Isolation of Human Hepatocuprein and Cerebrocuprein

THEIR IDENTITY WITH ERYTHROCUPREIN*

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SUMMARY

Cerebrocuprein and hepatocuprein have been isolated from extracts of human brain and liver, respectively, by a method which utilizes ion exchange chromatography and gel filtration procedures. Detailed studies of the physical, chemical, and immunological properties of the purified proteins were conducted. A comparison of the results with similar data obtained for erythrocuprein from human erythrocytes shows that the proteins from the three tissue sources are identical.

Two copper-containing proteins, now known as erythrocuprein and hepatocuprein, were first isolated from bovine erythrocytes and liver, respectively, by Mann and Keilin (1). Later a somewhat similar protein, designated cerebrocuprein, was isolated from human brain by Porter and Folch (2). Although most studies on these three proteins have indicated that they possess many properties in common, they have generally been regarded as different entities. A detailed systematic investigation appeared to be necessary to clarify this point. The results of such a study are presented here and indicate that the proteins from human liver, brain, and erythrocytes are identical.

This report deals mainly with the purification and properties of human hepatocuprein and cerebrocuprein, since human erythrocuprein has already been examined in detail (3-6). Previous immunological studies in which extracts of brain and liver were reacted with antibody to erythrocuprein have indicated that the three proteins are identical (7, 8). The availability of such antibody and the application of various fractionation techniques utilized in the purification of erythrocuprein (3, 5) greatly aided the development of a procedure for isolating the analogous proteins from liver and brain. All methods which have been previously used for isolating hepatocuprein and cerebrocuprein employed organic solvents and salts of heavy metals (1, 2, 9, 10). Since organic solvents have been shown to induce changes in several physical properties of erythrocuprein (4), only ion exchange chromatography and gel filtration have been used in our procedure.

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METHODS

Amino acid analyses were performed by the methods of Moore and Stein (11) on protein samples hydrolyzed for 20 hours at 110° in evacuated tubes. Analyses were conducted with a Spinco model 120 amino acid analyzer modified for accelerated operation (12). The results were corrected for the destruction of serine and threonine, and for the incomplete hydrolysis of valine and isoleucine, with the use of factors previously determined for erythrocuprein (5).

Results of micro-Kjeldahl analyses (13) were used to calculate protein concentrations assuming 16.9% nitrogen (4). Cystine analyses were performed by the method of Van De Bogart and Beinert (14). Prior to the analyses the protein samples were dialyzed at 3-5° for at least 48 hours against 0.15 M NaCl which had been passed over a column of Dowex A-1 chelating resin. The distilled water used in all experiments was passed through a mixed bed ion exchange resin (Continental Deionized Water Service, Chicago, Illinois).

Equilibrium sedimentation experiments were conducted essentially by the method of Yphantis (20) except that the concentration gradients were measured with the photoelectric scanning system.

Proteins to be used in peptide mapping experiments were dialyzed overnight near 4° against a pH 8.0, 3.0 M, Tris-hydrochloride buffer which was made 7.4 M in urea. These solutions, containing 5 to 10 mg of the denatured protein per ml, were incubated with 0.5 M 2-mercaptoethanol at 37° for 48 hours following which a 2-fold molar excess of iodoacetamide over the sulfhydryl content was added. The alkylated protein was dialyzed exhaustively against 0.2 M NH4HCO3 and then digested with trypsin (2%, w/w, of the sample) at 37° for 12 hours. A second addition of trypsin was made and the incubation continued for an additional 12 hours. About 2 mg of the lyophilized digests were fingerprinted by the method of Bennett (21). Pep-
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of erythrocuprein derived from residual blood in the tissues.

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and brain were obtained at autopsy within 20 hours after death.

Isolation of Hepatocuprein and Cerebrocuprein—Human liver
and brain were obtained at autopsy within 20 hours after death.
Organs which showed no gross pathological changes were utilized.
All preparative steps were carried out in a cold room near 5°.
Tissues were cut into 2- to 8-mm thick slices and the blood was
removed as thoroughly as possible by gently kneading the slices
in cold 0.15 M NaCl. The washed slices were then ground in a
meat grinder and soluble proteins were extracted by stirring the
homogenate in pH 7.0, 0.01 Γ/2, sodium cacodylate buffer (1
liter per kg of wet tissue) for 1 hour. The larger cell fragments
were removed by centrifugation at 6500 × g for 20 min and re-
extracted twice with the same amount of buffer. The hemo-
globin concentration in the combined extracts was measured as
cyanohemoglobin (25) for the purpose of calculating the amount
of erythrocuprein derived from residual blood in the tissues.

The extract was dialyzed to equilibrium against pH 7.0, 0.01
Γ/2, sodium cacodylate buffer and then centrifuged at 18,000 ×
g for at least 1 hour to remove small particulate matter which
would interfere with the subsequent chromatographic step. The
clear supernatant solution was applied to a column (5.8 × 50
em) of DEAE-cellulose (Whatman Fibrous D-22, Reeve Angel
Inc., Clifton, New Jersey) which was previously equilibrated
with the above buffer. Elution of absorbed proteins was ac-
complished with a linear salt gradient formed by adding 3 liters
of pH 7.0, 0.01 Γ/2, sodium cacodylate which was 0.3 m in NaCl
to 3 liters of this buffer without NaCl. Fractions of 16 to 20
ml were collected and examined for copper by a spot test method
employing 2,2′-biquinoline (5). The low levels of copper and
the presence of colored materials in some fractions made the
evaluation of these spot tests difficult. The presence of hepato-
cuprein or cerebrocuprein could be recognized more readily by
precipitin tests using antibody to erythrocuprein. Fractions
giving positive immunological tests coincided with those con-
taining copper. However, the brain extracts also contained
relatively large amounts of an acidic copper-containing protein
which did not react with antibody to erythrocuprein and which
was eluted only at Γ/2 greater than 0.2.

The pooled hepatocuprein or cerebrocuprein fractions from the
pH 7 chromatographic step were dialyzed against pH 5.5, 0.02
Γ/2, sodium acetate-acetic acid buffer. Precipitate which formed
was removed by centrifugation and the supernatant solution was
applied to a column (4.3 × 50 cm) of DEAE-cellulose which had
been equilibrated with the above buffer. The proteins were
eluted with a linear salt gradient which used 2 liters of pH 5.5,
0.02 Γ/2, sodium acetate-acetic acid buffer and 2 liters of this
buffer which was 0.25 m in NaCl. The fractions were examined
for copper and for reactivity with antibody and the results,
along with the optical densities at 280 mυ, were used as a guide
in selecting material for further fractionation. Suitable frac-
tions were pooled, dialyzed against pH 8.0, 0.02 Γ/2, triethanol-
amine-hydrochloride buffer and applied to a column (3.5 × 50
em) of DEAE-cellulose equilibrated with this buffer. The
chromatogram was developed with a linear salt gradient prepared
with 1.5 liters of pH 8.0, 0.02 Γ/2, triethanolamine-hydrochloride
and 1.5 liters of this buffer made 0.3 m in NaCl. Fractions of
10 to 15 ml were examined as described for the previous chro-
amatographic experiment and those suitable for further purifi-
cation were pooled.

After dialysis against 0.02 m sodium acetate (pH near 7.0), the
pooled protein was absorbed on a column (2 × 10 cm) of DEAE-
cellulose equilibrated with 0.02 m sodium acetate and then eluted sharply in less than 50 ml with 0.5 m NaCl. This solution
was applied directly to a column (5.4 × 110 cm) of Sephadex
G-75 equilibrated with pH 7.0, 0.01 Γ/2, sodium cacodylate buffer made 0.14 m in NaCl. A typical elution pattern is shown
in Fig. 1 and the fractions which were pooled are indicated. The
protein was concentrated to near 1% on a small DEAE-cellulose
column as described above and then dialyzed against 0.15 m NaCl.
The procedure was satisfactory for processing from 2 to 5 kg
of tissue. The yields were about 65 mg of hepatocuprein and
25 mg of cerebrocuprein per kg of liver and brain, respectively.
The amount of erythrocuprein in the extracts was calculated as-
suming that 100 ml of erythrocytes contained 34 g of hemoglobin
and 10 mg of erythrocuprein (26). The maximum quantity of
erythrocuprein in the initial extracts was less than 5% of the
amounts of hepatocuprein or cerebrocuprein isolated. Since sub-
stances in the tissue extracts other than cyanohemoglobin ab-
sorb at 540 mυ, and since erythrocuprein would not be expected
to be recovered quantitatively, these estimates for erythrocuprein
contamination are high.
Fig. 2. A comparison of erythrocuprein (A), hepatocuprein (B), and cerebrocuprein (C) by the Ouchterlony technique (17). The center well contained rabbit antibody to erythrocuprein and well D contained buffer. The same result was obtained with rabbit antibody to hepatocuprein.

Fig. 3. A comparison of the immunological properties of erythrocuprein, hepatocuprein, and cerebrocuprein by means of quantitative precipitin reactions utilizing rabbit antibody to erythrocuprein.

**RESULTS**

**Immunological Studies**—The results of immunodiffusion reactions between the purified erythrocuprein, hepatocuprein, and cerebrocuprein preparations and rabbit antibody to erythrocuprein are shown in Fig. 2. Reactions of complete identity were obtained for the three proteins. The same result was obtained when rabbit antibody to hepatocuprein was employed. The immunological identity of the proteins was confirmed by quantitative precipitin reactions. Coincident precipitin curves are obtained for the reaction between each of the three proteins and rabbit antibody to erythrocuprein (see Fig. 3). The results of these immunological studies are in agreement with those obtained earlier with crude extracts of liver and of brain (7, 8).

**Physical Studies**—The three proteins displayed identical electrophoretic behavior in starch gel at pH 5.5 and 8.0 (see Fig. 4 and 5).
FIG. 6. Peptide maps of tryptic digests of erythrocuprein (EC, upper), hepatocuprein (HC, center), and cerebrocuprein (CC, lower). The chromatography employed the 1-butanol-

acetic acid-water (4:1:5) solvent for 18 hours. The electrophoresis was conducted for 70 min at about 160 ma and 55 volts per cm with the pH 3.6 pyridine acetate buffer.

TABLE I

Some physical properties of hepatocuprein, cerebrocuprein, and erythrocuprein

<table>
<thead>
<tr>
<th>Protein</th>
<th>Isoelectric point</th>
<th>Molecular weight</th>
<th>$m_{w}^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatocuprein</td>
<td>4.76</td>
<td>35,600 ± 1,800</td>
<td>2.84 (0.31)</td>
</tr>
<tr>
<td>Cerebrocuprein</td>
<td>4.74</td>
<td>35,700 ± 1,900</td>
<td>2.70 (0.41)</td>
</tr>
<tr>
<td>Erythrocuprein</td>
<td>4.75</td>
<td>33,200 ± 1,800</td>
<td>2.94 (0.21)</td>
</tr>
</tbody>
</table>

* The values in parentheses are the concentrations in milligrams per ml for the protein solutions examined.

+ One standard deviation from the average molecular weight calculated from three gradient curves.

+ Data taken from Hartz and Deutsch (5).

TABLE II

Amino acid and copper contents of hepatocuprein, cerebrocuprein, and erythrocuprein

The values are expressed as moles per 33,600 g of protein and are averages of four, two, and three analyses for hepatocuprein, cerebrocuprein, and erythrocuprein, respectively, except where numbers in parentheses indicate the number of analyses.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Hepatocuprein</th>
<th>Cerebrocuprein</th>
<th>Erythrocuprein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>22.5</td>
<td>22.1</td>
<td>23.2</td>
</tr>
<tr>
<td>Histidine</td>
<td>13.3</td>
<td>13.7</td>
<td>12.8</td>
</tr>
<tr>
<td>Arginine</td>
<td>8.4</td>
<td>8.1</td>
<td>7.8</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>36.9</td>
<td>30.6</td>
<td>36.2</td>
</tr>
<tr>
<td>Threonine</td>
<td>16.1</td>
<td>16.4</td>
<td>16.2</td>
</tr>
<tr>
<td>Serine</td>
<td>20.9</td>
<td>20.7</td>
<td>20.3</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>28.7</td>
<td>27.6</td>
<td>27.4</td>
</tr>
<tr>
<td>Proline</td>
<td>10.7</td>
<td>10.0</td>
<td>11.3</td>
</tr>
<tr>
<td>Glycine</td>
<td>48.0</td>
<td>47.5</td>
<td>47.1</td>
</tr>
<tr>
<td>Alanine</td>
<td>21.2</td>
<td>20.9</td>
<td>20.6</td>
</tr>
<tr>
<td>Valine</td>
<td>28.2</td>
<td>29.7</td>
<td>29.6</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.3</td>
<td>0.0</td>
<td>0.1</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>16.1</td>
<td>16.8</td>
<td>16.5</td>
</tr>
<tr>
<td>Leucine</td>
<td>19.4</td>
<td>18.5</td>
<td>19.4</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.3</td>
<td>0.0</td>
<td>0.1</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>8.2</td>
<td>8.4</td>
<td>8.9</td>
</tr>
<tr>
<td>Half-cystine</td>
<td>7.7 (2)</td>
<td>6.3 (1)</td>
<td>6.7 (1)</td>
</tr>
<tr>
<td>Copper</td>
<td>1.83 (8)</td>
<td>1.86 (4)</td>
<td>2.02 (5)</td>
</tr>
</tbody>
</table>

4). They resolved into two components at pH 5.5, and into three at pH 8.0. Studies on erythrocuprein indicate that these components probably arise from small changes in the tertiary structure of the protein (27).

The charge properties of hepatocuprein and cerebrocuprein were examined further by the electrofocusing technique. The results presented in Fig. 5 show no evidence of the multiple components resolved by starch gel electrophoresis. Isoelectric points determined from the electrofocusing experiments were near 4.75 which is the same as the value obtained for erythrocuprein (see Table I).

One symmetrical boundary was obtained with both hepatocuprein and cerebrocuprein on velocity sedimentation analysis. Their sedimentation coefficients measured at low protein concentrations do not differ significantly but are slightly lower than...
that reported for erythrocuprein (see Table I). An earlier report gave \( \bar{s}_{20,w} \) values of 2.79 S for native erythrocuprein and 3.01 S after treatment with chloroform-ethanol (4). Sedimentation coefficients of 2.79 and 3.24 S have been reported for electrophoretic variants of erythrocuprein (27). Apparently shape changes occur which influence the sedimentation properties. Therefore, the small variations seen among the \( \bar{s}_{20,w} \) values for erythrocuprein, hepatocuprein, and cerebrocuprein reflect only minor differences between the proteins.

Molecular weights measured for hepatocuprein and cerebrocuprein by the equilibrium sedimentation method are nearly identical (see Table I). They are higher than those generally obtained for erythrocuprein by this technique, but all values obtained are within the range of experimental variation. The molecular weight data together with the velocity sedimentation coefficients indicate that the three proteins are the same size.

**Chemical Studies**—The amino acid compositions of erythrocuprein, hepatocuprein, and cerebrocuprein are presented in Table II. Their compositions are identical within experimental variation, except that the half-cystine content of hepatocuprein is significantly higher than those of the other two proteins. Previous amino acid analyses of erythrocuprein (4-6) have given widely variant results for half-cystine and the values presented here are within the range of those previously reported. Since much difficulty has been experienced in determining the level of this amino acid in erythrocuprein, it is doubtful that the variations in the half-cystine levels reflect significant differences between the three proteins.

Tyrosine was not detectable in the hydrolysate of cerebrocuprein and this result is in agreement with the compositions reported for erythrocuprein where very low or undetectable levels of tyrosine were found (4, 5). The 0.3 mole of tyrosine found in the hepatocuprein hydrolysate was probably derived from contaminating proteins. The relatively low copper content of hepatocuprein to be discussed below may reflect such contamination.

The primary structures of the three proteins were compared by means of maps of their tryptic peptides. Repeated experiments with three tryptic digests from each of the proteins did not reveal significant differences. The results of typical fingerprint experiments are shown in Fig. 6. Similar studies with chymotryptic digests also indicated that the proteins were identical; however, fewer discrete peptides were resolved from these digests.

Results of copper analyses on erythrocuprein, hepatocuprein, and cerebrocuprein are included in the data of Table II. The levels of cerebrocuprein and erythrocuprein do not differ significantly from 2 g atoms per mole of protein but the value for hepatocuprein is low. The amino acid data presented above suggest that contamination of the hepatocuprein by other proteins might account for the low copper content. The level of copper in the hepatocuprein preparations indicates the presence of 10% impurities. This degree of contamination, however, is not evident from starch gel electrophoresis patterns (Fig. 4) or from the results of the electrofocusing experiments shown in Fig.

**Fig. 7.** Visible and ultraviolet spectra of hepatocuprein and cerebrocuprein. The protein concentrations in the solutions used for the measurements were near 1.2 and 11 mg per ml for the ultraviolet and visible regions, respectively.

**Fig. 8.** The EPR spectra of (A) erythrocuprein, (B) hepatocuprein, and (C) cerebrocuprein in 0.15 M NaCl. The copper concentrations were 5.5 \( \pm 1.0 \) \( \times 10^{-4} \) M. The EPR conditions were: microwave frequency 9215 MHz, microwave power, 27 mwatts; modulation amplitude, 5 gauss; scan rate, 400 gauss per min; time constant, 0.25 sec; and sample temperature, 102°K. The amplifier gains were (A) 100, (B) 125, and (C) 160. The value of \( g_m \) is 2.063 (28).
Thus, the low copper level may be caused partly by the presence of hepatocuprein containing less than 2 atoms of copper per molecule.

**Spectral Studies**—The absorption spectra of hepatocuprein and cerebrocuprein presented in Fig. 7 have broad maximum centered near 675 mµ as previously noted for erythrocuprein (4, 5). The extinction coefficients, $E_{660}$ of 0.075 (±0.002) at this wave length are the same as that reported for erythrocuprein by Hartz and Deutsch (5).

Maxima near 280 mµ are not seen in the spectra of hepatocuprein and cerebrocuprein. This indicates that the proteins do not contain significant amounts of tryptophan or tyrosine. The spectrum obtained for hepatocuprein is very similar to that reported for erythrocuprein (5) in that the maximum occurs at 265 mµ and there is a shoulder at 258 mµ. The values of $E_{265}^{max}$ for two hepatocuprein preparations were 5.87 and 7.00. The spectrum obtained with cerebrocuprein had a maximum at 258 mµ and a shoulder at 265 mµ. At 265 mµ the $E_{265}^{max}$ value was 7.33. The extinction coefficients reported for erythrocuprein at 265 mµ vary between 5.06 and 9.02 (27). Thus, the variations in the ultraviolet spectra noted for the three proteins are not greater than those seen among spectra of different erythrocuprein preparations.

The EPR spectra for erythrocuprein, hepatocuprein, and cerebrocuprein are indistinguishable (see Fig. 8). This result together with the absorption spectra in the visible region indicate that the copper ligands of the proteins are very similar.

**DISCUSSION**

The possibility that erythrocuprein, hepatocuprein, and cerebrocuprein are identical has been evident since their discovery. The data presented in the literature do not lend themselves to a satisfactory comparison of these proteins because highly purified preparations were not employed in some studies and data presented for each protein were not obtained under identical experimental conditions. Furthermore, most of the studies had utilized proteins prepared by methods which appear to produce unpredictable alterations in their physical properties. Experience obtained from studies on erythrocuprein in this laboratory has aided in the solution of some of these problems. By conducting detailed studies simultaneously on protein preparations from each of the three tissue sources we have demonstrated unequivocally that erythrocuprein, hepatocuprein, and cerebrocuprein are identical.

The amount of hepatocuprein and cerebrocuprein isolated are low in terms of the total copper content of liver and brain, respectively. It is evident that these tissues possess other copper-containing proteins. These latter components segregate into various fractions in the isolation of hepatocuprein and cerebrocuprein and it appears feasible to isolate some of them and thus further extend our knowledge of the distribution of copper in these tissues.

Only a small amount of the hepatocuprein and cerebrocuprein isolated can be the result of the residual blood in the washed tissues used as source material. Furthermore, the properties of the isolated protein of liver and brain are similar to the preparations of other investigators. This clearly indicates that we are not dealing with protein entities of a different type than those hitherto explored by others.

The results of amino acid analyses and peptide mapping studies of tryptic and chymotryptic digests provide very strong evidence in support of the identity of erythrocuprein, hepatocuprein, and cerebrocuprein. These data are substantiated by molecular weight and velocity sedimentation measurements, by immunological and electrophoretic studies, and by copper analyses. Differences were seen in the absorption spectra and the half cystine content of the proteins but these variations were not greater than those seen among different erythrocuprein preparations.

Porter (29) noted that the three proteins were similar in many respects but they had different charge properties. The latter conclusion was based on the observation that the proteins were eluted at different ionic strengths when extracts of the respective tissues were chromatographed on columns of DEAE-cellulose. This method of comparing the charge properties of proteins can be misleading because the ionic strength at which elution occurs is influenced by the presence of other proteins.

In addition to the EPR studies conducted on solutions of the purified proteins, the spectrum of a specific precipitate of erythrocuprein was examined in order to determine whether the reaction with antibody induced changes in the copper ligands. Significant differences were found between the spectra obtained for the specific precipitate and that of the untreated protein. Thus, the isolation of erythrocuprein, hepatocuprein, and cerebrocuprein from tissue extracts for EPR studies cannot be accomplished satisfactorily with antibodies as was done earlier with ceruloplasmin (30, 31).

Most of the copper in brain extracts was associated with an acidic yellow protein which was well separated from the cerebrocuprein by the first DEAE-cellulose chromatographic step. Preliminary studies on this protein indicated that it is poorly soluble at pH 5.5 and readily converts to several polymeric species at neutral pH. It might be related to the copper-containing precipitate which Porter and Folch (2) obtained at pH 5.5 from extracts of bovine brain. Because a large portion of the copper in human brain appeared to be associated with this protein further investigations of its properties and physiological role could be important.

Since erythrocuprein, hepatocuprein, and cerebrocuprein are identical we suggest that this metalloprotein be designated cytocuprein. It does not appear desirable to refer to the same protein by different names.

**Acknowledgments**—We are grateful to Drs. H. Beinert and W. H. Orme-Johnson at the Institute for Enzyme Research for performing the electron paramagnetic resonance measurements.

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