AGOR, AHOR en HRP bleek sterk afhankelijk van de aanwezigheid van calcium in het incubatiemedium, de opname van colloïdaal albumine slechts in geringe mate. De opname van alle andere geteste stoffen was volkomen onafhankelijk van calcium. Remming van de mitochondriële ademhaling veroorzaakte een sterke vermindering van de opname van AGOR, AHOR, HRP en colloïdaal albumine, terwijl remming van de glycolyse slechts een geringe reductie tot gevolg had. Verstoring van de werking van microfilamenten veroorzaakte een lichte vermindering in de opname van AGOR en AHOR. De opname van colloïdaal albumine werd hierdoor sterker geremd. Remming van de werking van microtubuli had geen invloed op de opname van AGOR en AHOR, terwijl hierdoor de opname van colloïdaal albumine duidelijk verminderd was. Beïnvloeding van de pH van intracellulaire endocytoseblaasjes door toevoeging van chloroquine aan het incubatiemedium veroorzaakte een vermindering in de opname van AGOR en AHOR van ongeveer 50%.

Wanneer de cellen geïncubeerd werden met toenemende hoeveelheden van de testsubstanties, bleek de opname van AGOR, AHOR en colloïdaal albumine te voldoen aan Michaelis-Menten verzadigingskinetiek. Dit wees erop dat de substanties gedurende de opname aan de celmembraan geadsorbeerd werden. Op ultrastructureel niveau kon worden vastgesteld dat eveneens adsorptie plaatsvond tijdens de opname van HRP, colloïdaal zilver, colloïdaal koolstof en latex bolletjes.

Deze resultaten duiden erop dat zowel Kupffer- als endotheelcellen bindingsplaatsen of receptoren op hun celmembraan bezitten die in staat zijn de geteste stoffen te herkennen. Indien de cellen echter bij 37°C werden behandeld met pronase waren ze niet meer in staat om een van de geteste stoffen — met uitzondering van latex — te endocyteren. Hieruit kan worden geconcludeerd dat de meeste bindingsplaatsen of receptoren door deze behandeling worden afgebroken. In afwezigheid van serum was de opname van alle geteste stoffen minstens even efficiënt als in aanwezigheid ervan. De herkenning van de stoffen door de cellen was dus kennelijk niet afhankelijk van serumfactoren. De endocytose van AGOR, AHOR en HRP werd sterk geremd door de toevoeging van mannan, een polysaccharide dat is opgebouwd uit mannose-eenheden. Deze resultaten duiden erop dat de opname van AGOR, AHOR en HRP plaatsvindt via receptoren die specifiek mannose-eenheden kunnen herkennen.

Een behandeling van de cellen met pronase bij 37°C resulteerde in de totale afbraak van receptoren, terwijl een zelfde behandeling bij 10°C dit niet tot gevolg had. Hieruit kan worden geconcludeerd dat slechts een deel van het totale aantal receptoren zich op de celmembraan bevindt en dat de intracellulair aanwezige receptoren waarschijnlijk deel uitmaken van een receptor-recirculatie-systeem dat geremd wordt bij een temperatuur van 10°C.

De mechanismen waarmee Kupffer- en endotheelcellen de verschillende testsubstanties endocyteerden werden in detail bestudeerd op ultrastructureel niveau. In endotheelcellen werd slechts één endocytosemechanisme waargenomen, de zogenaamde bristle-coated micropinocytose. Bij deze wijze van opname werd vanuit een instulping van de celmembraan een intracellulair blaasje gevormd met een diameter van 0,1-0,15 µm. Deze blaasjes bezaten een borstelachtige membraan. Bij de opname van latex bolletjes groter dan 0,15 µm waren meerdere instulpingen tegelijk betrokken. De bristle-coated micropinocytoseblaasjes bleken zeer snel met elkaar te fuseren, waardoor grotere blaasjes ontstonden die over een gladde membraan beschikten. In Kupffercellen werden twee verschillende endocytosemechanismen geobserveerd. Alle testsubstanties — met uitzondering van latex bolletjes ≥ 0,29 µm — werden opgenomen via bristle-coated micropinocytose. De grotere latex bolletjes werden via fagocytose opgenomen. Fagocytose werd ook waargenomen bij de opname van aggregaten van kleine latex bolletjes, colloïdaal zilver en colloïdaal koolstof.

In conclusie: de klaringskinetiek van een stof waarvan wordt aangenomen dat hij door het RES wordt geklaard, geeft niet aan door welk mechanisme en door welk celtype de stof wordt opgenomen. Afhankelijk van een aantal factoren, die volkomen los staan van de intrinsieke endocytische capaciteit van de te bestuderen cellen, kunnen totaal verschillende klaringskinetieken gevonden worden voor verschillende testsubstanties. Colloïden worden niet, zoals vaak is aangenomen, uitsluitend door Kupffercellen en door middel van fagocytose geklaard. Endotheelcellen bleken actief te zijn in de klaring van alle geteste substanties. Het onderscheid tussen fagocytose en andere endocytosemechanismen kan niet uitsluitend aan de hand van klaringsexperimenten worden verkregen, noch door de invloed te bepalen van diverse inhibitoren van cellulaire processen op de endocytische capaciteiten van een cel. Indien door middel van klaringsexperimenten specifiek de fagocytose door Kupffercellen gemeten moet worden, is het noodzakelijk een juiste testsubstantie te kiezen, zoals grote latex bolletjes. Men moet zich echter wel bewust zijn van de algemene nadelen van klaringsstudies.

Het RES wordt nu gedefiniëerd als een populatie van cellen die colloïden, deeltjes, of andere stoffen kan klaren die algemeen als "RES" testsubstanties gebruikt worden. Deze definitie is onbruikbaar gebleken. Er wordt daarom in dit proefschrift een andere definitie voorgesteld waarin het RES gelimiteerd is tot die populatie van macrofagen die aan de bloedbaan grenzen en actief deelnemen aan de klaring van bepaalde substanties via één specifiek mechanisme, namelijk fagocytose. Hoewel leverendotheelcellen niet onder deze definitie zouden vallen, vormen deze cellen een aparte celpopulatie die desalniettemin een belangrijke rol speelt in de klaring van diverse stoffen uit het bloed.

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