

Liver Endothelium Mediates the Hepatocyte's Uptake of Ceruloplasmin

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Abstract. The mode of transport of ceruloplasmin (CP) into the liver was investigated in fractionated liver cell suspensions. Incubation of ^{125}I -CP at 4°C with these different fractions led to its binding only to endothelial cells but not Kupffer cells and hepatocytes. Incubation at 37°C led to rapid uptake of ^{125}I -CP by endothelium, but cell-associated radioactivity declined after 15 min, which suggests the release of the labeled substance. Internalization was confirmed by fractionation of surface-bound and internalized ligand. The released label now acquired binding potential for fresh target hepatocytes, and the binding was inhibitable with asialoceruloplasmin but not native CP. This suggested that the released molecule was modified in the endothelium by desialation.

tion was confirmed by incubation of endothelium with double-labeled CP (^3H label on sialic acid and ^{125}I on the protein part). We conclude that in the liver, CP is first recognized and taken up by endothelial cells that are endowed with appropriate surface receptors for the protein. Endothelium then modifies the molecule by desialation to expose the penultimate galactosyl residues. The modified molecule is then released, recognized, and taken up by hepatocytes through their membrane galactosyl-recognition system. These findings are consistent with the role of endothelium as an active mediator of molecular transport between blood and tissue, and further assign a biological role for the galactosyl-recognition system in hepatocytes.

HEPATOCYTE membrane contains a well-characterized receptor that can bind galactosyl residues of glycoproteins (2, 12). Thus, while native ceruloplasmin (CP)¹ is cleared from the circulation with a half-life of 2–3 d (1), the clearance rate is remarkably enhanced by desialation, which exposes its penultimate galactose residues (2, 14). The functional significance of these galactosyl receptors is not well-studied. Moreover, the mode of CP transport from the circulation into the liver is not exactly known.

We have recently developed a visual probe by covalently coupling various proteins to submicron sized amide-modified latex particles (minibeads) (24). This probe permits topographic observations and semiquantitation of receptors on individual cells in scanning electron microscopy (SEM). Internalization of the probe could also be visualized by transmission electron microscopy (TEM). When this method was applied to study the uptake of CP by liver cell suspensions (8), the binding was found to be entirely limited to endothelial cells. Neither hepatocytes nor Kupffer cells bound the probe.

In the present study, we provide evidence to suggest that, in the liver, native CP is first recognized and removed by the endothelium and is then released (diacytosis or transcytosis [28]). In the course of its endothelial passage, CP may be modified by desialation, a process that can expose its penul-

imate galactosyl residues making it recognizable to hepatocytes. Suggestive evidence is also presented to indicate that the molecule, so released, may indeed be taken up by hepatocytes. Thus, liver endothelium may mediate the uptake of CP by hepatocytes.

Materials and Methods

Materials

CP (Sigma Chemical Co., St. Louis, MO) was purified in our laboratory on a Sephadex G-200 column and its purity was then demonstrated by polyacrylamide gel electrophoresis (PAGE). Lyophilized metrizamide (grade 1), collagenase type IV, bovine serum albumin (fraction V powder, BSA), and neuraminidase from *Clostridium perfringens* attached to beaded agarose were also obtained from Sigma Chemical Co. Carrier-free Na^{125}I , NaB^3H_4 , and Bolton-Hunter reagent were obtained from ICN Pharmaceuticals, Inc. (Irvine, CA) and immobilized lactoperoxidase from Worthington Biochemical Corp. (Freehold, NJ).

Fractionation of Liver Cell Suspensions

Liver cell suspensions were prepared from male Sprague-Dawley rats (200–250 g) by a collagenase perfusion method that has been described in detail (8, 18, 19). Fractionation of these suspensions was done first on metrizamide gradients by a modified method of Seglen (18), also described previously in detail (19). This fractionation yielded two cellular fractions, one that consisted of 99% hepatocytes (identified in SEM and TEM by counting 1,000 cells) and only 1% nonparenchymal cells with a viability of >90% (Table I). The second fraction, which consisted of 99% nonparenchymal cells (Kupffer and endothelial cells), was further fractionated by centrifugal elutriation.

Cells were suspended in phosphate-buffered saline (PBS), and loaded in a

¹ Abbreviations used in this paper: ASCP, asialoceruloplasmin; CP, ceruloplasmin; SEM, scanning electron microscopy; TCA, trichloroacetic acid; TEM, transmission electron microscopy.

Table I. Cellular Comparison of the Final Preparation of Fractionated Liver Cell Suspensions Used in This Study

| | Purity* | Viability |
|-------------------|---------|-----------|
| Hepatocytes | ≥99% | ≥99% |
| Kupffer cells | 95–99% | ≥99% |
| Endothelial cells | ≥90% | ≥97% |

* Cell identification was made by counting 1,000 cells in SEM and TEM preparations (see the text).

Beckman JE6-B standard elutriator rotor (4.2 ml) using a J2-21 centrifuge (30). Before cell loading, the rotor was eluted with PBS at a flow rate of 11 ml/min. The rotor was then loaded with the cell suspension in PBS at a concentration of 10^7 /ml using a total volume of no more than 5 ml, maintaining the same flow rate. After the cell loading, elutriation was carried out at 750 g (2,500 rpm) at 20°C. The flow rate was increased to 20 ml/min, and 250 ml of eluant fluid was collected. This fraction contained 95–99% endothelium (Table I) as determined by examination of 1,000 cells (for each differential cell count) in SEM and TEM where endothelial cells have characteristic surface and cytoplasmic features (19). This was further confirmed by the reaction with factor VIII antibody (using an indirect method [23]). The viability was ~99%. The second fraction was obtained at the flow rate of 42 ml/min. This fraction consisted of >90% (Table I) Kupffer cells as identified by SEM and TEM. The contaminant was mostly endothelial cells but also a few bile epithelial cells. Viability of recovered cells was above 97%.

Radioisotope Labeling Methods

Iodination of CP was done by the lactoperoxidase method as described (4, 8). The final specific activity of the labeled protein was 0.25 mCi/mg. CP was also double-labeled with ^3H (sialic acid residues) and ^{125}I (protein moiety) as described elsewhere (11). ^3H labeling was done by the consecutive oxidation-reduction method of Van Lenten and Ashwell (27). CP (1 mg/ml) was dissolved in 0.1 M sodium acetate that contained 0.15 M NaCl (pH 5.6). This was oxidized by incubation with 0.24 ml of 0.012 M NaIO_4 for 10 min at 0°C. Oxidation was stopped by the addition of excess ethylene glycol (1 ml), and the solution was dialyzed overnight at 4°C against PBS, pH 7.4. Periodate consumption, determined by the method of Dyer (5), indicated more than 1.8 mol of periodate consumed per mole of sialic acid, remarkably close to 2 mol per mole which is theoretically ideal. Tritium was then introduced into sialic acid residues by adding 0.1 mg tritiated NaBH_4 dissolved in 0.02 ml of 0.01 M NaOH. After mixing, the solution was warmed to room temperature, and the reduction was allowed to continue for 30 min with stirring. To ensure the completeness of reduction, 0.3 mg of nonradioactive KBH_4 was added and the incubation was continued for another 30 min. Excess borohydride was removed by dialysis against 0.1 M Na acetate and 0.15 M NaCl (pH 5.6) for several hours before exhaustive dialysis against PBS (pH 7.4). Subsequently hydrolysis of the sialic acid (27) by either sulfuric acid or enzymatic method (neuraminidase) demonstrated that, respectively, 99.5% or 98.7% of the label was sialic acid.

This tritiated CP was then iodinated by using the Bolton-Hunter reagent (3) since the usual peroxidation method could remove the ^3H label from sialic acid. To obtain maximum labeling, tritiated CP was dialyzed overnight against 0.1 M borate buffer (pH 8.5) and to this solution 1 mCi of Bolton-Hunter reagent in 0.1 M borate buffer was added and incubated for 15 min at 0°C. Unused reagent was reacted with 0.5 ml of 0.2 M glycine in 0.1 M borate buffer for 15 min at 0°C. The preparation was passed through a G-25 Sephadex column (0.7 × 10 cm) to remove glycine and unattached Bolton-Hunter reagent. The final specific activities of ^3H and ^{125}I were 0.12 mCi and 0.11 mCi per milligram of protein.

Removal of Sialic Acid

CP was desialated by incubating 10 mg/ml of the protein with 0.5 μg of neuraminidase attached to beaded agarose (29) in a buffer (pH 5.5) that contained 0.1 M sodium acetate with 5 mM calcium chloride for 24 h at room temperature in a mechanical shaker. Neuraminidase was then removed by centrifugation. Asialoceruloplasmin (ASCP) was separated from free sialic acid by passing through the column of Sephadex G-25 (2 × 25 cm). The removal of sialic acid was confirmed by the thiobarbituric acid method (26) and calculation indicated that more than 90% of the sialic acid content of the molecule was removed.

Incubations

Incubations were done in microcentrifuge tubes. Suspensions of endothelium or hepatocyte fractions (1×10^6 cells/tube) in Dulbecco's PBS, pH 7.0, supplemented with 0.5% BSA were incubated with the radiolabeled ligand in a total volume of 250 μl at the desired temperature for the desired period. The reaction was terminated by the addition of 1 ml of cold PBS. Nonspecific binding was determined in the presence of a 100-fold excess unlabeled ligand. The nonspecific binding was subtracted from total binding, and the result was expressed as specific binding. The tubes were centrifuged in a microcentrifuge for 10 s. The pellets were washed twice with 1 ml of cold PBS. The washed cell pellets and the combined supernates were counted for the radioactivities.

Separation of Surface-bound and Internalized Ligand

This was done by acetic acid dissociation of surface-bound ligand according to the method of Haigler et al. (7). Briefly, after incubation with the ligand, cells were washed three times in PBS and incubated for 6 min at 4°C with 0.7 ml of equal proportions of 0.2 M acetic acid and 0.5 M NaCl (pH 2.5). The cells were then centrifuged in a microcentrifuge, and the supernate was collected. The cells were further incubated for another 6 min with 0.4 ml of the same acid solution, further centrifuged, and the supernate was again collected. The combined supernates were counted to determine the surface-bound ligand, while the cell pellet was counted after lysis with 1 N NaOH to determine the internalized ligand.

Results

The Binding and Uptake of ^{125}I -CP by Liver Cell Fractions

Incubation of liver cell fractions with ^{125}I -CP at 4°C led to the binding of CP only by the endothelium. Hepatocytes and Kupffer cells did not bind ^{125}I -CP significantly at 4°C (Fig. 1). The binding of the endothelium fraction was ligand concentration-dependent, and from the Scatchard analysis (17), the maximum binding was calculated to be ~347 fmol per 10^6

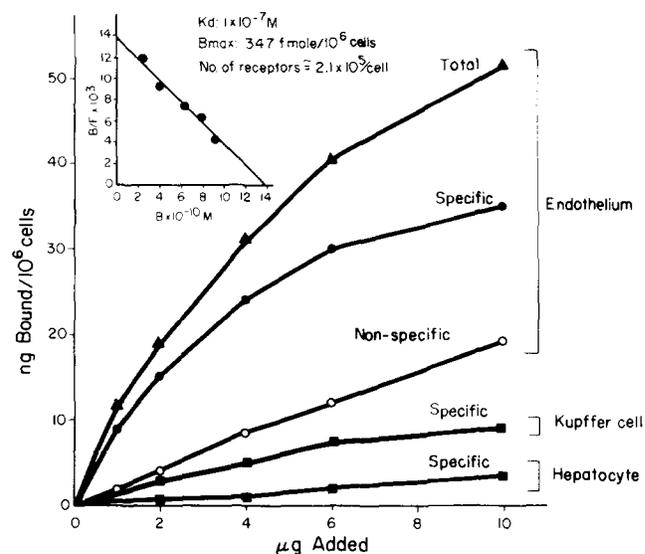


Figure 1. The binding of ^{125}I -CP to endothelium, Kupffer cell, and hepatocyte fractions of the liver cell suspension. Cells in each fraction (1×10^6) were incubated at 4°C for 60 min with an increasing concentration of ^{125}I -CP in 250 μl (total volume) of Dulbecco's PBS that contained 0.5% BSA. Inhibition was done in the presence of 100-fold excess (4 mg/ml) of unlabeled CP. Specific binding was calculated by subtracting nonspecific binding from total binding. Inset shows a Scatchard plot constructed from the specific binding data for the endothelium. Minimal binding was noted by hepatocytes and Kupffer cells and can be explained by contamination with endothelial cells.

cells with dissociation constant (K_d) of 1×10^{-7} M. The number of receptors per cell was 2.1×10^5 . The specificity of the binding of ^{125}I -CP was demonstrated by inhibition in the presence of a 100-fold excess (4 mg/ml) native CP. The magnitude of specific binding of ^{125}I -CP was a function of cell number (Fig. 2).

Specific binding of ^{125}I -CP to the endothelium as a function of time is shown in Fig. 3. At 4°C the binding was saturable, reaching a steady-state level between 5 and 30 min (data not shown). At 37°C (Fig. 3), however, the maximum uptake by endothelium occurred after 15 min. This was considerably higher than the binding plateau at 4°C (135 ng vs 35 ng per

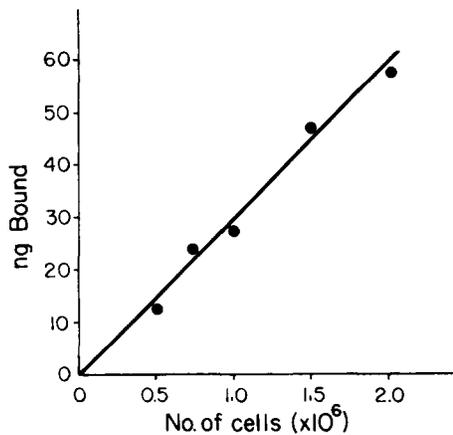


Figure 2. Effect of cell number on the specific binding of ^{125}I -CP to the endothelial-rich fraction. Variable numbers of endothelial cells (0.5 – 2.0×10^6 cells per $250 \mu\text{l}$) were incubated with $10 \mu\text{g}$ of ^{125}I -CP for 60 min at 4°C . Specific binding was calculated as before. The binding is a linear function of the cell number.

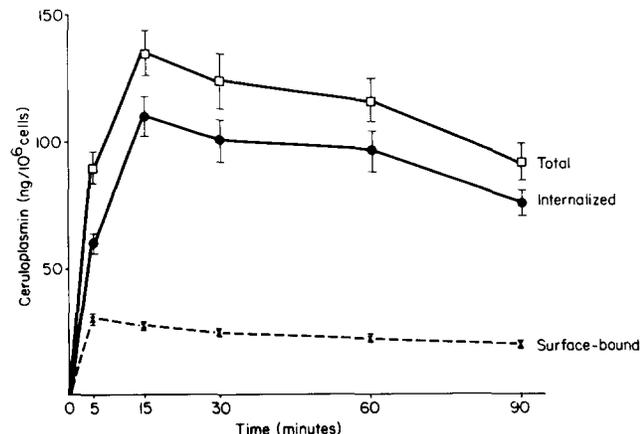


Figure 3. Uptake of ^{125}I -CP to endothelium fraction at 37°C . Cells (1×10^6) were incubated with $10 \mu\text{g}$ of ^{125}I -CP at 37°C for indicated periods in a final volume of $250 \mu\text{l}$. The reaction was terminated by the addition of 1 ml of cold PBS after the desired period. Inhibition was done in the presence of unlabeled CP (4 mg/ml). Specific uptake is shown as a function of time. Total uptake reaches a peak within 15 min and then appears to decline somewhat, which suggests the release of the label at a rate higher than that of uptake. Separation of radioactivity into surface-bound and internalized fractions was done as described in the text. The internalized fraction reaches a peak within 15 min and then begins to decline in a curve that parallels that of total uptake. The surface bound fraction reaches a peak within 5 min and then slightly declines, which suggests that some down regulation might have occurred.

10^6 cells). Cell-associated radioactivity somewhat declined thereafter, which suggests the release of the label from the cells at a rate exceeding that of its uptake. To assure that this decline was not the result of degradation of CP through proteolysis, the proteins in the supernate were precipitated with 20% trichloroacetic acid (TCA), and the precipitant as well as the supernate were counted for their radioactivity. At no time did the TCA nonprecipitable (soluble) radioactivity exceed 3% of total radioactivity, which suggests the absence of significant CP degradation. Moreover, the supernate was subjected to column chromatography on either Sephadex G-25 or G-200. The radioactivity remained in a single peak corresponding to that of CP. Cell viability at the end of the 90-min incubation was unchanged. Dissociation of total ligand uptake into surface-bound and internalized compartments is also shown in Fig. 3. The internalized ligand as a function of time followed a curve parallel to total uptake, reaching a maximum after 15 min and somewhat declining thereafter. Surface-bound ligand, on the other hand, reached a maximum within 5 min and remained stable or even declined slightly.

Further evidence for binding and release of ^{125}I -CP by endothelium was demonstrated in an experiment in which endothelium was first incubated with labeled ligand at 4°C for 30 min. Cells were then washed and transferred to 37°C for various periods. After completion of incubation, cells were centrifuged and radioactivity was counted in both cells and the supernate. Cell-bound radioactivity, which after incubation at 4°C had reached the same magnitude as in Fig. 1, began to decline as a function of time after the transfer of cells to 37°C (Fig. 4). A reciprocal increase in the radioactivity of the supernate indicated the release of radioactivity.

To assure the absence of proteolysis, the proteins in the supernate were precipitated with 20% TCA and the precipitant as well as the supernate were counted for their radioac-

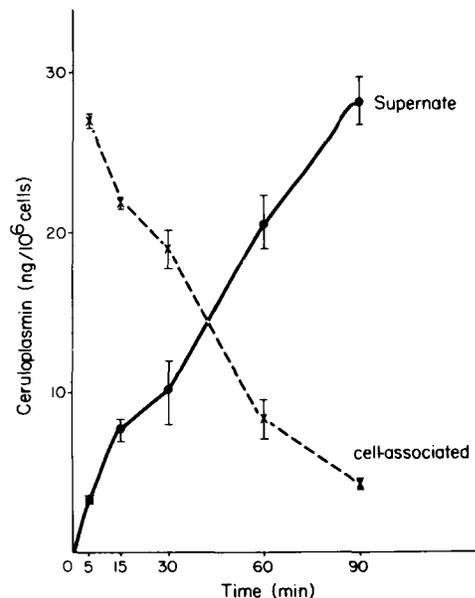


Figure 4. Endothelial cells (1×10^6) were incubated with $10 \mu\text{g}$ ^{125}I -CP at 4°C for 30 min. The cells were then washed three times in PBS and further incubated at 37°C for various periods. The cell-associated radioactivity at time zero (surface-bound) declines with time, and this decline is associated with a corresponding increase in the radioactivity in the supernate.

tivity. At no time did the TCA nonprecipitable (soluble) radioactivity exceed 3%, which suggests the absence of significant CP degradation. Moreover, the supernate was subjected to column chromatography on either Sephadex G-25 or G-200. The radioactivity remained in a single peak corresponding to that of CP. No significant specific uptake of CP by hepatocytes or Kupffer cells was noted at 37°C either (data not shown).

The Binding Capacity of the Supernate

Incubation of the supernate, "conditioned" by previous incubation with endothelium, with fresh target hepatocytes now led to the binding of the radioactive ligand in the supernate to cells (Fig. 5), which suggests that the radioactive ligand released from the endothelium might have acquired a new site recognizable by hepatocytes. This binding was not inhibited by excess unlabeled CP but was significantly inhibited by the galactosyl-containing molecules ASCP (Fig. 5), galactosyl-BSA synthesized in our laboratory (9), as well as asialofetuin (25).

Desialation of CP by Endothelium

Double-labeled CP (tritiated sialic acid, iodinated protein moiety) was incubated with endothelium fraction at 37°C for various periods, and the cell-associated radioactivities were counted separately. The results, shown in Fig. 6, demonstrated that for the first 15 min the curves for both ^3H and ^{125}I were overlapping, which indicated the uptake of the molecule that contained both labels. Thereafter, the two radioactivities dissociated with the ^{125}I curve slightly declined in a pattern similar to Fig. 3, while the ^3H curve remained stable or even slightly increased. The data provided evidence for desialization of CP molecule by the endothelium.

Mixed Cell Experiments

The results described above suggested that CP is removed by

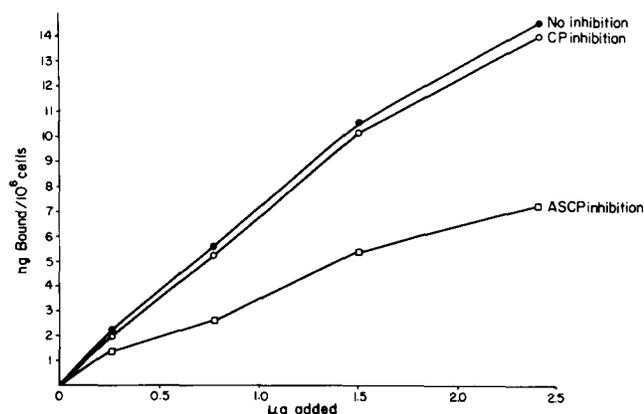


Figure 5. Binding of endothelium-conditioned supernate to fresh target hepatocytes. ^{125}I -CP ($10\ \mu\text{g}$) was first incubated with endothelial cells (1×10^6) for 90 min at 37°C in serum-free Dulbecco's PBS. The reaction was then terminated by the addition of 150 μl of cold PBS. The endothelium-conditioned supernate was then obtained by centrifugation, and its protein concentration was determined by calculation from the radioactivity. Fresh target hepatocytes (1×10^6 in PBS) were incubated with increasing concentration of the putative ligand for 60 min at 4°C in a final volume of 250 μl . Inhibition studies were done in the presence of unlabeled CP (4 mg/ml) or ASCP (4 mg/ml). The putative ligand binds to the hepatocyte fraction and the binding is partially inhibitable with ASCP but not with CP.

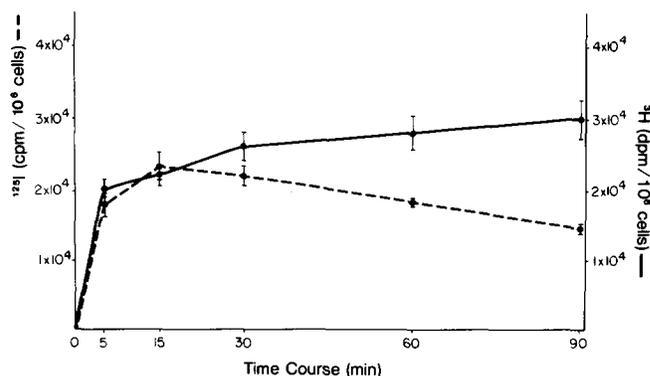


Figure 6. CP was double-labeled with ^3H (sialic acid residues) and ^{125}I (protein moiety) as described in the text. This preparation ($10\ \mu\text{g}$) was incubated with endothelium fraction (1×10^6) at 37°C for various time. The two radioactivities were counted separately. During the first 15 min the two curves are overlapping and suggest the uptake of sialated molecule. Then the two curves dissociate with ^3H being retained and ^{125}I beginning to decline.

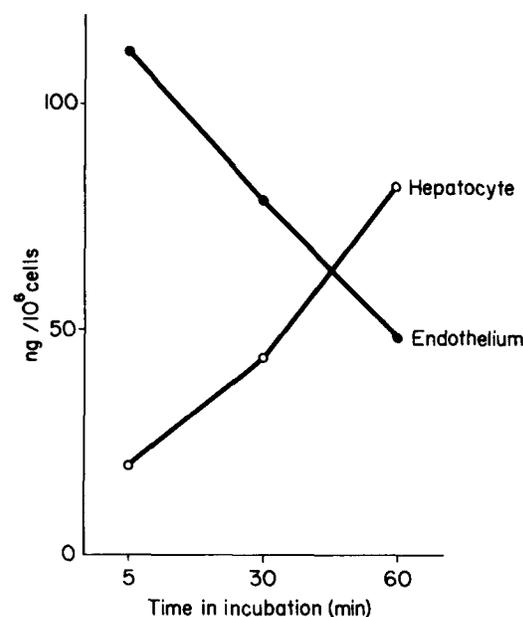


Figure 7. Cell-associated ^{125}I -CP in endothelium and hepatocytes in mixed cell system. A mixture of endothelium and hepatocytes fractions (1×10^6 cells each) were incubated with 40 $\mu\text{g}/\text{ml}$ of ^{125}I -CP for the indicated periods at 37°C in a total volume of 250 μl of Dulbecco's PBS that contained 0.5% BSA. Cells were then refractionated by centrifugation on 15% metrizamide solution. The endothelium fraction was recovered on the interface of metrizamide and buffer and the hepatocyte fraction from the bottom of the tube. Radioactivity and the cell number were determined. Specific uptake was calculated by subtracting the uptake in the presence of CP from total uptake. The decline in the radioactivity in the endothelium is associated with a reciprocal uptake of hepatocyte fraction.

endothelium and then released only to be taken up by hepatocytes (i.e., the endothelium mediates the uptake of CP by hepatocytes). To further evaluate this, mixtures of endothelium and hepatocytes in equal ratios (1×10^6 cells each) were incubated at 37°C with the radiolabeled CP for various periods of time. The mixture was then refractionated, and the cell-associated radioactivity was counted separately for each fraction. The results are shown in Figs. 7 and 8. It will be noted

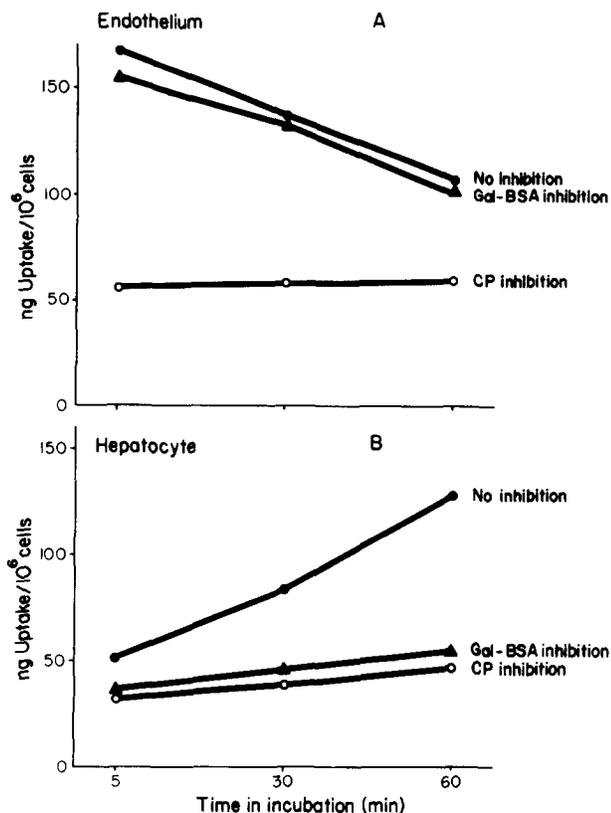


Figure 8. ¹²⁵I-CP uptake in mixed cell system. Inhibition studies were done in the presence of galactose-BSA (4 mg/ml) or unlabeled CP (4 mg/ml). Experimental conditions were described in Fig. 7. The uptake by hepatocytes, but not by endothelium, is inhibitable by galactose-BSA. CP inhibits the uptake by both hepatocytes and endothelium, which suggests that the initial endothelial uptake is necessary for the subsequent hepatocyte uptake.

that the ligand rapidly bound to the endothelium but gradually dissociated from this fraction. During the same period, there was a reciprocal increase in the hepatocyte-associated ligand (Fig. 7). Both the initial radioactivity associated with endothelium and the subsequent hepatocyte-associated radioactivity were inhibitable with excess native CP, indicating that the initial association with endothelium was necessary for the subsequent association with hepatocytes. However, its subsequent association with hepatocytes, but not the initial association with the endothelium, was partially inhibitable by galactose-BSA (Fig. 8).

Discussion

The results presented here indicate that in the liver cell suspensions, CP uptake is limited to endothelial cells that are endowed with appropriate specific membrane receptors. Hepatocytes and Kupffer cells do not take up CP significantly, and the small amount of binding observed could be explained by contamination with a small number of endothelial cells. This is consistent with our previous observation using a visual minibead probe (8). The endothelial binding of CP is further evident by the observation that binding is a linear function of endothelial cell number. That only a fraction of CP present in the medium is taken up by the cells may be due to down regulation of receptor as a consequence of continued exposure to the ligand (25). It is also possible that the receptor and

ligand is transported in a complex form by the endothelium precluding receptor recycling. This area deserves further study.

By fractionating the ligand taken up by cells into surface-bound and internalized fractions, we have further confirmed that the protein is indeed internalized by endothelial cells. This also confirms our previous observations using the minibead probe. In addition, we have demonstrated that the internalized ligand is then released by the endothelium into the medium, probably through a process of transcytosis. We have obtained confirmatory evidence for transcytosis of CP (10) by *in situ* perfusion of liver with colloidal gold-labeled CP. The label binds to the luminal surface of liver sinus endothelium at 4°C and is internalized at 37°C. Morphometric studies indicate that the probes then move via a vesicular transport system and are externalized on the abluminal side. During this process, the endothelium appears to desialate the molecule. This is suggested by dissociation of ³H and ¹²⁵I after incubation with the endothelium in those experiments in which sialic acid residues were labeled with ³H while the protein part of the molecule was iodinated. Additional support for desialation of the molecule was obtained by electrophoresis of the supernate, which demonstrates the generation of a radioactive band co-migrating with ASCP. This evidence has been presented elsewhere (25). Desialation can expose the penultimate galactosyl residues of the glycoprotein. Since hepatocyte membrane is known to possess specific receptors for galactosyl termini of glycoproteins, the release of desialated glycoprotein can lead to its binding and uptake by hepatocytes. Thus, the endothelium-conditioned molecule acquires the capacity to bind to fresh target hepatocytes as we have demonstrated here.

That the galactosyl receptors of hepatocyte membrane may be involved in the binding of endothelium-conditioned molecules is suggested by the lack of inhibition of hepatocyte binding with excess native CP, but the presence of inhibition with galactosyl-terminating molecules (ASCP, asialofetuin, galactosyl-BSA).

Incubation of radioactive ligand with a mixture of endothelial cells and hepatocytes indicated that the endothelial cell processing of the ligand may be necessary for its uptake by hepatocytes: cell-associated radioactivity rapidly increased in the endothelial cell fraction and this was followed by a slow drop and a reciprocal slow increase in the hepatocyte fraction. Moreover, in this system, the uptake of ligand by both fractions was inhibited in the presence of excess unlabeled CP, whereas the uptake of endothelium-processed CP by hepatocyte was not.

Our work, therefore, suggests a physiological function for galactosyl receptors of hepatocyte membrane. These studies further emphasize the function of endothelial barrier in the uptake of proteins. Similar endothelial transcytosis has been reported for another plasma protein, low density lipoprotein, in aorta (28), for lactoferrin in the liver (13), and by our own group for transferrin in the bone marrow (20). We have also observed endothelial mediation of transport in the liver for three other plasma proteins thus far studied: insulin, transferrin, and transcobalamin II (19, 21, 22). This mode of tissue uptake of plasma proteins may, therefore, be a general phenomenon and, if so, galactosyl receptors of hepatocytes could be considered as universal acceptor for many glycoproteins.

Further support for this view is provided by the work of

Regoezi et al. (15, 16) who have used infusion of human asialotransferrin type 3 in vivo as well as binding studies in vitro with purified rat liver plasma membrane. They demonstrated that while the liver uptake of asialotransferrin is via galactosyl receptors, competition with native protein can reduce the half-life of the ligand, which suggests that both receptors for native glycoprotein and asialoglycoprotein are needed for the uptake. They then concluded that diacytosis of asialotransferrin could explain their data.

Modification of the ligand, as suggested by our work, is further in line with the work of Greenspan and St. Clair (6) who have described the modification of low density lipoprotein during its passage through the skin fibroblasts. This process, called retroendocytosis, is very similar to modification of CP during its transcytosis across liver endothelium as described here. Thus, cellular modification of proteins for subsequent use may have other examples in cell biology.

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