Immunochemical Studies of Human Erythrocyte Proteins: Erythrocuprein and Catalase*

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SUMMARY

The immunochemical properties of chromatographically prepared human erythrocuprein differ from those of the chloroform-ethanol-treated protein. Losses of copper also appear to modify its immunochemical reactivity. The result of the immunochemical reactions of rabbit antibody to human erythrocuprein with human liver and brain extracts suggests a common antigen in the two tissues. Human erythrocyte catalase is not immunochemically identical with human liver catalase. Rabbit antisera to erythrocyte catalase fail to precipitate with hemolysates from some higher primates but give a cross-reaction with lysates from the cat and pig. Human erythrocuprein antisera give a cross-reaction only with hemolysate from the higher primates.

Immunochemical methods provide a powerful tool to delineate various properties of proteins such as purity, existence of allotypic forms, and changes in configuration related to their antigenicity, and to establish inter- and intraspecies relationships.

Various nonhemoglobin erythrocyte proteins have been the objects of extensive investigation. Of these, erythrocuprein (1, 2) and catalase (3–8) have been previously subjected to relatively extensive immunochemical study. The relationship of the erythrocyte and liver forms of the latter protein in a given species, however, is still unclarified (3–5). Several reports have indicated that catalases from these tissues of the cow (4) and the horse (5) are identical, but these conclusions are not based on extensive investigation.

Previous immunochemical studies of human erythrocuprein (1, 2) have been concerned primarily with the determination of the concentration of this protein in erythrocytes and its relationship to the copper-containing proteins of liver and brain. In all of the earlier studies, this protein, as well as catalase, has been isolated with the aid of chloroform-ethanol fractionation procedures. Recent investigations (9, 10) indicate that they are modified by such treatment. It was of interest to subject the more native forms of them to immunochemical characterization. In addition, the effects of removal of copper on the antigenic properties of erythrocuprein, the immunochemical relationships of the liver and brain forms of these proteins to the erythrocyte protein, and the immunological cross-reactions of rabbit antisera to erythrocyte catalase and erythrocuprein with the corresponding proteins of various animal species have been explored.

EXPERIMENTAL PROCEDURE

Catalase crystallized four times and highly purified noncrystalline erythrocuprein prepared from human erythrocytes (9) were initially employed as antigens. Rabbits were given weekly subcutaneous injections of 1 to 2 mg of these proteins in complete Freund's adjuvant (11) until suitable antibody titers were obtained. The y-globulin fractions of these antisera were isolated by chromatography on DEAE-cellulose (12) and diluted to solutions of the desired strength in a pH 7.4, 0.15 M NaCl-borrate buffer employed in all of our immunological studies.

The initial antisera also contained antibody directed against hemoglobin. It was removed by absorption with small amounts of purified hemoglobin. The precipitin reactions of the resultant antisera were highly specific as judged by immunoelectrophoretic procedures (13), by Ouchterlony tests (14), and by quantitative precipitin reactions.

Erythrocuprein and catalase of antigenic purity could be prepared by dissolving them in 0.01 M/2 sodium acetate buffers of pH 5.5 and 6.0, respectively, and by passing the solutions over columns of CM-cellulose equilibrated with these buffers. Small amounts of contaminating hemoglobin were retained on the columns by such treatment.

Quantitative precipitin reactions were carried out in the NaCl-borate buffer in a 3-ml volume essentially as described by Kabat and Mayer (15). Agar slide, single radial diffusion assays of erythrocuprein and of catalase were performed essentially by the method of Mancini et al. (16). Solutions of the catalase and erythrocuprein employed as antigen in the various precipitin studies were estimated by means of their previously determined extinction coefficients (9, 10).

Nitrogen analyses were performed on specific precipitates by the method of Miller and Houghton (17). Copper was assayed by the bishcybexalanone-oxalyldihydrazide method of Peterson and Bollier (18) and with the oxalyldihydrazide procedure of Markowitz et al. (19).

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Chromatographically prepared erythrocuprein was treated with chloroform-ethanol by a modification (10) of the method of Kimmel, Markowitz, and Brown (20) to give material analogous to that employed by previous investigators. Removal of copper from purified erythrocuprein was carried out by the methods listed in Table I. Approximately 50% of the protein in the samples subjected to the sodium diethylthiocarbamate and cyanide treatments at 21°C was insoluble in the pH 7.4, NaCl-borate buffer. The copper contents of the various treated proteins are also given in Table I.

Human liver and brain, shown by later histological examination to be normal, were obtained at autopsy within 30 min after death and immediately cooled in ice.1 Slices of the liver tissue were thoroughly washed with cold (1°C) 0.9% NaCl, minced, and washed again by repeated decantation in cold, NaCl-borate buffer. The mince was then homogenized and dialyzed extensively against NaCl-borate buffer. The copper contents of the various treated proteins are also given in Table I.

A portion of the brain of the same autopsy case was dissected free of superficial vascular tissue, and the outer gray matter was carefully separated from the inner white matter. These two tissues were then minced and further processed in a manner similar to that described for liver.

Hemolsates were prepared from erythrocytes that had been washed twice in 4 volumes of cold (1°C) NaCl-borate buffer; 3 volumes of this buffer containing 0.4% (w/v) saponin were added per volume of packed cells. The mixture was gently inverted 10 times and permitted to stand at 1°C for 60 min. The lysates were clarified by centrifugation at 15,000 × g for 60 min followed by filtration of the supernatant solutions through fine porosity, ash-free filter paper.

### Table I

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Conditions</td>
<td>Copper loss (%)</td>
</tr>
<tr>
<td>Penicillamine (0.05 m)</td>
<td>pH 7.4 at 1°C for 8 hrs</td>
<td>No detectable loss</td>
</tr>
<tr>
<td>Ascorbic acid (0.4 mg/ml)</td>
<td>pH 5.5 at 21°C for 1 hr</td>
<td>30</td>
</tr>
<tr>
<td>Ascorbic acid and diethylthiocarbamate (21)</td>
<td>pH 5.5 at 0°C for 8 hrs</td>
<td>85</td>
</tr>
<tr>
<td>Sodium cyanide (0.05 M)</td>
<td>pH 7.4 at 1°C for 8 hrs</td>
<td>60</td>
</tr>
</tbody>
</table>

1 We wish to thank the Pathology Department of Wilford Hall Hospital, Lackland Air Force Base, Texas, and the Vivarium Support Facility, United States Air Force School of Aerospace Medicine, Brooks Air Force Base, Texas, for providing us with fresh samples of human tissue and animal blood, respectively.

**RESULTS**

**Precipitin Studies of Erythrocuprein and Derivatives**—The results of quantitative precipitin reactions of chromatographically prepared erythrocuprein with rabbit antibody are presented in Fig. 1. A zone of equivalence was obtained at the points indicated by the arrows of this figure. A molecular ratio of antibody to antigen near 2 is found for the specific precipitate at the point of maximum precipitation. This is based on molecular weights of 33,500 and 160,000 for erythrocuprein and rabbit antibody, respectively.

Results of the precipitin reactions of the chloroform-ethanol-treated erythrocuprein are included in Fig. 1, and the zone of equivalence is again designated by arrows. An increased specific precipitation is noted in the antigen excess region. The molecular composition of the specific precipitate has an antibody to antigen ratio near 2 only in the first portion of the equivalence zone. These results indicate that significant antigenic modification of erythrocuprein has been effected by the ethanol-chloroform treatment. Agar gel, double diffusion experiments by the Ouchterlony technique, however, do not distinguish native erythrocuprein from this form of the protein (see Fig. 2C).

Antigenic modification of erythrocuprein is also induced by the removal of copper. The results of agar gel, double diffusion precipitin studies with erythrocuprein modified as shown in Table I are presented in Fig. 2. Immunologic identity is seen to obtain for the major zone of precipitation for all of the preparations. However, a minor precipitin band is observed with the proteins that have been allowed to react with sodium diethylthiocarbamate, with cyanide, with ascorbic acid, and with penicillamine (see Fig. 2, A and B). This secondary zone of precipitation appears to fuse with the major precipitate band. This conversion of erythrocuprein to an antigenically heterogeneous system as the result of copper removal is somewhat similar to the result obtained by Kasper and Deutsch (22) in their studies of ceruloplasmin.

These modified erythrocupreins were also employed as antigens in quantitative precipitin reactions. Representative data for the proteins from which copper was removed at 0°C and at 21°C are shown in Fig. 3. In the former case the sodium diethylthiocarbamate-treated protein exhibits a marked decrease in the amount of maximum precipitation. The cyanide-treated
Fig. 2. Agar gel, double diffusion studies of modified erythrocupreins with rabbit antibody. Plates A and B are for samples in which copper was removed at 0°C, A and D at 21°C. The incubation period for all plates was 96 hours. The antibody is in the center well in every case. The wells contained the following components.

<table>
<thead>
<tr>
<th>Plate</th>
<th>Well 1</th>
<th>Well 2</th>
<th>Well 3</th>
<th>Well 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Sodium diethyldithiocarbamate</td>
<td>Ascorbate</td>
<td>NaCN</td>
<td>Penicillamine</td>
</tr>
<tr>
<td>B</td>
<td>Untreated</td>
<td>Ascorbate</td>
<td>Penicillamine</td>
<td>Sodium diethyldithiocarbamate</td>
</tr>
<tr>
<td>C</td>
<td>Untreated</td>
<td>CHCl₃-C₂H₅OH-treated</td>
<td>Ascorbate</td>
<td>Penicillamine</td>
</tr>
<tr>
<td>D</td>
<td>Untreated</td>
<td>Sodium diethyldithiocarbamate</td>
<td>Ascorbate</td>
<td>NaCN</td>
</tr>
</tbody>
</table>

-protein and the ascorbic acid reduction product both show similar decreases in the zone of maximum precipitation. The extent of the decreases in specific precipitation noted are in general related to the amount of copper removed.

The results of precipitin reactions with protein in which the removal of copper had been carried out at 21°C show interesting differences. The penicillamine-treated protein exhibits a considerably broader zone of maximum precipitation even though the amount of precipitate is only 5% less than that observed for native erythrocuprein. Protein treated with ascorbic acid, with ascorbic acid and sodium diethyldithiocarbamate, and with cyanide precipitates less antibody and gives a broader zone of precipitation at the maximum.

Comparative Immunological Studies of Human Erythrocuprein and Copper Proteins from Brain and Liver—A variety of low molecular weight copper proteins have been isolated from liver and brain tissue (23–26). These proteins, prepared by methods which include the use of organic solvents, bear a general physicochemical resemblance to erythrocuprein. It was of interest to compare the immunological reactivity of tissue extracts containing these proteins with antisera to erythrocuprein.

The results of Ouchterlony reactions with a hemolysate and
ERYTHROCUPREIN

**FIG. 3.** The results of quantitative precipitin reactions of chromatographically prepared erythrocuprein and of erythrocuprein derivatives prepared at (A) 0° and (B) 21° with rabbit antibody. DDC, diethyldithiocarbamate.

**FIG. 4.** Ouchterlony studies of human tissue extracts with rabbit antibody to human erythrocuprein (center well); Well 1, human hemolysate; Well 2, human liver extract; Well 3, human white brain matter extract; and Well 4, human gray brain matter extract.

with liver and with white and gray brain tissue extracts as antigens are shown in Fig. 4. Reactions of immunological identity are noted with each of these antigens. To rule out the possibility that these results might be due to erythrocyte contamination of the washed tissues, an experiment was conducted with an erythrocuprein antiserum also containing antibody to hemoglobin. If blood lysate had been present in the tissue extracts in an amount sufficient to provide for the precipitin bands observed (approximately 0.002% to 0.01% erythrocuprein), then the amount of hemoglobin in these extracts should have yielded a discrete precipitin band. Failure to observe this indicates that the precipitin reaction noted was due to a tissue component reacting with the antibody to erythrocuprein.

Quantitative precipitin tests were conducted on the liver and brain extracts. The relative immunological reactivity of each extract was first determined by the single radial diffusion procedure (16). The apparent concentration of erythrocuprein found in liver, gray matter, and white matter extracts was 0.125, 0.04, and 0.015 mg per ml, respectively. Solutions of pure erythrocuprein were diluted to these levels with 3% bovine serum albumin to also provide total protein levels near those of

**FIG. 5.** The results of quantitative precipitin reactions of human liver extracts and of pure erythrocuprein with rabbit antibody to erythrocuprein.

**FIG. 6.** The results of quantitative precipitin reactions of human cerebral gray matter extract and of pure erythrocuprein with rabbit antibody to erythrocuprein.

**FIG. 7.** The results of quantitative precipitin reactions of human cerebral white matter extract and of pure erythrocuprein with rabbit antibody to erythrocuprein.
FIG. 8. Ouchterlony studies of rabbit antibody to human erythrocyte catalase and to erythrocuprein with hemolysates from various animal species. The photos were taken after 120 hours of incubation at room temperature. The antibody is in the center well.

Plate antibody to

<table>
<thead>
<tr>
<th>Source of hemolysates in wells</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>A, erythrocuprein</td>
<td>Human</td>
<td><em>Macacus speciosa</em></td>
<td><em>M. m. mulatta</em></td>
<td>Baboon</td>
</tr>
<tr>
<td>B, catalase</td>
<td>Human</td>
<td>Baboon</td>
<td>Goat</td>
<td><em>Macacus speciosa</em></td>
</tr>
<tr>
<td>C, catalase</td>
<td>Human</td>
<td>Pig</td>
<td><em>M. m. mulatta</em></td>
<td>Cat</td>
</tr>
</tbody>
</table>

Quantitative precipitin reactions were then carried out with each extract and the diluted pure erythrocuprein solutions. The results are illustrated in Figs. 5 to 7. The near superimposition of the precipitin curves in each case suggests immunological identity between erythrocuprein and the reactive protein of the liver and of the brain extracts. The copper of the specific precipitates was found to account for 35%, 6%, and 17% of the total copper in the liver, white matter, and gray matter extracts, respectively. These relative levels of erythrocuprein copper are considerably higher than those reported by Shields et al. (2) in analogous studies.

Immunological Comparison of Erythrocupreins of Various Animal Species—Hemolysates from the baboon, *Macacus speciosa*, *M. m. mulatta*, beagle dog, collie dog, goat, pig, white rat, mouse, chicken, guinea pig, and rabbit were allowed to react with antisera to chromatographically prepared erythrocuprein by the Ouchterlony technique. The results of these experiments are illustrated in Fig. 8 A.

The lysate of each of the primates tested yielded a reaction of identity with human erythrocuprein, whereas those of all of the other animals tested exhibited no specific precipitation with the antibody preparation employed. These results are similar to those of Shields et al. (2).

Precipitin Studies of Erythrocyte Catalase—The results of the quantitative precipitin reactions of human erythrocyte catalase are shown in Fig. 9. The zone of equivalence is indicated by the arrows.

Fig. 9. The results of quantitative precipitin reactions of purified human erythrocyte catalase with rabbit antibody. The zone of equivalence is indicated by the arrows.

FIG. 10. Ouchterlony studies of human erythrocyte catalase and of extracts of human tissues with rabbit antibody to human erythrocyte catalase (center well); Well 1, human hemolysate; Well 2, human cerebral gray matter extract; Well 3, human cerebral white matter extract; Well 4, human liver extract.
with rabbit antibody are presented in Fig. 9. A definitive equivalence point is noted in the zone indicated by the arrows of the figure. A molecular ratio of antibody to antigen near 2 is found for the specific precipitate at the point of maximum precipitation.

A single precipitin band between purified erythrocyte catalase and its rabbit antibody is seen in Ouchterlony studies.

**Comparative Immunological Studies of Human Erythrocyte and Liver Catalases**—Immunochemical cross-reactivity between the catalase of liver and erythrocytes of the same species has been reported, but there has been disagreement concerning the immunological identity of this enzyme from the two sources (3–5). To aid in resolving this question, immunochemical studies were carried out with rabbit antiserum to chromatographically prepared crystalline human erythrocyte catalase. The results of qualitative agar gel, double diffusion experiments are presented in Fig. 10. A reaction of identity is not observed between the liver and erythrocyte enzyme since slight spurring is evident. The brain extracts failed to form a specific precipitate with this antibody.

The human liver extract was assayed by the single radial diffusion precipitin procedure. An apparent erythrocyte catalase concentration of 0.05 mg per ml of liver extract was found. Pure erythrocyte catalase was diluted with 3% bovine serum albumin to give the apparent concentration of erythrocyte catalase and of total protein in the liver extract. The results of quantitative precipitin reactions of these solutions are shown in Fig. 11. In agreement with the Ouchterlony results, they do not support an immunological identity between erythrocyte and liver catalases.

**Immunological Comparison of Erythrocyte Catalases from Various Animal Species**—Rabbit antibody to human erythrocyte catalase was allowed to react with the red cell lysates from the cat, pig, guat, dogs, rat, mouse, chicken, guinea pig, rabbit, baboon, Macacus speciosa, and Macacus mulatta mulatta. Only the cat and pig lysates were found to precipitate with this antibody. The results of the qualitative tests are shown in Fig. 8 and reveal that the precipitin reactions noted were not ones of identity. Inhibition studies were not performed to determine whether a nonprecipitating complex had formed between the antibody and the various hemolysates that failed to give a visible specific precipitin reaction.

**DISCUSSION**

Our results on the qualitative and quantitative immunological studies with antisera to erythrocuprein and erythrocyte catalase indicate a high degree of purity of these crystalline human proteins. The various precipitin reactions in agar gels gave evidence of only a single antigen-antibody reaction even when hemolysates and tissue extracts were used as antigen sources. The quantitative precipitin reactions gave clear equivalence zones, and the molecular compositions of the specific precipitates were indicative of reactions of a single antigen with its antibody. Modification of the antigenic properties of erythrocuprein was clearly evident following even partial removal of its copper or treatment of this protein with solutions of chloroform-ethanol under conditions used by previous workers in their preparation of this antigen.

The presence of a single zone of precipitation when several concentrations of erythrocuprein were allowed to react with its antibody by the Ouchterlony technique suggested to Markowitz, Cartwright, and Wintrobe (1) that the antigen system under study was homogeneous. The immunological heterogeneity of this protein preparation, however, was subsequently clearly demonstrated by Shields et al. (2). The result of the agar gel diffusion studies of the former workers (1) was interpreted to indicate the immunological identity of their erythrocuprein with a protein in erythrocyte lysates. This identity was presented as evidence that the erythrocuprein had not been modified during its isolation. The results of the quantitative precipitin reactions illustrated by Fig. 1 strongly suggest that chloroform-ethanol treatment modifies the immunological reactivity of the chromatographically isolated erythrocuprein even though simple agar gel diffusion techniques do not reveal this (Fig. 2C).

The close immunochemical similarity or identity of erythrocuprein to copper-containing proteins in extracts of liver and of brain is interesting. Proof of the identity of erythrocuprein, cerebrocuprein (25, 26), and hepatocuprein (23, 24) must await critical comparisons of the purified materials. The need for purification of the latter two copper proteins by milder procedures than those previously employed (23–26) is suggested by the results of the present and previous studies (9, 10). The use of antibody to erythrocuprein for the evaluation of fractionation procedures for the separation of the corresponding liver and brain proteins may be an important aid in their purification.

Cerebrocuprein, hepatocuprein, and erythrocuprein have been thought to be related to ceruloplasmin. Various immunological studies performed in our laboratory reveal a complete lack of reaction between human ceruloplasmin and rabbit antiserum to human erythrocuprein. Similar results have been observed by Markowitz et al. (1). The lack of oxidase activity, the divergent physicochemical properties, and the amino acid and carbohydrate contents of erythrocuprein (10) are further indications of the dissimilarity between it and ceruloplasmin.

The immunochemical reactions observed for catalase in the present studies show distinct differences in the erythrocyte and liver forms of this enzyme. Variations in the amino acid content of equine erythrocyte and equine liver catalases have been noted (3). Peptide maps of the tryptic hydrolysates of equine liver and blood catalases show the presence of some peptides common to each enzyme and also of peptides unique to a specific catalase. This confirms the noted amino acid differences and indicates

**FIG. 11.** The results of quantitative precipitin reactions of crystalline human erythrocyte catalase and of human liver extract with rabbit antibody to human erythrocyte catalase.
variations in the structures of these two catalases (3). Saha, Campbell, and Schroeder (3) reported that equine and bovine liver catalases differ in reactivity with antibody to bovine liver catalase. The horse liver enzyme, however, exhibits a closer resemblance to the bovine liver enzyme than to horse erythrocyte catalase. This observation agrees with the amino acid and peptide differences noted for the catalases from the different tissues. Bonnichsen (5) has reported that equine blood catalase was serologically identical with equine liver catalase. This finding was supported by amino acid analyses performed by Theorell and Åkeson (27) and by Bonnichsen (5). The results of the present study tend to support the findings of Saha et al. (3) rather than those of Bonnichsen (5). The degree of similarity of human liver and erythrocyte catalases will depend upon the results of further physicochemical and immunological studies of the purified enzymes.

The immunological cross-reactivity of antibody to human erythrocyte catalase with the corresponding pig and cat catalases is rather surprising in view of the lack of reactivity with the catalases of the phylogenetically closer related primate species studied. Antibody to human erythrocytecuprein gave reactions only with the higher primate erythrocytecupreins as might be anticipated. Detailed chemical comparisons of the various catalases will be required to see if those of the pig and cat possess greater similarities to human erythrocytecuprein than do those of the higher primates in keeping with the noted immunological results.

REFERENCES
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