Biochimica et Biophysica Acta, 645 (1981) 293–299
Elsevier/North-Holland Biomedical Press

BBA 79297

HEPATIC RECEPTOR FOR ASIALO-GLYCOPROTEINS

ULTRASTRUCTURAL DEMONSTRATION OF LIGAND-INDUCED MICROAGGREGATION OF RECEPTORS

VICTORIA KOLB-BACHOFEN

Institute of Biophysics and Electron Microscopy, Department of Medicine, University of Düsseldorf, Moorenstrasse 5, D-4000 Düsseldorf 1 (F.R.G.)

(Received January 20th, 1981)

Key words: D-Galactose; Lectin; Receptor; Endocytosis; Ultrastructure; (Rat hepatocyte)

Endocytosis of asialo-glycoproteins in hepatocytes is mediated by a lectin-like receptor with specificity for D-galactose. Early events of receptor-ligand interactions have been studied by ultrastructural analysis. Hepatocytes were isolated from the rat liver by collagenase perfusion and incubated with a galactosylated electron dense marker (gold-Gal-BSA, galactosylated bovine serum albumin adsorbed onto colloidal gold particles). Initial binding of gold-Gal-BSA particles occurs to receptors diffusely distributed at hepatic microvilli of the former space of Disse. No lectin activity was found in membrane areas that had formed in situ the region of hepatic cell contact or bile canaliculi. Microaggregation of receptor-ligand complexes is seen as an early consequence of particle binding. Microaggregates contain 2–5 particles and are located outside coated pits. After prolonged incubation larger clusters are formed, these are found associated with coated membrane areas. It is concluded that at least three steps precede the uptake of galactosylated proteins by hepatocytes. These are: (i) binding of ligand at diffusely distributed binding sites; (ii) local microaggregation of receptor-ligand complexes; (iii) formation of larger clusters and association with coated pits.

Introduction

A number of proteins and peptides have been shown recently to enter the cell by receptor-mediated endocytosis. This process involves the internalization of receptor-ligand complexes in specialized regions of the cell surface called coated pits. The coated pits invaginate to form the intracellular coated vesicles [1,2].

Little is known about the early events between formation of the receptor-ligand complex and its clustering in coated pits. In a few cases it has been shown that initially receptors are diffusely distributed in the plane of the membrane [3–8]. Evidence has accumulated that after binding of the ligand small clusters of receptors (microaggregates) are formed outside coated pits [9,10] with subsequent movement of aggregates to form larger clusters associated with coated pits.

In the present paper an ultrastructural analysis of the early events of receptor-mediated endocytosis of asialo-glycoproteins by hepatocytes is given. The hepatic receptor is a lectin with specificity for D-galactose residues [11] and has been found to mediate endocytosis via the coated pit/coated vesicle pathway [12]. I have found that in isolated rat hepatocytes initial binding of a D-galactosylated electron dense marker occurs randomly at hepatic microvilli outside coated pits. Ligand-binding induces the formation of receptor microaggregates and finally the association of larger clusters with coated pits. Thus the endocytosis of asialo-glycoproteins via the hepatic lectin shows characteristics similar to those described for some peptide hormones [13].

Material and Methods

Hepatocytes. Livers of male Wistar rats weighing 150–180 g were perfused with buffered collagenase (Boehringer, Mannheim, F.R.G.) and hepatocytes were isolated as described previously [14]. Freshly isolated hepatocytes were incubated for 1 h in Eagle's medium at 4°C before the start of experiments.

Galactosylated electron-dense marker. Colloidal gold particles covered with a layer of D-galactosylated protein were used as ligand. Gold granules of approx. 17 nm diameter were prepared as described by Horrisberger et al. [15] by reducing a 0.01% $AuCl_4$ solution with 1% sodium citrate. Galactosylated bovine serum albumin (Gal-BSA) was kindly provided by Dr. Herbertz (Diabetes Research Institute, Düsseldorf, F.R.G.). This synthetic glycoprotein was prepared following the method of Stowell et al. [16], it contained 5 mol D-galactose per mol albumin. Complexes of gold-Gal-BSA were prepared by incubating gold sol (20 ml) with Gal-BSA (0.5 mg in 0.1 ml 5 mM NaCl, pH adjusted to 7.0 with 0.2 M K_2CO_3). Complexes were stabilized by the addition of 0.2 ml of 1% poly(ethylene glycol). Gold particles were washed three times at 32 000 × *g* for 30 min and finally suspended in 0.5 ml PBS + 0.02% poly(ethylene glycol).

Analysis of receptor-ligand interactions. Hepatocytes, $2 \cdot 10^5$ cells in 100 µl, were mixed with an equal volume of the gold-Gal-BSA suspension. Control samples were incubated for 5 min with 25 mM sugar at 4°C prior to addition of gold-Gal-BSA. The reaction mixture was incubated at ice-bath temperature or at 37°C as indicated in Results. Cells were spun down and washed once with Eagle's medium (3 min at 40 × *g*).

Embedding and electron microscopy. The fixation procedure was carried out according to Franke et al. [17] with slight variations. Cells were fixed for 10 min at 4°C with 0.1% glutaraldehyde in 0.1 M S-collidine buffer containing 0.1 M saccharose, 2.0 mM $CaCl_2$ and 100 mM NaCl and post-fixed for 40 min with 2% OsO_4 in the same buffer. Specimens were dehydrated with ethanol and embedded in expoxy resin according to the method of Spurr [18]. Electron microscopy was done on dark grey sections (40–60 nm) contrasted with aqueous uranyl acetate for 5 min and lead citrate [19] for 1 min.

Results

(1) Localization of the galactose-binding receptor

The initial distribution of galactose-binding receptors on hepatocytes was studied after incubation of cells with excess gold-Gal-BSA at 4°C for 10 min: Single gold particles were found binding to the hepatic plasma membrane exclusively in the microvilli-rich region of the former space of Dissé (Fig. 1a). Particles are predominantly bound at microvilli, few are located at the base of microvilli and less than 1% are found in coated pits (Fig. 1b). Virtually no binding was observed at membrane regions devoid of microvilli, i.e., areas which in situ had been regions of cell contacts and of bile canaliculi (Fig. 1c).

The total number of bound gold particles was counted on cell sections through the nuclear equator. Between 25 and 55 particles were found per cell section in individual experiments. This leads to a rough estimate of $(2–5) \cdot 10^4$ ligands bound per total hepatocyte cell surface. Measurements with radioactive asialo-glycoproteins [20–22] gave a number of $(6–15) \cdot 10^4$ binding sites per hepatocyte.

Galactose-binding sites on the cytoplasmic side of interior cell membranes could be studied in sections through broken hepatocytes. Gold particles are found binding to the plasma side of Golgi-derived vesicle membranes (Fig. 2a) and to the outer membrane of the nuclear envelope (Fig. 2b). Tanabe et al. [23] described receptors on the plasma side of isolated lysosomal vesicles. Binding sites on the nuclear envelope have not been described so far.

The specificity of gold-Gal-BSA binding to hepatocytes was demonstrated as follows: The addition of 25 mM *N*-acetylgalactosamine to hepatocytes 5 min prior to the addition of gold-Gal-BSA particles specifically inhibited particle binding by 70–85% (Table I). Inhibition was seen for binding sites on the plasma membrane as well as binding sites on the Golgi apparatus and nuclear membrane. The addition of 25 mM mannose to hepatocytes did not prevent particle binding. When gold particles coated with BSA instead of Gal-BSA were used, binding was reduced by more than 95% (Table I).

(2) Aggregation of receptor ligand complexes

After the initial binding of gold-Gal-BSA particles at diffusely distributed sites at hepatic microvilli (see

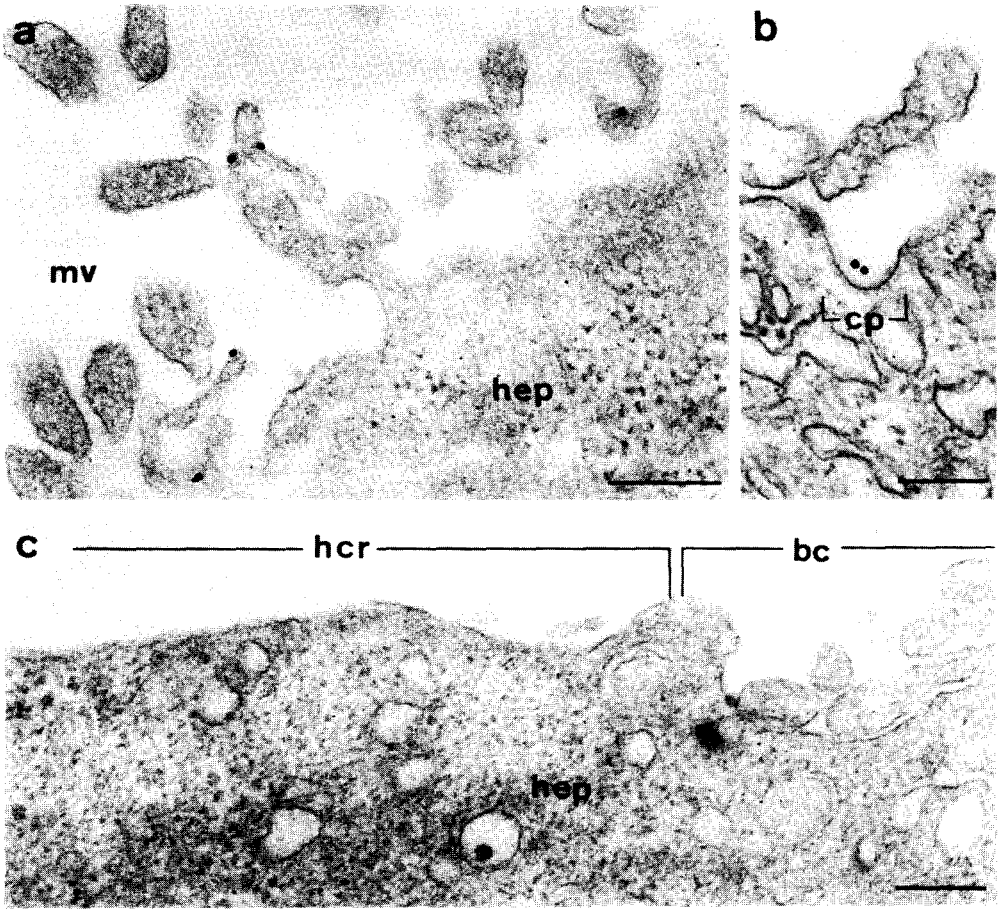

Fig. 1. Hepatocytes (hep) were incubated with gold-Gal-BSA for 10 min at ice-bath temperature (4°C). (a) Most particles are bound randomly to the microvillous (mv) region of the former space of Dissé. (b) Few (less than 1%) particles are located at specialized membrane regions, the coated pits (cp). (c) Virtually no binding of particles is observed in membrane regions with short microvilli (in situ: bile canaliculi) (bc) and in membrane areas devoid of microvilli (in situ: regions of hepatocyte contacts) (hcr). Bars represent 0.2 μm.

Fig. 1a), movement of receptor-ligand complexes can be observed. Prolonged incubation at 4°C leads to the microaggregation of particles into small clusters of 2 to 5 (Fig. 3a–c). Quantitative data on receptor-ligand microcluster formation are given in Fig. 4. Initially (after 10 min at 4°C) approx. 10% of particles are found in doublets or slightly larger aggregates. The percentage of microclusters increases to 34% after an incubation of 30 min at 4°C and to 67% after 60 min incubation at 4°C. Cluster formation is significantly enhanced when the incubation mixture is warmed up to 37°C during the last 5 min of the incubation period. The percentage of microclusters increases to 60% (25 min at 4°C + 5 min at 37°C) and to 75% (55 min at 4°C + 5 min at 37°C).

Concomittant with the increase in aggregate formation the number of particles found in coated pits or coated vesicles also increases. This process is again speeded up at 37°C (Fig. 5). Gold particles bound in coated membrane areas are aggregates of 3 to 16, single particles are rarely observed in these specialized membrane areas (Fig. 3d).

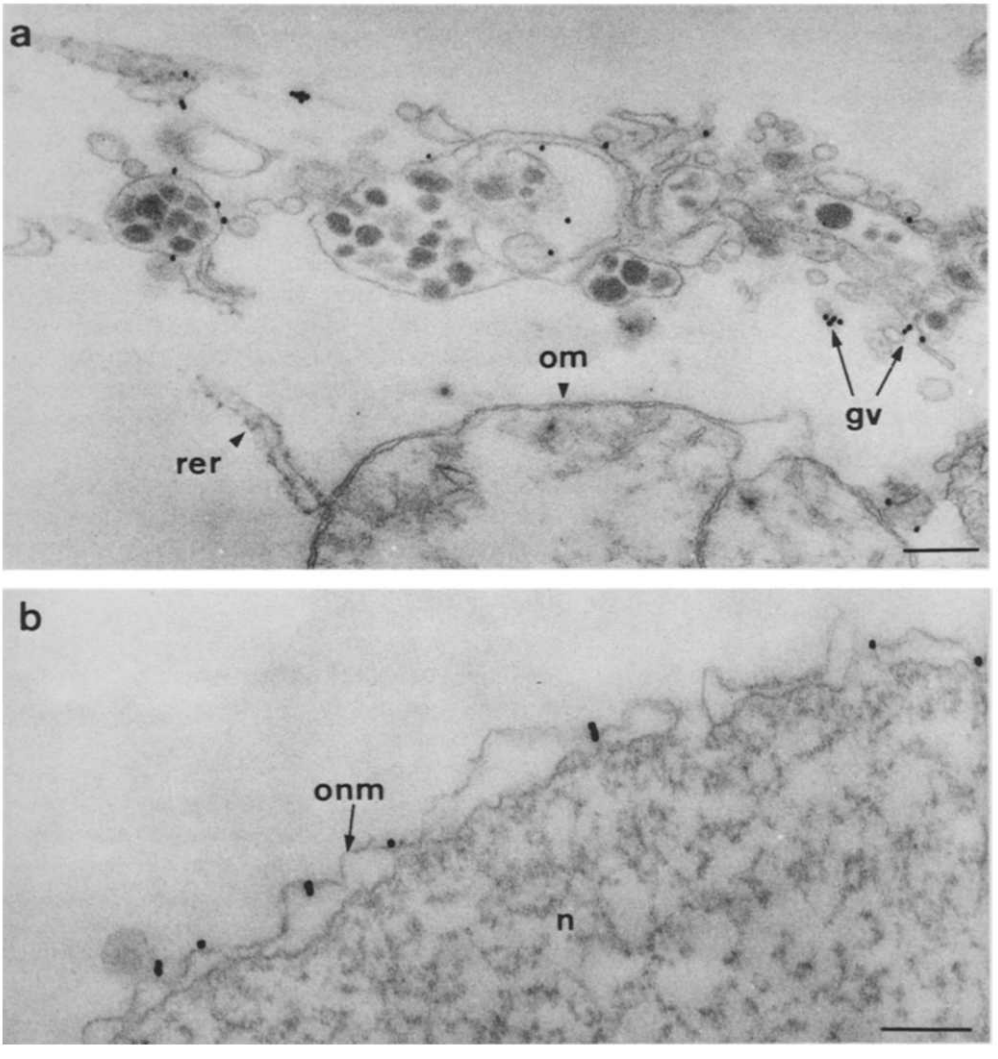

Fig. 2. Sections through broken hepatocytes reveal binding sites for gold-Gal-BSA at the cytoplasmic side of organelle membranes. (a) Binding of particles is seen with Golgi-derived vesicles (gv), whereas no binding was observed at the plasma side of rough endoplasmic reticulum membranes (rer) and at outer mitochondrial membranes (om). (b) Binding sites for gold-Gal-BSA are also found on the plasma side of the outer nuclear membrane (onm) of hepatocyte nuclei (n). Bars represent 0.2 μm.

Discussion

In the experiments reported in this paper early events of receptor-ligand interactions have been studied for the D-galactose-specific lectin on isolated hepatocytes. The initial binding of gold-Gal-BSA particles occurs randomly at hepatic microvilli of the former space of Dissé, no lectin activity is found at other membrane areas.

This finding allows the conclusion that free receptor units are not clustered and are localized outside coated membrane areas. Due to the size of the ligand (average diameter of gold particles 17 nm plus a cover of galactosylated albumin) it cannot be judged whether a receptor unit is equivalent to a monomer or an oligomer of galactose binding sites.

After the binding of gold-Gal-BSA, movement of receptor ligand complexes can be observed. Microaggregation of particles still occurs outside coated pits. Clusters contain 2–5 particles. Local aggregation

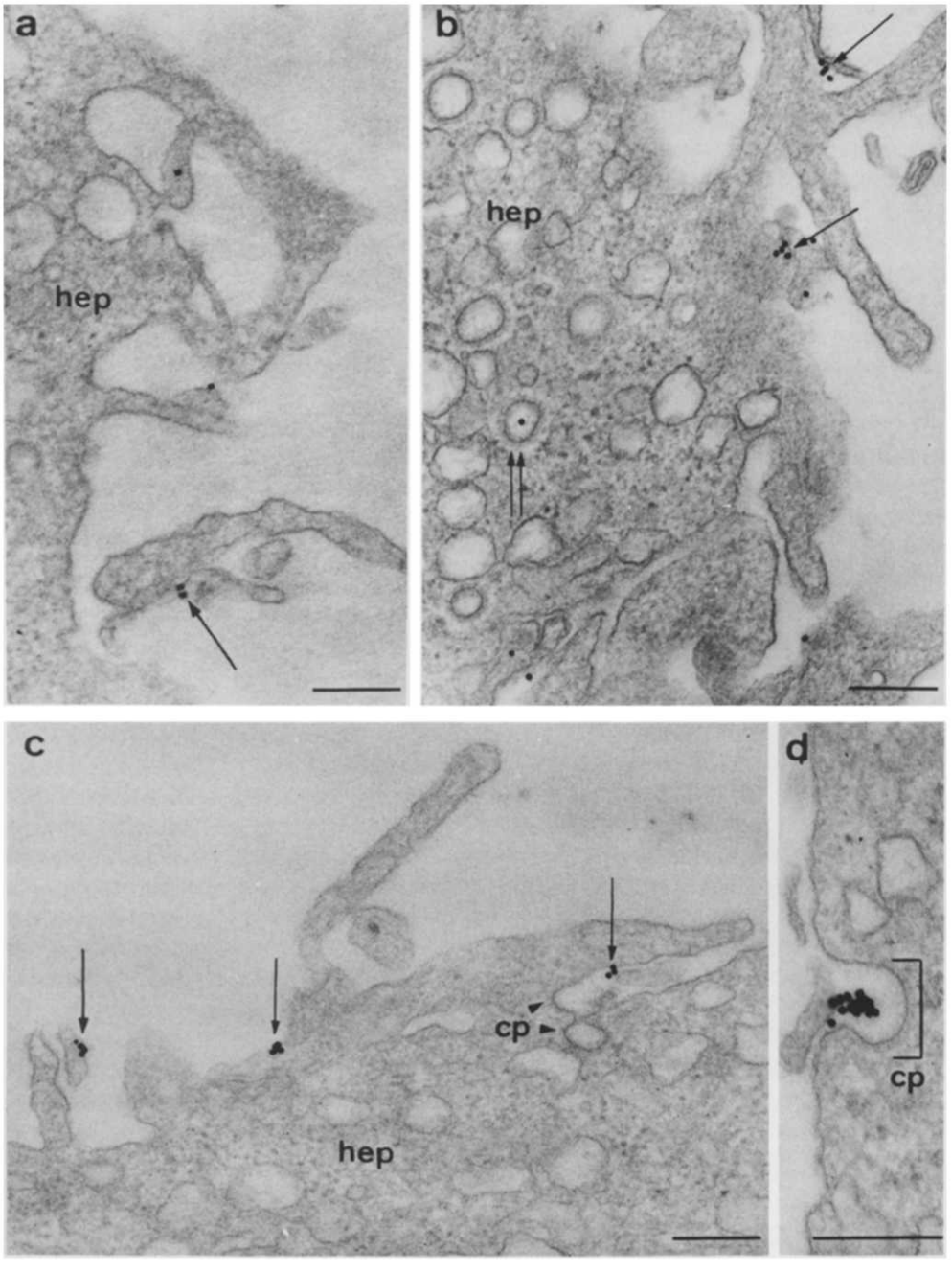

Fig. 3. Prolonged incubation of hepatocytes with gold-Gal-BSA particles results in aggregation of bound particles: (a) Hepatocytes (hep) were incubated with the ligand for 30 min at ice bath temperature (4°C). The majority of particles are still diffusely distributed at hepatic microvilli, 34% of ligands are found in microaggregates (arrow). (b) After incubation for 60 min at 4°C, 67% of bound particles are seen in microaggregates (arrows). The double arrows point to a particle associated with a tangentially sectioned coated pit. (c) Hepatocytes were incubated as in (b) but warmed up to 37°C during the last 5 min of the incubation period. Nearly 80% of bound particles are found in microaggregates (arrows) which are still located outside coated pits (cp). (d) Hepatocytes were incubated as in (c). 13% of bound particles are located in coated pits (cp) within larger aggregates of up to 16 particles seen per section. Bars represent 0.2 μm.

TABLE I

SPECIFICITY OF THE BINDING OF GOLD-Gal-BSA PARTICLES BY ISOLATED HEPATOCYTES

Ligand	Inhibitor [a] [25 mM]	Number of particles bound per hepatocytes section [b] (±S.E.), n = 15	Percentage Inhibition
gold-Gal-BSA	–	26 ± 6	
gold-Gal-BSA	*N*-acetyl-D-galactosamine	5 ± 3	81
gold-Gal-BSA	D-mannose	24 ± 5	8
gold-BSA	–	1	

[a] Hepatocytes were preincubated with the inhibitor for 5 min at 4°C and subsequently for 1 hour witl. gold particles.

[b] Only hepatocyte sections through the nuclear aequator (both nuclear membranes clearly discernible) were analysed.

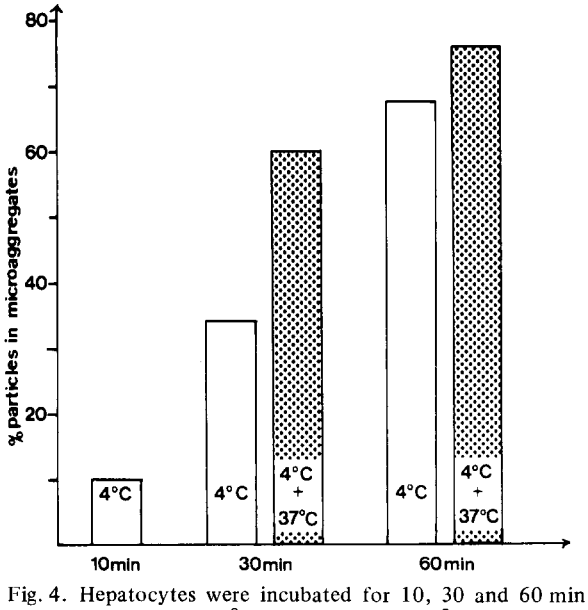

Fig. 4. Hepatocytes were incubated for 10, 30 and 60 min with gold-Gal-BSA at 4°C (open bars) or at 4°C and warmed up to 37°C during the last 5 min of the incubation period (dotted bars). The number of total bound particles and the number of particles found in aggregates (doublets or more) outside coated pits were counted. Only sections through the nuclear aequator (both nuclear membranes clearly vesible) were analysed (n = 15).

of complexes is slowed down but not abolished at 4°C. The formation of microclusters outside coated pits as a consequence of ligand binding has been suggested previously for the receptors for insulin and epidermal growth factor on cultured fibroblasts [9,10]. The experiments reported here represent the first direct evidence for ligand-induced microaggregation of receptors. The formation of microaggregates may be regarded as an essential step in receptor-mediated endocytosis since larger clusters are only seen at a later stage. These aggregates are regularly found at the base of hepatic microvilli associated with coated membrane areas. Thus for receptor-mediated endocytosis of gold-Gal-BSA particles by hepatocytes the following reaction scheme can be depicted:

(i) initial binding of ligand to diffusely distributed receptors;

(ii) local formation of mobile microclusters;

(iii) aggregation of microclusters and association of these aggregates with coated membrane areas.

It should, however, be kept in mind that in the present experiments receptor movement has been studied in cells cultivated in vitro. It has not yet been established whether the hepatic D-galactose receptor is also randomly distributed at hepatic microvilli in vivo. So

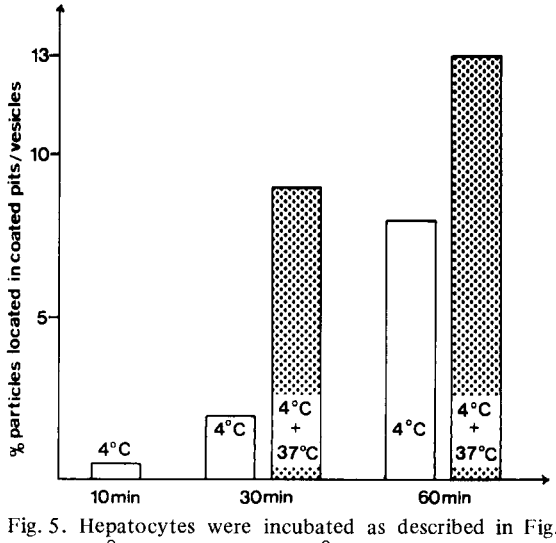

Fig. 5. Hepatocytes were incubated as described in Fig. 4, either at 4°C (open bars) or at 4°C and warmed up to 37°C during the last 5 min (dotted bars). The number of total bound particles and of particles located in coated pits or coated vesicles was counted on equatorial sections through hepatocytes (n = 25).

far studies performed in vivo have only shown late events of receptor-ligand interaction, i.e., the association of larger aggregates with coated pits and their uptake into coated vesicles [12]. Since, however, D-galactose binding sites are restricted to membrane areas of the space of Dissé in vivo as well as in vitro, no major membrane protein redistribution seems to occur during the isolation of hepatocytes.

I therefore assume that the characteristics of lectin-ligand interactions described here are also true for the intact organ.

Acknowledgements

I thank Prof. W. Vogell for generous support of my work, Anderea Schlömer for expert technical assistance in electron microscopy and Brigitte Renn for technical help in isolation procedures.

References

1 Goldstein, J.L., Anderson, R.G.W. and Brown, M.S. (1979) Nature 279, 679–685
2 Pearse, B. (1980) Trends Biochem. Sci. May 1980, 131–134
3 Gorden, P., Carpentier, J.L., Cohen, S. and Orci. L. (1978) Proc. Natl. Acad. Sci. USA 75, 5025–5029
4 Schlessinger, J., Shechter, Y., Willingham, M.C. and Pastan, I. (1978) Proc. Natl. Acad. Sci. USA 75, 2659–2663
5 Willingham, M.C., Maxfield, F.R. and Pastan, I. (1979) J. Cell Biol. 82, 614–625
6 Haigler, H. T., McKanna, J.A. and Cohen, S. (1979) J. Cell Biol. 81, 382–395
7 Willingham, M.C. and Pastan, I. (1980) Cell 21, 67–77
8 Salisbury, J.L., Condeelis, J.S. and Satir, P. (1980) J. Cell Biol. 87, 132–141
9 Kahn, C.R., Baird, K.L., Jarrett, D.B. and Flier, J.S. (1978) Proc. Natl. Acad. Sci. USA 75, 4209–4213
10 Shechter, Y., Hernaez, L., Schlessinger, J. and Quatrecasas, P. (1979) Nature 278, 835–838
11 Ashwell, G. and Morell, A.G. (1974) Adv. Enzymol. 41, 99–128
12 Wall, D.A., Wilson, G. and Hubbard, A. (1980) Cell 21, 79–93
13 Schlessinger, J. (1980) Trends. Biochem. Sci. August 1980, 210–214
14 Kolb, H., Schudt, C., Kolb-Bachofen, V. and Kolb, H.-A. (1978) Exptl. Cell Res. 113, 319–325
15 Horrisberger, M. and Rosset, J. (1977) J. Histochem. Cytochem. 25, 295–305
16 Stowell, C.P. and Lee, Y.C. (1978) J. Biol. Chem. 253, 6107–6110
17 Franke, W.W., Lüder, M.R., Kartenbeck, J., Zerban, H. and Keenan, T.W. (1976), J. Cell Biol. 69, 173–195
18 Spurr, A. (1969) J. Ultrastruct. Res. 26, 31–45
19 Venable, J.H. and Coggeshall, R. (1965) J Cell Biol. 25, 407–408
20 Steer, C.J. and Ashwell, G. (1980) J. Biol. Chem. 255, 3008–3013
21 Weigel, P.H. (1980) J. Biol. Chem. 255, 6111–6120
22 Tolleshaug, H. and Berg, T. (1980) Hoppe-Seyler's Z. Physiol. Chem. 361, 1155–1164
23 Tanabe, T., Pricer, W.E. and Ashwell, G. (1979) J. Biol. Chem. 254, 1038–1043