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# LIVER ENDOTHELIUM DESIALATES CERULOPLASMIN

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Our previous work indicated that ceruloplasmin (CP) is transported through liver endothelium via a receptor-mediated mechanism and is subsequently externalized on the abluminal side. In the present work $^{125}I$ or $^{3}H$-labeled ceruloplasmin was chased through the endothelium and the supernate was subjected to affinity chromatography using $RCA_{120}$ column. Evidence was obtained that liver endothelium completely desialates CP in the course of its transport. The results substantiated the view that desialated CP is subsequently removed by hepatocytes through asialoglycoprotein receptors. 

Ceruloplasmin is a multifunctional, copper-containing α-2-glycoprotein that contains 8% carbohydrate (1-3). Each CP molecule contains 9 sialic acid residues in the terminal position of its glycan moiety with galactosyl residues in penultimate position (1,4).

We have previously demonstrated that liver endothelium mediates the hepatocyte's uptake of CP (5,6,7). Endothelium takes up CP on the luminal side through a mechanism involving receptor-mediated endocytosis. The molecule moves across the endothelium via a vesicular pathway and is externalized on the abluminal side where it is recognized and taken up by hepatocytes, apparently through a mechanism involving asialoglycoprotein receptors (7). This scheme calls for desialation of CP by endothelium. We now have utilized lectin affinity chromatography to demonstrate that liver endothelium does indeed desialate CP.

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## MATERIALS AND METHODS

Liver cell suspensions were prepared from male Sprague-Dawley rats (200-250 g) by a collagenase perfusion method (5-10). Fractionation of these suspensions was done first on metrizamide gradients and then by centrifugal elutriation. These methods have been described in detail (5-10). The yield was $10^7$ endothelial cells per liver with 99% purity (judged by indirect immunofluorescent staining for factor VIII as well as their distinctive feature in electron microscopy) and more than 95% viability. Cells were suspended in Dulbecco PBS at a concentration of $10^7$ cell/0.5 ml.

CP (Type X, Sigma) was purified on a Sephadex G-200 column and its purity was demonstrated by polyacrylamide gel electrophoresis (PAGE). It was labeled with $^{125}I$ (protein moiety) or with $^{3}H$ (sialic acid residues). Iodination was done by the lactoperoxidase method as described (11). $^{3}H$ labeling was done by the consecutive oxidation-reduction method (12): To do this CP (1 mg/ml) was dissolved in 0.1M sodium acetate containing 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 (13), indicated that more than 1.8 mol of periodate consumed per mol of salic acid, remarkably close to 2 mol which is theoretically ideal. Tritium was then introducted 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 non-radioactive $KBH_4$ was added and the incubation was continued for another 30 min. Excess borohydride was removed by dialysis against PBS (pH 7.4). Subsequently hydrolysis of the sialic acid by either sulfuric acid or neuraminidase (12,14) demonstrated that, respectively, 99.5% or 98.7% of the label was on sialic acids. Specific activities were 293.2 µCi/nmol and 3.79 µCi/nmol CP respectively for $^{125}I$ and $^{3}H$. To remove small amounts of asialo-CP, present in these preparations, purified CP was subjected to $RCA_{120}$-agarose (Sigma) column chromatography (10 mm X 25 mm) equilibrated with Dulbecco PBS (pH 7.4) at a flow rate of 2 ml/h and was fractionated into test tubes containing 400 µl each (Fig 1). Fractions were first eluted with equilibration buffer (Dulbecco PBS) and desialated CP, retained by the column, was then completely eluted with 0.2 M β-D-galactose in Dulbecco PBS (pH 7.4). Fractions containing sialated CP were collected and concentrated into 300 µg/ml using Amicon (MW>10,000).

Endothelial cell suspension in 500 µl of Dulbecco PBS was then incubated with 500 µl of $^{125}I$ or $^{3}H$-labeled sialated CP (final concentration 150 µg/ml) at 4°C for 30 min. Cells were then washed thrice with cold Dulbecco PBS and resuspended in 300 µl of Dulbecco PBS and incubated at 37°C for 2 h in a microcentrifuge tube with gentle agitation preventing the settlement of the cells. After the incubation, the cells were centrifuged using a microcentrifuge and the supernate was obtained. The cell viability was above 90% throughout the incubation.

The supernate was then subjected to the $RCA_{120}$-agarose column chromatography under the same conditions as mentioned above and fractions were counted for radioactivities.

Asialo-CP was prepared by mild acid hydrolysis (14) and purified on Sephadex G-25 column eliminating free sialic acid. Subsequently it,

too, was labeled with $^{125}I$ or $^{3}H$, and subjected to $RCA_{120}$-agarose column chromatography. Sialic acid content of all peaks was then measured by thiobarbituric acid method (14).

## RESULTS

Native $^{125}I$-CP gave two radioactive peaks by $RCA_{120}$ chromatography (Fig 1). The first peak, containing 8.9 mol of sialic acid per mol CP, was not retained indicating the coverage of penultimate galactosyl residues by sialic acids. This peak demonstrated a shoulder suggesting the heterogeneity of th molecule with regard to its sialic acid content. The second peak, containing 5.9 mol of sialic acid per mol CP, appeared only after the elution with galactose and was considered to be adequately desialated to be retained by the column. This peak was much smaller in $^{3}H$-CP compared to $^{125}I$-CP indicating that while it was desialated, desialation was incomplete.

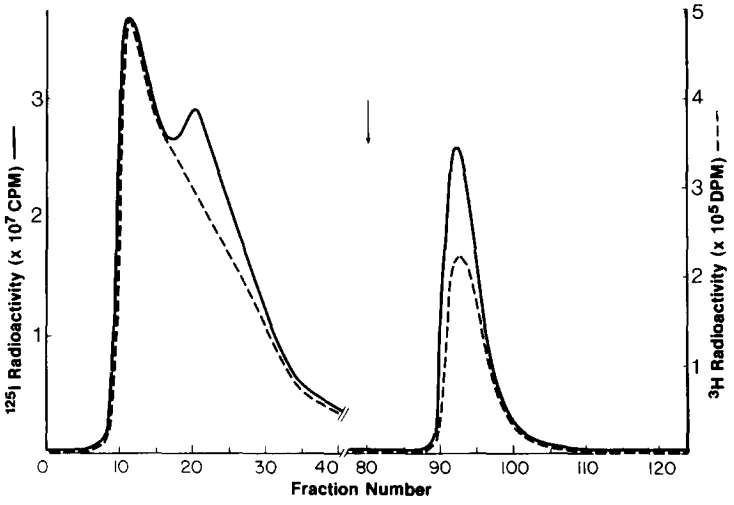


Fig. 1. Chromatography of native human ceruloplasmin (CP) labeled with either $^{125}I$ (protein moiety) or $^{3}H$ (sialic acid residues) on $RCA_{120}$ agarose. An $RCA_{120}$ agarose column (10 mm x 25 mm) was equilibrated at room temperature with Dulbecco PBS, pH 7.4. Native CP (300 µl) was applied to the column at 2 ml/h at 400 µl fractions were collected. The column was washed with equilibration buffer. Arrow indicates the application of buffer containing 0.2 M D-galactose. The first peak is the unbound protein constituting 80% of total protein and containing 8.9 mol of N-acetylneuraminic acid per mol CP. The second peak is that of bound protein constituting 20% of total protein and containing 5.9 mol of N-acetylneuraminic acid per mol CP, being consistent with $^{3}H$-curve.

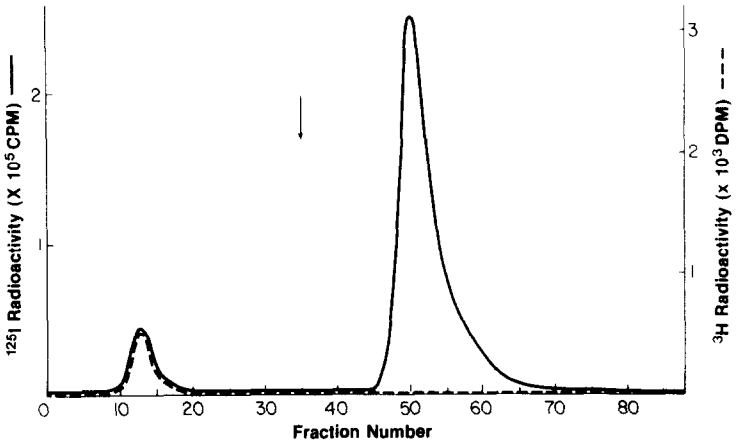


Fig. 2. Chromatography of supernate after incubation of sialated CP with liver endothelium (see text). Some 90% of the protein is retained by the column and eluted with 0.2 M galactose. This fraction contains no sialic acid, by $^3$H-radioactivity curve and thiobarbituric acid method.

The first peak (sialated CP) was separated, concentrated and incubated with liver endothelium at 4°C for 30 min to bind the ligand to the cell membrane. Free, unbound ligand was then washed off by centrifugation of the cells which were then resuspended in Dulbecco PBS. Bound ligand was chased by incubation of cells at 37°C for 2 h. The supernate was obtained by centrifugation and was subjected to $RCA_{120}$ column chromatography. Fig 2 shows the results. With $^{125}$I-CP, the majority of radioactivity appeared in a peak that was retained by the column indicating it was the desialated form of CP. Similar experiment with $^3$H-CP demonstrated no radioactive peak in this position and indicating that desialation was complete. This was further confirmed by thiobarbituric acid method. The smaller peak of $^{125}$I radioactivity was not retained by $RCA_{120}$ column and corresponded to $^3$H peak in the same position indicating that only a small fraction of CP was not desialated by endothelium. Desialation of CP by acid hydrolysis led to the appearance of similar peaks as after incubation with liver endothelium (Fig 3).

To ensure that the radioactive peaks were indeed associated with the protein, the absorbance of all fractions was determined at 280 nm

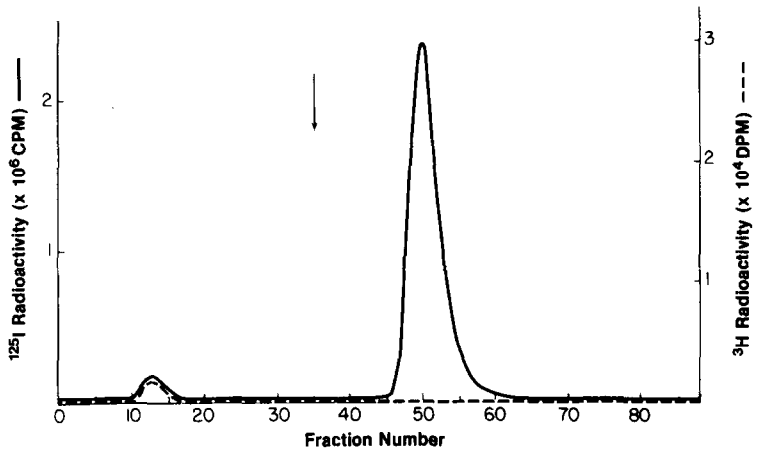


Fig. 3. Chromatography of asialo-CP prepared by mild acid hydrolysis. As is the case with the supernate, some 90% of the protein was retained by the column and eluted with 0.2 M D-galactose. This fraction was also proved to be completely desialated.

spectrophotometerically. The elution patterns were identical with those of $^{125}I$. Moveover, to ensure that radioactivity was not caused by degradation of CP, the protein in each fraction was precipitated with 20% trichloroacetic acid (TCA), and the radioactivity was determined in both the precipitate and the supernate. In all cases more than 80% of radioactivity was precipitated with TCA.

## DISCUSSION

$RCA_{120}$ has an affinity for terminal galactosyl residues of glycoconjugates (15,16). And in this regard it may be considered similar to asialoglycoprotein receptors of hepatocyte membrane. In the latter situation, at least 2 exposed galactosyl residues are said to be required for binding (17), although recently more restricted three-dimensional arrangement of individual galactosyl residues have also been shown to be essential (18). This may also be true of $RCA_{120}$. Thus, when native $^{125}I$-CP is passed through $RCA_{120}$ column, the peak that is not retained consists of two components, one may be interpreted as fully sialated molecules and the second one containing only one of its 9 penultimate galactosyl residues exposed. The smaller peak that is retained may be interpreted as

having two or more exposed galactosyl residues. That this peak is not fully desialated is indicated by the fact that $^{3}H$-labeled molecule (in which sialic acids are labeled) also gives a peak in this position.

When the sialated (unretained) fraction is collected, concentrated, bound to purified liver endothelium at 4°C and chased at 37°C through the cell, the majority of what is externalized is fully desialated CP, indicating that liver endothelium desialates CP. This is consistent with our previous work using double-labeled CP and polyacrylamide gel electrophoresis that suggested the generation of asialoceruloplasmin after incubation with liver endothelium (7). In this regard liver endothelium appears to be as effective in desialation of CP as is acid hydrolysis. The site of desialation in the endothelium remains to be elucidated. Binding of the putative ligand in the supernate to fractionated hepatocytes has been demonstrated (7). The binding affinity is similar to that of asialo-CP, suggesting that the binding is mediated by asialoglycoprotein receptors.

Similar desialation and endothelial mediation for tissue transport has been reported in the liver for another glycoprotein, transferrin (19). Thus, this present work gives further credence to the concept that liver endothelium can mediate tissue transport of glycoprotein through specific receptor-mediated endocytosis and consequent to its desialation of these glycoproteins, asialoglycoprotein receptors of hepatocyte membrane may serve as universal acceptors for these molecules.

## ACKNOWLEDGEMENT

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