Physical and Chemical Studies on Ceruloplasmin

V. METABOLIC STUDIES ON SIALIC ACID-FREE CERULOPLASMIN IN VIVO*

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SUMMARY

Injection into rabbits of radioactive ceruloplasmin from which sialic acid had been removed enzymatically resulted in a rapid disappearance of the asialoceruloplasmin from the serum and its appearance in parenchymal cells, but not in Kupffer cells, of the liver. The rapidity of this transfer was dependent upon the presence of intact galactose residues as the terminal, nonreducing sugar of the carbohydrate moiety. Intact asialoceruloplasmin, doubly labeled with tritium and 14Cu, was isolated from the liver in good yield 24 min after intravenous injection.

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MATERIALS AND METHODS

14Cu, with a specific activity of about 20 mC per mg, was obtained as copper nitrate in 1 N HNO3 from Union Carbide Corporation, Tuxedo, New York. It was converted to copper sulfate by addition of H2SO4 and evaporation to dryness. The residue was dissolved in 0.10 M sodium acetate, pH 5.6, to a final concentration of 10-3 M copper. The content of 14Cu in protein solutions, serum, or tissues was assayed in a Nuclear-Chicago well type scintillation detector, DS-202, by means of a model 8725 analyzer scaler, with corrections for background and physical decay of the radioisotope.

Sodium borohydride-3H, obtained from International Corporation, City of Industry, California, had a measured specific activity of 340 mC per mmole (1). For assays of serum, in a Packard model 2375 Tri-Carb liquid scintillation spectrometer, up to 0.50 ml was added to 17.0 ml of Cabosil (2). Samples of liver were suspended in 4.0 ml of 33% KOH (w/w) per g of tissue, wet weight, and allowed to stand overnight at room temperature. The suspension was heated to 70° for 1 hour and cooled, and 0.50-ml aliquots were added to 17.0 ml portions of Cabosil. All assays were performed by adding 0.10 ml of water to one aliquot and 0.10 ml of standard H2O-3H solution to a second aliquot; counting efficiencies varied from 7 to 15% for serum and from 4 to 6% for liver.

Amberlite IRA-400 and Chelex 100 ion exchange resins were obtained from Calbiochem.

Neuraminidase of Vibrio cholerae, strain Z-4, 500 units per ml, and galactose oxidase, 10 units per mg, were obtained from General Biochemicals. Horseradish peroxidase, 400 units per mg, was supplied by Worthington.

β-Galactosidase was isolated and purified by the method of Hughes and Jeanloz (3) from cultures of Diplococcus pneumoniae, type 1-F, kindly given to us by Miss Leslie Wetterlow of the State Biological Station, Jamaica Plain, Massachusetts. The purified enzyme contained 1970 units of β-galactosidase per mg, and was completely devoid of β-N-acetylglucosaminidase and of both α- and β-L-fucosidase activities.
Antihuman ceruloplasmin goat serum was kindly supplied by Dr. John Robbins.

All other reagents were the same as those used previously (1).

**Purification of Rabbit Ceruloplasmin and Preparation of its Derivatives**

Ceruloplasmin (Preparation 1)—Rabbit serum from 1- to 3-year-old animals (Pel Freeze Biologicals, Inc., Rogers, Arkansas) was dialyzed in the cold for 48 hours against two changes of 20 volumes of buffer (pH 5.6) containing 0.05 M sodium chloride (Buffer A). After centrifugation for 20 min at 20,000 x g, the 3 volumes of this serum were passed through a column containing 1 volume of DEAE-cellulose equilibrated with the same buffer. The column was washed with the same buffer until A_{540} at 280 m\u00b6 was less than 0.200, after which the crude ceruloplasmin solution was eluted with a buffer (pH 5.6) containing 0.05 M sodium acetate and 1% sodium chloride (Buffer B). To 1 volume of the eluate were added 3 volumes of ethanol-chloroform solution (9:1, v/v). The resulting suspension was kept at 25°C for 30 min and the blue precipitate, separated by centrifugation for 10 min at 3,000 x g, was dissolved in 1 volume of Buffer B for each 10 volumes of serum. Some insoluble white material was removed by centrifugation at 35,000 x g for 15 min, and the blue solution was dialyzed overnight in the cold against 100 volumes of Buffer B. To each 100 ml of the dialyzed solution were added 33 g of crystalline ammonium sulfate at 3°C. The blue precipitate was separated by centrifugation at 20,000 x g for 20 min, dissolved in a minimal volume of Buffer B, and dialyzed overnight in the cold against 200 volumes of this buffer. Ceruloplasmin was precipitated from the dialyzed solution by the addition of ethanol-chloroform solution. The precipitate was dissolved again in a minimal amount of Buffer B, and the solution was clarified by centrifugation at 35,000 x g for 15 min, dialyzed overnight in the cold against 200 volumes of Buffer B, sterilized by passage through a Millipore filter, and stored at 3°C in sealed vials. An example of this procedure is given in Table I. The properties of purified rabbit ceruloplasmin, as well as those of its various modifications, are summarized in Table II.

**Asialoceruloplasmin (Preparation 2)—Ceruloplasmin (Preparation 1) and neuraminidase, in final concentrations of 0.45% and 42 units per ml, respectively, were incubated at 25°C for 75 hours in a buffer (pH 5.6) containing 0.05 M sodium acetate, 0.165 M sodium chloride, 2 x 10^{-3} M calcium chloride, and 1% tolue. The protein was then precipitated by the addition of 3 volumes of ethanol-chloroform, 9:1, and incubated at 25°C for 30 min. The blue precipitate, separated by centrifugation for 10 min at 3,000 x g, was dissolved in 1 volume of Buffer B, clarified by centrifugation for 10 min at 3,000 x g, and dialyzed overnight in the cold against 100 volumes of this buffer. This treatment removed 98% of the sialic acid.

Oxidized Asialoceruloplasmin (Preparation 3)—Asialoceruloplasmin (Preparation 2), galactose oxidase, and horseradish peroxidase, in final concentrations of 0.54%, 12.7 units per ml, and 0.07%, respectively, were added to this buffer, and the mixture was incubated at 25°C for 30 min. The blue solution was dialyzed overnight in the cold against 100 volumes of this buffer. This treatment removed 98% of the sialic acid.

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**Table I**

*Purification of ceruloplasmin from rabbit serum (Preparation 1)*

<table>
<thead>
<tr>
<th>Material</th>
<th>Volume</th>
<th>Total ceruloplasmin-bound copper</th>
<th>Recovery</th>
<th>A_{540}/A_{280}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dialyzed serum</td>
<td>300 ml</td>
<td>327 μg</td>
<td>100.0%</td>
<td>0.013</td>
</tr>
<tr>
<td>Eluate from DEAE</td>
<td>55.8 ml</td>
<td>293 μg</td>
<td>89.6%</td>
<td>0.038</td>
</tr>
<tr>
<td>After first ethanol-chloroform precipitation</td>
<td>34.5 ml</td>
<td>237 μg</td>
<td>72.5%</td>
<td>0.042</td>
</tr>
<tr>
<td>Ammonium sulfate precipitation</td>
<td>8.0 ml</td>
<td>160 μg</td>
<td>48.9%</td>
<td>0.047</td>
</tr>
<tr>
<td>After second ethanol-chloroform precipitations</td>
<td>4.8 ml</td>
<td>114 μg</td>
<td>34.9%</td>
<td>0.047</td>
</tr>
</tbody>
</table>

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**Table II**

*Properties of purified native and modified rabbit ceruloplasmin*

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Ratio, A_{420}/A_{280}</th>
<th>Enzymatic activity&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Sialic acid&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Hexoses&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Galactose&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Hexosamine&lt;sup&gt;e&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Native ceruloplasmin</td>
<td>0.047</td>
<td>0.023</td>
<td>0.48</td>
<td>0.0180</td>
<td>2.16</td>
<td>2.52</td>
</tr>
<tr>
<td>2. Asialoceruloplasmin</td>
<td>0.048</td>
<td>0.023</td>
<td>0.48</td>
<td>0.0170</td>
<td>0.85</td>
<td>2.52</td>
</tr>
<tr>
<td>3. Oxidized asialoceruloplasmin</td>
<td>0.041</td>
<td>0.092</td>
<td>0.54</td>
<td>0.0173</td>
<td>2.68</td>
<td>1.31</td>
</tr>
<tr>
<td>4. Trinitiated asialoceruloplasmin</td>
<td>0.041</td>
<td>0.018</td>
<td>0.44</td>
<td>0.0173</td>
<td>1.34</td>
<td>0.67</td>
</tr>
<tr>
<td>5. Asialongalactogalactoceruloplasmin</td>
<td>0.049</td>
<td>0.023</td>
<td>0.47</td>
<td>0.0179</td>
<td>1.34</td>
<td>0.67</td>
</tr>
</tbody>
</table>

<sup>a</sup> Expressed as ΔA_{420} at 530 m\u00b6 per min per μg of protein-bound copper at 30°C, determined as in Reference 4, except that the assay medium contained 1.5 x 10^{-3} M CaCl<sub>2</sub> in addition to its other components.

<sup>b</sup> Determined by the method of Warren (5).

<sup>c</sup> Determined on the neutral sugar fraction described in Reference 1; standard curves were made with solutions of equimolar amounts of m-mannose and m-galactose. For this and for the four individual carbohydrate analyses, the protein concentration was calculated from the absorbance at 280 m\u00b6 with the extinction coefficient, 13.1, obtained for native ceruloplasmin.

<sup>d</sup> Determined on the neutral sugar fraction by Method I of Roth, Segal, and Bertoli (6).

<sup>e</sup> Determined by the method of Elson and Morgan (7) following digestion of the protein in 2 N HCl at 100°C for 6 hr's in sealed Pyrex tubes with glucosamine-HCl as standard.

<sup>f</sup> This preparation of ceruloplasmin migrated as a single, symmetrical peak in free electrophoresis in a sodium phosphate buffer (pH 7.6) of ionic strength 0.10, with a mobility of -3.73 x 10^{-4} cm² volt⁻¹ sec⁻¹. Dry weight was determined as described in Reference 1. The copper content of the preparation (determined as in Reference 1) was 0.271%; A_{540} at 610 m\u00b6 was 0.618; A_{420} at 280 m\u00b6 was 13.1; and the fucose content (determined on the neutral sugar fraction by the method of Dische and Shettle (8)) was 0.11%.
acetate, 0.165 M sodium chloride, and 1% toluene. After dilution with 2 volumes of water, the pH was adjusted to 5.6 with 1.0 M acetic acid and the solution was passed through a column of one-third its volume of DEAE-cellulose equilibrated with Buffer A. The column was washed with the same buffer, and the blue protein was eluted with Buffer B. Treatment with ethanol-chloroform and dialysis, as in the preparation of asialoceruloplasmin, effected further purification. Quantitative determination of intact D-galactose in Preparation 3 indicated that 94% of the galactose residues originally present in the asialoceruloplasmin had been oxidized.

Tritiated Asialoceruloplasmin (Preparation 4)—Tritiation of Preparation 3 with sodium borohydride- 3H was carried out as described previously for human ceruloplasmin (1).

Asialoagalactoceruloplasmin (Preparation 5)—Asialoceruloplasmin (Preparation 2) and β-galactosidase, in final concentrations of 0.54% and 890 units per ml, respectively, were incubated at 25°C for 71 hours in a buffer (pH 6.3) containing 0.05 M sodium acetate, 0.165 M sodium chloride, and 1% toluene. After the addition of 2 volumes of 0.05 M sodium acetate buffer (pH 5.6) the solution was passed through a column made of one-third its volume of DEAE-cellulose which had been equilibrated with Buffer A. The column was then washed with the same buffer until A 1 cm at 280 nm of the effluent was less than 0.020, and the blue protein was eluted with Buffer B. The eluate was treated with ethanol-chloroform and dialyzed as in Preparation 2. Of the galactose residues originally present in asialoceruloplasmin, 95% were removed.

Preparation of 64Cu-Labeled Ceruloplasmin and its Derivatives—A 0.25 to 1.0% protein solution in Buffer B (2 to 4 ml) was placed in a Thunberg tube and cooled in ice. To this was added sufficient 1% ascorbic acid, in a 1.0 M sodium acetate buffer (pH 5.6), to give a final ascorbic acid concentration of 0.2 mg per ml. A solution of 64Cu was then added, in an amount equal to that of the protein-bound copper, and the tube was immediately evacuated and incubated at 25°C for 1 hour. The solution was then passed through a column equilibrated with Buffer B containing the following ion exchange resins: Chelex 100, 1.0 ml; Amberlite IRA-400, 3.0 ml; Chelex 100, 1.0 ml. To effect removal of traces of free,ionic copper, it was necessary to keep the effluent solution at room temperature for 18 hours, treat it with ethanol-chloroform as above, dissolve the precipitate in a minimal amount of Buffer B, and filter the solution through a column containing 1.0 ml of Chelex 100 equilibrated with this buffer. Such preparations were free of detectable ionic copper and had an A 400:A 280 ratio between 0.040 and 0.047. The specific activity of the protein-bound copper was between 0.21 and 0.36 that of the protein-bound copper in Preparation 3. The specific activity of the protein-bound copper in Preparation 4 was 3.0 to 4.0 that of the protein-bound copper in Preparation 3. The specific activity of the protein-bound copper in Preparation 4 was 3.0 to 4.0 that of the protein-bound copper in Preparation 3.

Preparation of Doubly Labeled Human Asialoceruloplasmin—Tritiated human asialoceruloplasmin, prepared by the method previously described (1), was labeled with 64Cu by the procedure used for rabbit asialoceruloplasmin.

Animal Experiments

From 0.4 to 1.0 ml of solutions of the various protein preparations was injected rapidly into the marginal ear vein of male, albino rabbits weighing 2 to 2.5 kg. At 3, 6, 12, and 24 min after injection, approximately 2 ml of blood were allowed to drip into a test tube from a cut vein in the opposite ear. The blood was permitted to clot at room temperature, and the radioactivity of the serum was determined after centrifugation. Immediately after withdrawal of the 24-min sample, the animal was killed by an injection of 2.5 ml of a 6% solution of sodium pentobarbital (Sedasol) and the liver, kidneys, spleen, lungs, and heart were removed. The tissues were washed with cold water, blotted dry, and weighed, after which samples were taken for counting.

RESULTS

All of the native and modified rabbit ceruloplasmin preparations listed in Table II, including Preparation 4, were labeled with 64Cu prior to injection. The rates of disappearance from the serum of the radioactive label following intravenous injection of these proteins are shown in Fig. 1. At 15 min after injection, over 90% of the native ceruloplasmin (Curve 1) remained in the serum, whereas less than 10% of asialoceruloplasmin (Curve 2) was detectable. Because it was possible that the rapid disappearance of label in the latter case had resulted from cleavage of
2. Asialoceruloplasmin
3. Oxidized asialoceruloplasmin
4. Asialoceruloplasmin labeled with $^{64}$Cu and with tritium
sonically treated at 10 kc for 60 set (Branson Sonic Power, Danbury, Connecticut), and centrifuged at 100,000 X g for 60 min.

The slides were then treated with Kodak Developer D-19 at 0° for 4 min and with 5yo sodium thiosulfate at 0° for 7 min, and stained with hematoxylin and eosin. The illustration (X 300) shows part of a liver lobule from the biopsy obtained 8 hours and then imbedded in paraffin. At the time the rabbit was killed, 26 min after injection, the liver, weighing 83 g, contained 72.3% and the serum, 4.401, of the injected radioactivity.

1. Ceruloplasmin
   Rabbit 1, 2.20 kg ......................................
   Rabbit 2, 2.00 kg ......................................

2. Asialoceruloplasmin
   Rabbit 3, 2.35 kg ......................................
   Rabbit 4, 2.25 kg ......................................

3. Oxidized asialoceruloplasmin
   Rabbit 5, 2.25 kg ......................................
   Rabbit 6, 2.40 kg ......................................

4. Asialoceruloplasmin labeled with $^{64}$Cu and with tritium
   Rabbit 7, 2.15 kg ......................................
   Rabbit 8, 2.30 kg ......................................
   Rabbit 9, 2.20 kg ......................................
   Rabbit 10, 2.25 kg ......................................

5. Asialoagalactoceruloplasmin
   Rabbit 11, 2.20 kg .......................................
   Rabbit 12, 2.25 kg .......................................

**Table III**

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Protein injected (mg)</th>
<th>Label</th>
<th>Percentage of total injected label recovered in</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Serum</td>
<td>Liver</td>
</tr>
<tr>
<td>1. Ceruloplasmin</td>
<td>2.14</td>
<td>$^{64}$Cu</td>
<td>88.5</td>
</tr>
<tr>
<td></td>
<td>2.11</td>
<td>$^{64}$Cu</td>
<td>86.7</td>
</tr>
<tr>
<td>2. Asialoceruloplasmin</td>
<td>2.18</td>
<td>$^{64}$Cu</td>
<td>4.3</td>
</tr>
<tr>
<td>3. Oxidized asialoceruloplasmin</td>
<td>1.98</td>
<td>$^{64}$Cu</td>
<td>3.1</td>
</tr>
<tr>
<td>4. Asialoceruloplasmin labeled with $^{64}$Cu and with tritium</td>
<td>1.76</td>
<td>$^{64}$Cu</td>
<td>72.0</td>
</tr>
<tr>
<td></td>
<td>1.55</td>
<td>$^{64}$Cu</td>
<td>62.5</td>
</tr>
<tr>
<td>5. Asialoagalactoceruloplasmin</td>
<td>2.17</td>
<td>$^{64}$Cu</td>
<td>61.5</td>
</tr>
</tbody>
</table>

**Table IV**

**Immunochemical recovery of doubly labeled asialoceruloplasmin from rabbit liver**

Human asialoceruloplasmin, 0.70 mg, doubly labeled with tritium and $^{64}$Cu, was injected into the marginal ear vein of a rabbit weighing 2.05 kg. After 24 min, the animal was killed and the liver, which weighed 83 g and contained 72% of the injected $^{64}$Cu, was removed (less than 3% of the $^{64}$Cu was in the blood at the time of death). The entire liver was homogenized with 5 volumes of 0.165 NaCl in a mixer (Osterizer) for 3 min, sonically treated at 10 kc for 60 sec (Branson Sonic Power, Danbury, Connecticut), and centrifuged at 100,000 X g for 60 min. The supernatant fraction contained 79% of the $^{64}$Cu present in the intact organ, but without sonic treatment less than 10% was in the supernatant. To a 16-ml aliquot of the supernatant were added, in the cold, 1.0 ml of human serum, containing 0.3 mg of ceruloplasmin, and 5.0 ml of anti-human ceruloplasmin goat serum. The mixture was incubated for 2 hours at 3° and centrifuged at 3,000 rpm for 10 min. After washing with cold 0.9% NaCl solution, the precipitate was dissolved in 4.0 ml of 50% acetic acid and the solution was assayed for radioactivity.

**Table V**

<table>
<thead>
<tr>
<th>Sample</th>
<th>$^3$H content $^d$pm</th>
<th>$^{64}$Cu content $^c$pm</th>
<th>Ratio $^b$, $^3$H content to $^{64}$Cu content</th>
<th>Recovery $^a$</th>
<th>$^3$H</th>
<th>$^{64}$Cu</th>
</tr>
</thead>
<tbody>
<tr>
<td>Injected asialoceruloplasmin</td>
<td>$2.3 \times 10^4$</td>
<td>$5.3 \times 10^4$</td>
<td>43.4</td>
<td>33</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>Recovered asialoceruloplasmin</td>
<td>$7.6 \times 10^4$</td>
<td>$1.75 \times 10^4$</td>
<td>43.8</td>
<td>33</td>
<td>33</td>
<td></td>
</tr>
</tbody>
</table>

$^{64}$Cu from the protein, doubly labeled asialoceruloplasmin was prepared and injected. Curve 4 illustrates that the rates of disappearance from the serum of tritium and $^{64}$Cu are essentially identical.

The unique role of the terminal galactosyl residues in effecting this rapid clearance of asialoceruloplasmin was shown by the markedly increased survival time which resulted when the

![Fig. 2. Histotradioautograph of liver following injection of tritium-labeled asialoceruloplasmin. A male albino rabbit, weighing 2.05 kg, was anesthetized with a solution of 25% urethane and the liver was exposed by laparotomy. Titrated rabbit asialoceruloplasmin, 3.3 mg (11.9 X $10^6$ dpm), was injected into the marginal ear vein. Wedge biopsy specimens of liver, obtained at 3, 8, and 24 min, were fixed in 10% formaldehyde solution for 18 hours and then imbedded in paraffin. At the time the rabbit was killed, 26 min after injection, the liver, weighing 83 g, contained 72.3% and the serum, 4.4% of the injected radioactivity. Sections of tissue of approximately 6 μ thickness, on glass slides, were sequentially dipped in NTB emulsion No. 3 (Eastman Kodak Company) at 45°, dried, and exposed for 14 days at 3°, all in the dark. The slides were then treated with Kodak Developer D-19 at 0° for 4 min and with 5% sodium thiosulfate at 0° for 7 min, and stained with hematoxylin and eosin. The illustration (X 300) shows part of a liver lobule from the biopsy obtained 8 min after injection. Note the portal space in the right lower corner, the presence of silver granules overlying the parenchymal cells, and their absence in Kupffer cells (arrows).
galactose moiety was either modified or removed. Treatment of asialoceruloplasmin with galactose oxidase, with conversion of 94% of the primary carbinol groups of galactose residues to aldehyde functions, resulted in a striking reversal in the pattern of its survival following injection (Curve 3) as compared with that of the untreated asialoceruloplasmin (Curve 2). A similarly prolonged survival time was observed following injection of asialoceruloplasmin from which 95% of the galactosyl residues had been removed with purified β-galactosidase (Curve 5).

The prompt disappearance of asialoceruloplasmin from the serum was found to be accompanied by a rapid accumulation of untreated asialoceruloplasmin (Curve 2). A similarly prolonged survival time was observed following injection of asialoceruloplasmin with galactose oxidase, with conversion of galactose moiety was either modified or removed. Treatment of ceruloplasmin from which 95% of the galactosyl residues had been removed with purified β-galactosidase (Curve 5).

A third experiment showing deposition of the intact protein within the liver utilized doubly labeled human asialoceruloplasmin. At 30 min after its injection, the liver was removed and its tissue was disrupted by homogenization and sonication. Following centrifugation, the supernatant fraction was allowed to react with anti-human ceruloplasmin goat serum. The specific precipitate formed was collected, washed, and dissolved in 50% acetic acid. The recovery of both 3H and 64Cu was 33%, and the ratio of 3H to 64Cu was 43.4. This ratio was 43.8 in the original, injected material (Table IV).

Cellular localization of the radioactivity within the liver, provided by historadiography of biopsy specimens obtained at 3, 8, and 24 min after the injection of tritiated asialoceruloplasmin, indicated that it was exclusively in parenchymal liver cells; no activity was found in the Kupffer cells at any time (Fig. 2).

DISCUSSION

Asialoceruloplasmin labeled with 64Cu disappears from a rabbit's circulation within minutes (Fig. 1), whereas 64Cu-labeled native rabbit ceruloplasmin survives much longer (Fig. 1) and, indeed, has been shown to have a half-life of 56 hours (9). The almost parallel rates of disappearance from serum of 3H and 64Cu in doubly labeled asialoceruloplasmin (Fig. 1), the recovery, from the liver, of the same proportion of both radioactive markers as that present in injected rabbit asialoceruloplasmin (Table III), and the immunochemical recovery of a significant fraction of injected human asialoceruloplasmin, in which both radioactive labels are also present in the same ratio as in the injected protein (Table IV), constitute strong evidence that cleavage of either label is not occurring and that the entire protein is being transferred from plasma into liver.

This rapid clearance of asialoceruloplasmin by the liver occurs only when the intact galactose residues of the protein are exposed by the removal of sialic acid from native ceruloplasmin. Fig. 1 shows that either removal of these galactose residues, by treatment with β-galactosidase, or oxidation of them, by galactose oxidase, increases the survival time of the protein toward that exhibited by the native protein.

The disappearance of asialoceruloplasmin from the circulation is followed by its appearance in parenchymal cells of the liver exclusively; none is seen in Kupffer cells. This contrasts to the localization of heat-denatured serum proteins and of foreign colloids which, although equally rapidly removed from the circulation, appear almost exclusively in reticuloendothelial cells of liver and spleen.

It is possible that the exposed galactose residues of asialoceruloplasmin are bound to specific receptor sites in the liver or that the removal of sialic acid results in a changed conformation of the protein, either of which could be followed by the prompt movement of the protein from serum to liver. It is unlikely that a change in charge of the protein plays a role in this transfer, since the survival time of asialoceruloplasmin is reversed in large measure toward that of the native protein by modifications involving no further change in charge. Whatever mechanism effects the rapid removal of asialoceruloplasmin, it appears not to be operative for all plasma proteins: in preliminary experiments we have observed that, in rabbits, human asialotransferrin has a half-life entirely comparable to that of native rabbit transferrin (11).

It has never been explained how the majority of patients with Wilson's disease exhibit a deficiency of ceruloplasmin which is qualitatively indistinguishable from that found in normal individuals or how an analogous situation may exist in any other disease associated with a pair of abnormal, autosomal, recessive genes. The data reported here suggest the possibility, in relation specifically to Wilson's disease, of the presence of a genetically defective enzyme system which normally catalyzes the transfer of sialic acid to an otherwise complete ceruloplasmin molecule. The consequence of this defect would be a deficiency in normal ceruloplasmin molecules; those molecules synthesized with incomplete carbohydrate chains either would not be released from their site of synthesis or, if secreted, would not survive in the circulation.

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REFERENCES

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