Immunoochemical Studies of Crystalline Human Ceruloplasmin and Derivatives*

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Previous immunoochemical investigations of ceruloplasmin have been primarily concerned with the assay of this substance in human sera and its localization by the technique of immunoelectrophoresis. The proteins used in the preparation of antisera appear to have been impure or poorly defined. The ready availability of crystalline human ceruloplasmin (1, 2) prompted us to study its immunoochemical properties as an adjunct to its physicochemical characterization (3). The lability of this protein to rather mild treatment is reflected in a pronounced modification of its immunoochemical properties. Most of these changes appear to be related to a partial loss of copper from the molecule.

EXPERIMENTAL PROCEDURE

In initial experiments, four times crystallized ceruloplasmin prepared from human plasma (1) was used to immunize rabbits. The antisera obtained reacted only weakly with purified ceruloplasmin but strongly with crude preparations and with serum. The major portion of the antibody produced was found to be directed against γ1-macroglobulins.

The rabbit γ-globulin fraction of this antisera prepared by ethanol fractionation (4) was used to purify further the crystalline antigen. This was accomplished by reacting crystalline ceruloplasmin with the antisera and allowing the mixture to incubate overnight at 2°C. The small amount of white precipitate that formed was removed by centrifugation in the cold. The supernatant was dialyzed against 0.05 M sodium acetate at pH 7.2 and then chromatographed on a DEAE-cellulose* column that had been equilibrated against this solution. The column was successively washed with (a) 0.05 M sodium acetate, (b) 0.05 M sodium acetate containing 0.05 M NaCl, and (c) 0.5 M NaCl. Step a removed the uncombined rabbit γ-globulins, while Step b eluted a small amount of what apparently was a soluble blue antigen antibody complex. The purified ceruloplasmin that was eluted with the 0.5 M NaCl was dialyzed against a pH 7.4 buffer of 0.7% NaCl solution and borate and was then mixed with Freund's adjuvant (5). Rabbit antisera to ceruloplasmin prepared in this manner showed no detectable reactions with other serum proteins when tested by immunoelectrophoretic methods and by the Ouchterlony technique.

The γ-globulin portion of the antisera was separated and reconstituted into a 0.7% NaCl solution-borate buffer, pH 7.4. Quantitative precipitin reactions were carried out in the same buffer in a 3-ml reaction volume as described by Cohn, Wetter, and Deutsch (6).

Microimmunoelectrohoresis experiments were performed by the technique of Scheidigger (7). Double diffusion studies of the immunological cross reactivities of various animal sera with rabbit anticeruloplasmin were carried out by the Ouchterlony technique (8). The details of the precipitin bands could usually be better visualized if stained. This was accomplished by treating the gels with Amido schwarz following extensive washing with 0.7% NaCl solution to remove soluble proteins. After staining, the excess dye was removed by washing with a methanol water acetic acid (50:50:10) mixture.

Vertical starch gel electrophoresis was performed by the method of Smithies (9). The position of ceruloplasmin in the gel was localized by utilizing the well known oxidase activity of this protein to convert benzidine to a colored derivative. Immunoochemical localization of ceruloplasmin in these gels was carried out by the technique of double diffusion as described by Poulak (10).

The apoprotein of ceruloplasmin was prepared by treatments with ascorbic acid and DDC essentially by the method of Morell and Scheinberg (11) to provide DDC apoceruloplasmin and also by dialysis against NaCN at pH 7 to 8 to give cyanoapoceruloplasmin. The blue and colorless proteins in the reaction mixture of ascorbic acid with ceruloplasmin were separated as previously described (3).

Ceruloplasmin was also modified by reaction with 0.05 M mercaptoethanol, cysteine, and penicillamine at pH 7.4 for 1 to 2 hours at room temperature. The mercaptan was removed by dialysis in the cold before immunoochemical analysis.

RESULTS

Quantitative Precipitin Reactions—The results of the quantitative precipitin reactions of rabbit antibody with native ceruloplasmin and for various derivatives produced by chemical modification are presented in Fig. 1. Detailed data for the reactions of native ceruloplasmin are given in Table I. An apparent equivalence point for the native protein was found in the range of 40 to 60 μg of antigen nitrogen per ml of antibody preparation. The equivalence point for the DDC apoprotein was detected in the range of 10 to 29 μg of antigen nitrogen. The apoproteins showed a broad zone of specific precipitation in the antigen excess region. This indicates a heterogeneous immunoochemical system even though an apparent equivalence point had been obtained.

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‡ The abbreviations used are: DEAE-cellulose, diethylaminoethyl cellulose; DDC, N,N'-diethylthiocarbamate.
protein (160,000) behaves similarly to the apoproteins in giving a diminished amount of specific precipitate and in showing a broad zone of precipitation in the antigen excess region.

The supernatants to the specific precipitates were divided into two portions for tests for excess antigen and antibody. A test for antigen excess was made by adding 0.1 ml of the antibody preparation to a third of it. From 2 to 3 μg of antigen nitrogen were added to the remainder to test for antibody excess. These reaction mixtures were stored for 24 hours in the cold, centrifuged, and inspected carefully for traces of specific precipitates.

The results for the quantitative precipitin reactions of the apoprotein suggested that a relatively large amount of antibody had been produced against a minor antigenic component. Supernatants to specific precipitates in the apparent equivalence point region of the apoprotein reacted positively when tested for antibody excess by the addition of 100 to 200 μg of apoceruloplasmin nitrogen whereas the addition of 2 to 3 μg gave negative results. A test for antibody excess in response to these large antigen additions was also found to obtain into the region of apparent antigen excess. However, when the supernatants to the specific precipitates of native ceruloplasmin were tested for antibody excess with increased amounts of antigen nitrogen, an equivalence zone was still obtained.

Removal of antibody to the major antigenic component was performed by absorption of the standardized antiserum with an amount of apoceruloplasmin (40 μg of antigen N per ml of antibody) that gave a supernatant which failed to form a specific precipitate on further addition of a small amount of antigen or on addition of antibody. This preparation will be referred to as the "absorbed antibody."

### Immunochemical Reactions in Gels

The results of the reaction of crystalline ceruloplasmin and two normal human sera with ceruloplasmin antibody by the Ouchterlony technique are shown in Fig. 2A. A single precipitin band is seen and a reaction of identity obtains between the serum- and ceruloplasmin-containing reservoirs. However, when different concentrations of ceruloplasmin are reacted with this antibody preparation, the higher level of antigen shows as many as four precipitin bands (Fig. 2B, C, and D). It is difficult to interpret this experiment even though various investigators have suggested that a single antigen may give several precipitin bands in immunodiffusion experiments (13-16). Other evidence to be presented will show that several antibodies to ceruloplasmin are formed. However, the majority of the experimental evidence suggests that the antigenic determinants responsible for these antibodies are localized on a single molecule.

Ouchterlony type experiments with DDC- and cyanoceruloplasmin are shown in Fig. 3A and indicate two distinct precipitin bands. Fig. 3B shows that when the absorbed antibody is reacted with the apoprotein only one of the precipitin bands persists. In this experiment, the second precipitin band of the apoprotein with unabsorbed antibody was a rather broad zone of precipitation. This may be due to a relatively high concentration of the responsible antigen. It can be seen that the zone of precipitation is near the antibody reservoir.

Starch gel electrophoretograms of crystalline ceruloplasmin in pH 8.5 borate and in Tris-EDTA-borate buffers are shown in Fig. 4. Two components are partially resolved in the latter system, whereas in borate buffer only a single protein zone is seen. Immunodiffusion analyses of these starch gel strips show a single precipitin band for the borate buffer experiment, whereas

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**Table I**

Data for quantitative precipitin reactions of native ceruloplasmin with rabbit antibody

<table>
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<th>Antibody N</th>
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Fig. 1 indicates that there are differences in the immunological reactivities of the DDC- and cyanoapoproteins. These apoproteins have been also found to show some differences in sedimentation and electrophoretic behavior (3).
two precipitin lines are seen after electrophoresis in the Tris-EDTAA-borate buffer. The component of highest electrophoretic mobility has been shown to be a colorless protein produced as the result of chelation of some of the copper by the Tris and EDTA components of the buffer (3).

Chromatography of crystalline ceruloplasmin has been previously shown to modify its properties. A protein with an increased negative charge and with a lowered spectrophotometric absorption at 610 nm (3) is obtained. The results of a starch gel immunoelectrophoretic experiment on protein so treated is shown in Fig. 4C and reveals a splitting of the precipitin band.

Starch gel immunoelectrophoretic examination of cyanoapo-ceruloplasmin shows a single precipitin band (Fig. 5.1). The immunochemical reactions of DDC apoceruloplasmin, however, are quite complex. The experiment shown in Fig. 5B indicates three antigenic components. The precipitin lines formed by Components a and b fuse. This indicates immunological identity and suggests that b, because of its slower migration in the gel, may be a polymeric form of a. Absence of cross-reactivity of component c with either a or b is additional evidence that the apoprotein is composed of a population of molecules which are heterogeneous with respect to their antigenic determinants. Fig. 5C shows that further changes in electrophoretic properties occur on aging of the apoprotein. Both slower and faster migrating components are produced. Apoprotein solutions that have been aged contain considerable amounts of faster sedimenting components (3). This observation suggests that the components of low electrophoretic mobility noted in Fig. 5C are due to high molecular weight aggregates of DDC apoceruloplasmin.

The protein stained portion of the same gels used in the immunoelectrophoresis experiments show the marked similarity of the two apoproteins (Figs. 5.1 and B). In addition to the main protein zone, one fast and at least one slow electrophoretic component is observed in each case. In agreement with the results of moving boundary electrophoresis experiments (3), the apoprotein prepared by the DDC method was found to possess larger amounts of the slower migrating component. None of the components resolved by electrophoresis in starch gel gave a positive oxidase test.

It was of interest to determine if differences in ceruloplasmins isolated from individual sera could be found by immunochemical study of the apoprotein derivative. Apoprotein was prepared by the DDC method from crude ceruloplasmin preparations isolated from 10 ml of individual sera. Examination of 20 preparations did not reveal any significant differences. The results of Ouchterlony tests on 6 of the preparations are shown in Fig. 6A and show that each gives two precipitin bands. The double diffusion reactions of 4 of the sera from which apoceruloplasmin was prepared show a single sharp precipitin band (see Fig. 6B).

The reactions of ascorbic acid-treated ceruloplasmin with
FIG. 3. Ouchterlony experiments of native and DDC- and cyanoapoceruloplasmin with (A) unabsorbed antiserum. The antiserum is in the center reservoir; 1 and 2 contain DDC- and cyanoapoprotein, respectively; 2 and 4 contain native ceruloplasmin. (B) The center reservoir contained DDC apoceruloplasmin, 1 and 3 contained unabsorbed antiserum, while 2 and 4 contained absorbed antiserum.

FIG. 4. Starch gel electrophoretograms of crystalline ceruloplasmin in pH 8.5 borate buffer (A) and in Tris-EDTA-borate buffer (B). The upper strip of these photos is the protein-stained portion of the gel, the lower strip shows the immunoelectrophoretic result. (C) The immunoelectrophoretic (pH 8.5 borate buffer) result for chromatographed crystalline ceruloplasmin.

FIG. 5. Starch gel electrophoresis experiments in pH 8.5 sodium borate buffer of (A) cyanoapoceruloplasmin, (B) DDC apoceruloplasmin, and (C) aged DDC apoceruloplasmin. The upper strip of A and B is the protein-stained gel, while the lower strip shows the immunoelectrophoretic result. Only the immunoelectrophoretic result is shown in C.

FIG. 6. Ouchterlony experiments on (A) DDC apoceruloplasmin preparations from six individual normal human sera and on (B) four normal human sera.
ceruloplasmin antiserum by the Ouchterlony technique are shown in Fig. 7A. The unfractionated protein gives two precipitin lines, whereas a single band is given by the blue and by the colorless component.

The colorless ascorbate reduction product does not react completely with the antibody, and its precipitin band is weak and not clearly defined. The blue component gives a single precipitin band. Quantitative precipitin studies (Fig. 1) and agar gel double diffusion methods indicate that it is identical with native ceruloplasmin.

Starch gel immunoelectrophoresis experiments on the ascorbate reduction products are shown in Fig. 7B and C. The chromatographically isolated colorless component gives three precipitin bands which are designated a, b, and c (Fig. 7C). From the protein-stained starch gel strip and other physicochemical studies, it would appear that the a component is aggregated protein, and that b is the four copper reduction product.

The c component of Fig. 7C has a higher mobility than apo-protein but gives a reaction of identity with band b. It could represent b molecules which have not undergone marked unfolding and, therefore, the effect of their increased negative charge on electrophoretic mobility has not been cancelled by an increase in axial ratio. The starch gel immunoelectrophoresis pattern of the unfractionated ascorbic acid-treated ceruloplasmin is quite similar to the pattern for the DDC apoceruloplasmin shown in Fig. 5B.

Microimmunoelectrophoresis studies of ascorbic acid-treated ceruloplasmin and its fractions are shown in Fig. 8. The slowest moving protein is the residual unmodified ceruloplasmin. It is apparent that the anodic mobility of the colorless component is greater than the native material and that it cross-reacts strongly with the native protein. Fig. 8 also shows that the colorless

Fig. 7. Double diffusion studies of ascorbic acid treated ceruloplasmin. (A) Ouchterlony experiment. The antiserum is in the center reservoir, 1 contains the isolated blue component, 2 contains the isolated colorless component, and 3 and 4 contain unfractionated ascorbic acid-treated ceruloplasmin.

The result of starch gel electrophoresis in pH 8.5 sodium borate buffer for unfractionated ascorbic acid-treated ceruloplasmin is shown in B; the result for the chromatographically isolated colorless component is shown in C. The upper strip of B and C is the protein-stained gel while the lower portion shows the immunoelectrophoretic result.

Fig. 8. Microimmunoelectrophoretic analysis of ascorbate-treated ceruloplasmin and of the chromatographically resolved components. The left portion of the agar gel contained the chromatographically isolated colorless component in each case. The anodic migration is upwards. The right reservoir contained: 22, unfractionated ascorbate-treated ceruloplasmin, antiserum unabsorbed; 24, unfractionated ascorbate-treated ceruloplasmin, antiserum absorbed; 26, isolated blue component, antiserum unabsorbed; 27, isolated blue component, antiserum absorbed.

Fig. 9. Microimmunoelectrophoresis studies of ceruloplasmin treated with various sulphydryl compounds. The abbreviations used are: DEDTC, DDC; ME, 2-mercaptoethanol; and PA, DL-penicillamine. The treated ceruloplasmins were in the left reservoir; the right reservoir contained native ceruloplasmin in each case. The median and lateral channels contained antiserum.
Component reacts with that portion of the antibody preparation that was removed in the absorption procedure described earlier. The data also show that the colorless reduction product and residual native protein are effectively separated by chromatography of an ascorbate-ceruloplasmin reaction mixture.

Fig. 9 shows that modifications of ceruloplasmin leading to changes in immunological reactivity are effected by treatment with mercaptans. Variable amounts of protein of increased anodic mobility are produced.

Cross-reactivity Studies—Various mammalian sera show strong immunological cross-reactions with rabbit antibody to human ceruloplasmin. Ouchterlony type studies indicative of this are shown in Fig. 10. A faint spur is seen in the precipitin lines of human and monkey serum (Fig. 10A, C, and D). The monkey serum also shows a strong spur to the goat, sheep, hog, dog, and white-tailed deer serum (Fig. 10A to D). The chicken, rat, and guinea pig sera react only weakly (Fig. 10A and D). The results of Fig. 10 indicate that rabbit antisera can demonstrate a minimum of four antigenic determinants in human ceruloplasmin.

The sera of four species of deer and of one goat cross-reacted strongly with rabbit anti-ceruloplasmin (Fig. 10E) but failed to give visible precipitation with the absorbed antibody (Fig. 10F). Fig. 10E shows complete fusion of the precipitin bands of the deer serum and indicates that the ceruloplasmins of these species possess some common antigenic determinants. This does not mean that the ceruloplasmins present in these sera are antigenically identical but indicates that the portions of these ceruloplasmins which cross-react with the rabbit antibody to human ceruloplasmin are quite similar. The ceruloplasmins of these species, are, however, antigenically deficient to the human pro-
tein. Immunoelectrophoretic examination of various deer sera indicated that the antigenic components in the serum of the white-tailed, the red, and the fallow deer have a lower anodic mobility than human ceruloplasmin, whereas serum from the Sitka deer and the goat were indistinguishable from the human by this method.

Fig. 11 shows starch gel electrophoresis experiments on various animal sera in which the gels were stained with benzidine to demonstrate oxidase activity. It is apparent that the ceruloplasmins of various animals show some differences in mobility and in concentration. The latter supposes that the intensity of the oxidase stain is proportional to the ceruloplasmin concentration of the different sera. No apparent correlations appear to exist between the electrophoretic mobilities of the various ceruloplasmins, their apparent concentration as indicated by the oxidase stain, and the immunochemical results shown in Fig. 10.

**DISCUSSION**

Antibodies to crystalline human ceruloplasmin may be directed largely against impurities. Such antisera, however, can be used in conjunction with chromatographic techniques to prepare ceruloplasmin sufficiently pure for use as an antigen. Various workers have prepared rabbit antibody to human ceruloplasmin that was sufficiently specific to be used for quantitative immunchemical assay of this protein. The nature and purity of the antigen employed, however, was often not clearly indicated. The crystalline antigen of Scheinberg and Gitlin (17) incited antibody production to many other serum proteins. Markowitz et al. (18) immunized rabbits with a ceruloplasmin preparation that was approximately 75% pure. Both groups of investigators absorbed their antisera with serum of patients with Wilson's disease.

The ceruloplasmin of Schultze and Schwick (19) appears to have been approximately 94% pure and rabbit antisera to it was found by Berggård (20) to show two precipitin lines when examined by the Ouchterlony technique. The antigens and antisera used by other investigators in numerous quantitative and semiquantitative studies were generally ill defined (21-26). In view of the difficulties experienced in the present investigations in preparing a specific antiserum to ceruloplasmin, it is surprising that the preparations of this protein used by the above workers stimulated antibody production so readily.

It is difficult to interpret the agar gel immunodiffusion results that show that apoceruloplasmin can form two distinct precipitin bands while the native protein forms only one. One of the precipitogens of the apoprotein is immunologically similar to a component formed from ceruloplasmin on treatment with ascorbate and with mercaptans. It is known that these components have lost some or all of their copper and have undergone a change in structure leading to increased asymmetry and to an increased net negative charge. These components have antigenic properties in common with the ceruloplasmins of various species which cross-react with rabbit antibody to human ceruloplasmin.

It was noted that the sera of various animals show strong reactions with the rabbit antibody to human ceruloplasmin. Rhesus monkey ceruloplasmin apparently possesses most of the antigenic determinants of the human protein. Most of the ungulate sera react quite strongly with the rabbit antibody but to a lesser degree than the monkey. The Ouchterlony cross-reaction experiments indicate that the rabbit has formed at least four different antibodies to human ceruloplasmin. The immunochromatographic reactions of the modified human ceruloplasmins with this antibody also gave evidence for the presence of three or four antibodies.

**SUMMARY**

Immunochromatographic studies of crystalline human ceruloplasmin indicate that it is antigenically complex. This protein and certain of its derivatives readily undergo changes that are associated with modification of their immunological behavior. The immunochromatographic reactions of ceruloplasmin derivatives with rabbit antibody to ceruloplasmin indicate that three or four antibodies are present. Cross-reactivity studies with various animal sera suggest the presence of a similar number of antibodies.