In 1924, Hart, Elvehjem and coworkers demonstrated that copper is essential for erythropoiesis in mice, if not all, mammalian species (1). Since that time many studies have been performed in attempts to elucidate the manner in which copper functions in erythropoiesis, but as yet this mechanism is not understood.

Recent studies in this laboratory (2) have indicated that the amount of copper in the erythrocytes of copper-deficient pigs is reduced and that the life span of these copper-deficient erythrocytes in the circulation is shortened. This suggests that copper may be an essential component of adult red cells and, therefore, it seemed of importance to isolate and characterize the copper-containing component or components of erythrocytes.

The only previous study of this nature was that of Mann and Keilin (3). These workers isolated a blue copper protein from ox erythrocytes. The protein contained 0.34 per cent copper and had a molecular weight of about 34,000. Attempts to isolate a pure copper protein from human erythrocytes were unsuccessful, although a preparation containing 0.21 per cent copper was obtained.

The purpose of this paper is to describe the isolation of a copper protein from human erythrocytes and to outline an immunologic technique for the quantitative estimation of this protein in homolysates of erythrocytes. In addition, immunologic, chemical and physical properties of the protein are described in this and in another paper (4).

**EXPERIMENTAL**

"Copper-free" distilled water was obtained by passage of distilled water through a Deeminizer (Crystal Research Laboratories, Inc., Hartford, Connecticut) column of exchange resins. Saturated lead acetate solution was adjusted to pH 5.7 and stored at 0° as were all other reagents, except where noted. The chloroform-ethanol solution (1:14) was stored at -5° until used. In the later stages of purification, all tubes and vials were capped with Parafilm (Marathon Corporation, Menasha, Wisconsin), which has been found to be copper-free. Calcium triphosphate gel was prepared according to Tsuboi and Hudson (5) except that the final suspension was in copper-free distilled water. In the preparation of the gel it is important that it be washed thoroughly with distilled water because erythrocuprein is eluted from the gel in the presence of even small amounts of phosphate buffer. This gel was stable for at least several months when stored at 0-5°. Each freshly prepared batch was tested for its ability to adsorb erythrocuprein from solution.

The various buffers used were all prepared in copper-free distilled water and stored at 0-5°. Analytical reagent grade ammonium sulfate was recrystallized and a saturated solution in copper-free distilled water was prepared and adjusted to pH 6.8 to 6.4 with NH₂OH and then stored at 0°. All fractionations with ammonium sulfate were at 0°.

Nitrogen analyses were performed by a micro-Kjeldahl method (6). Absorption spectra were determined with a Beckman model DU spectrophotometer. Paper electrophoreses were performed in the Beckman Spinco model R cell. The papers were stained with bromphenol blue for protein and with aizarin blue S (7) for detection of protein-bound copper. Copper analyses were performed colorimetrically with diethyldithiocarbamate (8) or with oxalyldihydrazide (9).

Antibody against purified erythrocuprein was prepared by immunizing rabbits intradermally with a Freund adjuvant mixture (10). Three courses of immunization, each consisting of three weekly injections of antigen (1.0 mg.), were necessary in order to obtain antisera containing about 0.3 mg. of antibody N per ml. The antiserum was preserved with 1:10,000 Merthiolate² or stored in the frozen state. Homogeneity of antisera was demonstrated by Oudin determinations (11) which were performed in a constant temperature bath. Ouchterlony (12) plates were run at room temperature. Quantitative precipitin analyses were done as outlined by Kabat and Mayer (13).

**Isolation and Purification of Erythrocuprein**

*Step 1. Preparation of Hemolysate—Human blood,© preserved in acid-citrate-dextrose solution, was used in these studies. Blood samples were pooled without regard to blood group and Rh factor. In a typical run 6 liters of blood were used and the plasma separated by centrifugation and discarded. The erythrocytes were washed three times in the cold with isotonic saline and

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2 Eli Lilly and Company, Indianapolis, Indiana. A 1 per cent solution in 0.85 per cent NaCl was stored in the dark.
3 Cells which were more than 6 weeks old were usually unsatisfactory, due to extensive hemolysis. Erythrocytes were washed immediately after removal of plasma since storage for 24 hours or longer led to greatly increased fragility.
then hemolyzed with an equal volume of cold distilled water. The total copper was 3030 µg. (Table I).

Step 2. Removal of Hemoglobin—0.88 volume of cold chloroform-ethanol solution was added rapidly with vigorous stirring to the cold hemolysate (3). A massive precipitate developed immediately and it was necessary to agitate the suspension for at least 5 minutes to ensure thorough mixing. After another 5 minutes, the suspension was filtered through Hy-Flo Super Cel (Johns-Manville Products, New York, New York) and the filter cake was washed with cold distilled water. The filtrate was again filtered through a fresh cake of Hy-Flo Super Cel in order to remove the last traces of precipitated hemoglobin. The volume of the clear, light yellow filtrate was 6.84 liters and it contained 821 µg. of copper (27 per cent recovery).

Step 3. Lead Precipitation—240 ml. of saturated lead acetate solution were added slowly to the chilled filtrate, with stirring. The precipitate was centrifuged off and the supernatant solution was tested with additional lead acetate solution to determine the completeness of precipitation. The precipitate was then extracted with 400 ml. of 0.33 M phosphate buffer, pH 6.0, for 2 hours at 0°, and after centrifugation the residue was reextracted with 200 ml. of buffer. The extracts were pooled and dialyzed in the cold against distilled water until they were free of phosphate. This procedure extracted all of the copper-containing material from the lead complex.

Step 4. Tricalcium Phosphate Gel Adsorption—200 ml. of cold tricalcium phosphate gel suspension were added and after 10 minutes the gel was removed by centrifugation. After adsorption, the gel was washed three times in the centrifuge with cold copper-free distilled water and then extracted with two 200-ml. portions of 0.2 M phosphate buffer, pH 6.0. Following this the extracts were first dialyzed against distilled water until free of phosphate, and finally against two changes of copper-free distilled water, after which they were lyophilized.

Adsorption with phosphate gel removed about 60 per cent of the contaminating nitrogenous material. For example, in one experiment the preparation before adsorption contained 270 µg. of Cu and 146 mg. of N, whereas after elution the solution contained 258 µg. of Cu and 58 mg. of N. The yield at this stage was 436 mg. of lyophilized material (698 µg. of Cu). This represented a recovery of 23 per cent of the Cu present in the original hemolysate (Table I).

Step 5. Ethanol Fractionation—Ethanol fractionation was carried out in a refrigerated bath maintained at -5°, the ethanol having been prechilled to -20°. Pilot experiments were done to determine the optimal ionic strength and pH. Best fractionation was obtained at pH 5.2, ionic strength = 0.10. Some dissociation of copper occurred below pH 4.5.

415 µg. of lyophilized material from Step 4 were dissolved in 30 ml. of chilled distilled water and 30 ml. of acetate buffer, ionic strength 0.20, and pH 5.2, were added. The turbid, somewhat green solution was then chilled to 0° and cold ethanol was added dropwise with stirring. After addition of enough ethanol to prevent freezing, the temperature was lowered to -5° and maintained at this point throughout the fractionation and centrifugation procedures. Fractions were separated by centrifugation at 45 per cent and 65 per cent ethanol concentration, volume for volume, dissolved in cold, copper-free distilled water, dialyzed in the cold for 24 hours and then lyophilized. 198 mg. of material with a copper content of 0.25 per cent was obtained in this fashion from the material precipitating at 65 per cent ethanol concentration. The 45 per cent fraction yielded 104 mg. of material containing 0.10 per cent Cu, whereas the supernatant solution from the 65 per cent precipitate yielded 48 mg. containing 0.05 per cent Cu. This represented a total recovery of 21 per cent of the copper in the starting material (Table I).

Step 6. Ammonium Sulfate Fractionation—138 mg. of material precipitating from solution 65 per cent ethanol (Step 5) were dissolved in 10 ml. of copper-free distilled water and chilled to 0°. A saturated solution of ammonium sulfate was added slowly with stirring and fractions were separated at 50, 66, 75, and 89 per cent of saturation. The precipitates were dissolved in distilled water, dialyzed until sulfate-free and lyophilized as described previously. The fraction precipitating at 75 per cent saturation weighed 28.5 mg. and contained 0.32 per cent Cu (Table I).

Some Properties of Purified Erythrocuprein

Copper Concentration and Copper to Nitrogen Ratios—The most highly purified preparations, i.e. those precipitating from solution 75 per cent saturated with ammonium sulfate, contained 0.32 to 0.36 per cent copper. This corresponds to a minimal molecular weight of about 17,900. Copper (µg.) to nitrogen (mg.) ratios were 19.2 to 21.6.

Clear, faintly greenish blue solutions were obtained in water, physiological saline or buffered solutions. None of the copper present was dialyzable. Less than 2.5 per cent of the copper reacted directly with diethyldithiocarbamate, an amount which is within the experimental error of the analytical method (14). Paper electrophoretic strips stained for copper with alizarin blue S revealed a single copper-containing spot.

Temperature Stability—Stability with respect to temperature

<table>
<thead>
<tr>
<th>Step</th>
<th>Fraction</th>
<th>Yield*</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Hemolysate</td>
<td>3030</td>
<td>Cu/mg</td>
</tr>
<tr>
<td>2</td>
<td>Filtrate</td>
<td>820</td>
<td>Cu/mg</td>
</tr>
<tr>
<td>3</td>
<td>Extract of Pb precipitate</td>
<td>436</td>
<td>Cu/mg</td>
</tr>
<tr>
<td>4</td>
<td>Ethanol supernatant</td>
<td>51</td>
<td>Cu/mg</td>
</tr>
<tr>
<td>5</td>
<td>45% ethanol precipitate</td>
<td>109</td>
<td>Cu/mg</td>
</tr>
<tr>
<td>6</td>
<td>65% ethanol precipitate</td>
<td>208</td>
<td>Cu/mg</td>
</tr>
<tr>
<td>7</td>
<td>50-66% ammonium sulfate precipitate</td>
<td>3</td>
<td>Cu/mg</td>
</tr>
<tr>
<td>8</td>
<td>66-78% ammonium sulfate precipitate</td>
<td>25</td>
<td>Cu/mg</td>
</tr>
<tr>
<td>9</td>
<td>78-89% ammonium sulfate precipitate</td>
<td>43</td>
<td>Cu/mg</td>
</tr>
<tr>
<td>10</td>
<td>89-99% ammonium sulfate precipitate</td>
<td>16</td>
<td>Cu/mg</td>
</tr>
</tbody>
</table>

* The yields have been adjusted so that they represent those that would have been expected had all of the material from each step been used for the isolation of the compound.
Table II

Temperature stability of erythrocuprein

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Copper released</th>
<th>Immunological reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>°C</td>
<td>hours</td>
<td>wt.</td>
</tr>
<tr>
<td>0</td>
<td>3</td>
<td>&lt;0.06†</td>
</tr>
<tr>
<td>38</td>
<td>3</td>
<td>&lt;0.06†</td>
</tr>
<tr>
<td>56</td>
<td>3</td>
<td>&lt;0.11†</td>
</tr>
<tr>
<td>56</td>
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<td></td>
</tr>
<tr>
<td></td>
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</tr>
</tbody>
</table>

* Reactivity of the heated sample toward homologous antiserum is expressed as:

Antibody N precipitated after heating \( \times 100. \)

Antibody N precipitated before heating \( \times 100. \)

† Below limits of the accuracy of the method.

Table III

pH Stability of erythrocuprein

<table>
<thead>
<tr>
<th>pH</th>
<th>Copper released</th>
<th>Immunological reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>wt.</td>
<td>%</td>
</tr>
<tr>
<td>1.0</td>
<td>1.1</td>
<td>100</td>
</tr>
</tbody>
</table>
| 1.2    | 0.73 | 74 | \( \times 100. \)
| 2.5    | 0.50 | 46 | 57 |
| 4.5    | <0.14† | <13 | 100 |
| 7.4    | 0 | 0 | 83 |
| 8.6    | <0.24† | <24 | 83 |
| 11.5   | 0.91 | 83 | \( \times 100. \)

* The reactivity of the acid treated samples is an average of values obtained at several points in the region of antibody excess. Reactivity of the heated sample toward homologous antiserum is expressed as:

Antibody N precipitated after heating \( \times 100. \)

Antibody N precipitated before heating \( \times 100. \)

† Below limits of the accuracy of the method.

Fig. 1. Quantitative precipitin reactions of purified ("native") and acid treated erythrocuprein with rabbit anti-erythrocuprein serum. The points in the upper section were all in the region of antibody excess.

was measured as a function of the amount of copper released and change in immunologic reactivity.

0.5-ml aliquots of an aqueous solution of erythrocuprein (1.54 mg. per ml.) were incubated at 0°, 38° and 56° for different periods of time (Table II). All solutions remained clear throughout all of the experiments. At the end of the incubation period the per cent of the copper present which reacted directly with sodium diethyldithiocarbamate was determined. Immunological reactivity was determined by dilution of the incubated samples with 0.9 per cent sodium chloride, followed by addition of aliquots to calibrated homologous antiserum and measurement of the amount of precipitated nitrogen. The results are presented in Table II.

Aqueous and saline solutions of the cuproprotein have been stored at 0-5° for periods up to one year without an increase in directly reacting copper or a decrease in immunological reactivity. Likewise, the protein was found to be stable for at least 3 hours at 39°. At 56°, approximately 7 per cent of the copper was released in 1 hour and immunological reactivity decreased 10 per cent. Further deterioration of the protein did not take place after 1 hour at this temperature.

pH Stability—Aqueous samples of erythrocuprein were adjusted with either dilute HCl or NaOH to the desired pH and after 1 hour at room temperature the solution was readjusted to pH 7.0. After dilution to volume, aliquots were used for directly reacting copper determinations and estimation of immunological reactivity. The results are given in Table III and Fig. 1.

At pH 2.5, 46 per cent of the copper was released but there was a decrease of only approximately 13 per cent in the immunological reactivity of the protein and the initial combining ratio of the acid-treated protein was not altered (Fig. 1). At pH 1.0, 100 per cent of the copper was released with an associated loss of only 30 per cent of the immunological reactivity. However, the initial combining ratio of the protein was markedly altered.

Influence of Cyanide—Three different erythrocuprein preparations, each with a copper content of 0.32 per cent, were treated at 0° for 24 hours with \( 5 \times 10^{-3} \) m NaCN. In two of the experiments the cyanide solutions were adjusted to pH 8; in the third experiment the solution of cyanide was adjusted to pH 7.5 in Tris buffer (tris(hydroxymethyl)aminomethane). After 24 hours the solutions were dialyzed at 0° for 24 hours against 200 volumes of copper-free distilled water. The erythrocuprein solutions were then lyophilized and the copper content and copper : nitrogen ratios determined.

At pH 8.0 exposure to cyanide resulted in a loss of 44 to 53 per cent of the copper. At pH 7.5, 22 per cent of the copper was lost and the copper : nitrogen ratio (µg. per mg.) decreased from 21.8 to 15.8. The immunological reactivity of the protein after treatment with cyanide at pH 7.5 decreased only 5 per cent.

Absorption Spectrum—The absorption spectrum for erythrocyocuprein is presented in Fig. 2. For comparison, the absorption spectrum of ceruloplasmin, the copper protein of plasma (15), is also shown.

Crude preparations of erythrocuprein had absorption maxima at 278 and 405 mµ. As purification proceeded, the absorption band in the visible range shifted to 655 mµ and the maximum in the ultraviolet region shifted from 278 to 265 mµ. In agreement with the report of Holmberg and Laurell (15), ceruloplasmin had absorption maxima at about 280 and 695 mµ.

The molecular extinction coefficient (e) of erythrocuprein at
January 1959 H. Markowitz, G. E. Cartwright, and M. M. Wintrobe

400 500 600 700 800
WAVELENGTH, \( \mu \text{m} \)

**FIG. 2.** Absorption spectra of erythrocuprein and ceruloplasmin. - - -, ceruloplasmin; ---, erythrocuprein. Densities were adjusted to 1.0 mg. of protein per ml. (1.0 cm. light path) in the ultraviolet range. In the visible range densities were adjusted to 5.0 mg. of protein per ml. (5.0 cm. light path).

655 \( \mu \text{m} \) is 284 and at 265 \( \mu \text{m} \), 18,400; the molecular extinction coefficient of ceruloplasmin at 605 \( \mu \text{m} \) is 10,000 and at 280 \( \mu \text{m} \), 81,500.

**Immunological Properties**—When two different concentrations of erythrocuprein (0.8 and 3.0 mg. per ml.) were tested against homologous antisem by the Oudin agar diffusion technique, only one zone was observed. This gave a straight line when its rate of migration was plotted against the square root of the time in hours (16). The supernatant solutions from the precipitin reactions contained either excess antibody or antigen, but in no case were both present in the same sample.

By Ouchterlony agar diffusion tests (12) all pure erythrocuprein preparations studied produced only one line of precipitation and appeared identical to one another. Hemolysates of adult erythrocytes likewise gave only one line of precipitation and this line appeared identical from one hemolysate to another. Direct comparison of purified preparations of erythrocuprein with adult red cell hemolysates also showed the “reaction of identity” (17). This is illustrated in Fig. 3.

A hemolysate of beef erythrocytes, when tested by the Ouchterlony technique, failed to give any reaction with antihuman erythrocuprein rabbit serum. Ceruloplasmin isolated from human serum by the procedure of Holmberg and Laurell (15), also failed to give a reaction with antierythrocuprein rabbit serum either by precipitin or by agar diffusion techniques. Erythrocuprein did not react with antisem to blood groups A or B or with Coombs’ antisem.

**Erythrocuprein Content of Human Erythrocytes**—Hemolysates of human erythrocytes were prepared as follows. 4 ml. of a 30 to 50 per cent suspension of washed erythrocytes were hemolysed by the addition of 0.45 mg. of saponin in isotonic saline and, after 2 hours at 0\(^\circ\), isotonic sodium chloride was added to make a final volume of 25 ml. The hemolysates were then centrifuged at 15,000 \( \times g \) for 30 minutes at 0\(^\circ\) and stored in the frozen state until used.

Hemolysates of human erythrocytes were added to a known volume of antisem. After 48 hours in the cold, the precipitates were removed by centrifugation at 0\(^\circ\) and washed with

![Fig. 3. Agar diffusion analysis (Ouchterlony). Upper left, hemolysate of normal human erythrocytes prepared by freezing and thawing packed red blood cells. Upper right, purified erythrocuprein (30 \( \mu \text{g.} \) per ml.). Lower center, rabbit antisem for purified erythrocuprein. All wells were charged with 0.1 ml. of the appropriate solution and the Petri dish sealed with Parafilm and allowed to remain in the dark. Immediately before photographing the plate was treated with 5 ml. of 20 per cent trichloroacetic acid for 5 minutes and then washed with distilled water. This increased the density of the precipitate bands.](http://www.jbc.org/)

<table>
<thead>
<tr>
<th>Subject</th>
<th>Red blood cell copper</th>
<th>Erythrocuprein</th>
<th>Erythrocuprein copper</th>
<th>Erythrocuprein Cu ( \times 10^7 ) in Red blood cell Cu</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>114</td>
<td>33.6</td>
<td>108</td>
<td>95</td>
</tr>
<tr>
<td>2</td>
<td>117</td>
<td>35.8</td>
<td>114</td>
<td>98</td>
</tr>
<tr>
<td>3</td>
<td>84</td>
<td>29.1</td>
<td>93</td>
<td>110</td>
</tr>
<tr>
<td>4</td>
<td>99</td>
<td>29.2</td>
<td>94</td>
<td>95</td>
</tr>
</tbody>
</table>

* Obtained from normal male subjects.
† Erythrocuprein in mg. \( \times 1000 \times 0.0002 \).
‡ Erythrocuprein copper divided by total red blood cell copper.

3.0 and then 2.5 ml. of cold 0.9 per cent sodium chloride. The nitrogen content of the specific precipitates was determined and from the calibration curve the amount of erythrocuprein nitrogen present in the hemolysate was calculated. From a nitrogen content of 16.5 per cent (4), the amount of erythrocuprein in 100 ml. of packed red cells could be calculated. The values obtained for the erythrocytes from four normal, adult male subjects are presented in Table IV.
Enzymic Activity—Tests for oxidase activity with p-phenylenediamine, ascorbic acid and tyrosine as substrates were negative. The preparation contained no carbonic anhydrase activity.

DISCUSSION

The presence of a single band when several different concentrations of erythrocuprein were tested against homologous antiserum by the agar diffusion techniques suggests that the system studied was homogeneous. The observation that the erythrocuprein isolated was immunologically identical with the protein in erythrocyte hemolysates, as evidenced by Ouchterlony agar diffusion tests, suggests that the properties of the naturally occurring cuproprotein were not modified. Had the protein been altered during purification, the appearance of several bands or an indication of some degree of cross reaction might have been expected (17). The physical homogeneity of the preparation will be discussed in more detail in the paper which follows (4).

When erythrocuprein was subjected to a pH of 2.5 for 1 hour at room temperature, 46 per cent of the copper-protein bonds were cleaved. The resulting product, however, still reacted strongly with antiserum to the native protein. It can be seen in the upper portion of Fig. 1 that the initial combining ratio of the acid-treated protein had not been altered. More drastic treatment at pH 1.1 produced a marked alteration in the initial combining ratio. Both preparations, however, were less capable of combining with antibody. Whether this was due specifically to removal of copper, to denaturation and spatial alteration of erythrocuprein alone, or to a combination of the two cannot be determined from the data available. It is evident, however, that a marked cleavage of copper bonds can take place with retention of a major portion of the immunological reactivity. This conclusion is strengthened by the experiments with buffered cyanide solutions in which one-fourth of the copper bonds were ruptured with certainty whether or not there is a small amount of nonerythrocyte copper protein.

Mann and Keilin (3) isolated a blue copper-containing protein from beef erythrocytes. This protein had a molecular weight of about 34,000 and contained 2 atoms of copper per molecule. The copper protein isolated by us from human erythrocytes differs in isoelectric point (4, 15) and in absorption spectra. Furthermore, there is complete lack of immunological cross reactivity between the two cuproproteins. Thus, in the human subject at least, there is more than one "hemocuprein." For this reason, we propose the name, erythrocuprein, for the human erythrocyte copper protein. Recent studies in our laboratory suggest that in certain disease states there may be several immunologically identifiable, but cross-reacting, cuproproteins.

Erythrocuprein is similar in color, copper content and molecular weight to hepatocuprein isolated from ox liver by Mann and Keilin (3) and from horse liver by Mohamed and Greenberg (18). The red cell copper protein differs from cerebrocuprein, the copper protein isolated from human brain (19), in that the copper in the latter compound reacts directly with dithyldithiocarbamate.

Preliminary studies indicate that normal adult human erythrocytes contain about 30 to 36 mg. of erythrocuprein per 100 ml. of packed cells, as measured by the precipitin technique. The concentration of copper in the erythrocytes analyzed was 93 to 114 μg./100 ml. Since erythrocuprein contains at least 3.2 μg. of Cu per mg. of protein, it is apparent that erythrocuprein accounts for most, if not all, of the copper in normal erythrocytes. Further studies will be necessary to ascertain with certainty whether or not there is a small amount of nonerythrocyte copper protein in normal erythrocytes.

SUMMARY

1. A procedure is described for the isolation of a cuproprotein (erythrocuprein) from human erythrocytes.

2. This protein contains 0.32 to 0.36 per cent copper and is relatively stable at 0° to 37° and in the range of pH 4.5 to 8.6. The absorption spectrum of erythrocuprein has maxima at 265 μm and 655 μm.

3. Erythrocuprein is the major, if not the only, cuproprotein present in normal, mature human erythrocytes.

Acknowledgments—We are indebted to the American Red Cross, Salt Lake City Chapter, for making available the outdated blood used in this study; to Dr. J. H. Hink, Jr., of Cutter Laboratories, Inc., Berkeley, California for the preparation of the crude erythrocuprein used as the starting material in one of the preparations; and to Dr. Emil L. Smith of the University of Utah for advice and assistance during the course of this study.

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