ISOLATION OF PURIFIED COPPER PROTEIN FROM HORSE LIVER

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Copper proteins have been isolated from both plant and animal sources. Those of plant origin functionally appear to be aerobic oxidases, notably, polyphenol oxidases of potatoes (1), of mushrooms (2), and of tea (3), laccase (4), and ascorbic acid oxidase (5).

The copper proteins of animals, in addition to the above, may also have an oxygen transport function or may merely serve for the storage of copper.

Mann and Keilin (6) isolated a crystalline copper protein from red blood corpuscles which they named hemocuprein. It was a blue protein with a molecular weight of 35,000, containing 0.34 per cent copper. These workers also isolated a colorless copper protein from liver which they were unable to crystallize. This protein, hepatocuprein, also contained 0.34 per cent copper. It appears to account for only part of the total copper present in liver.

Copper proteins have been isolated from cow's milk (7) and from blood plasma (8). Except for the copper protein of blood plasma, which has oxidative activity against *p*-phenylenediamine and benzidine, no particular enzymatic functions have been found for the above mentioned animal proteins.

In this communication there is reported the isolation of a bluish green colored protein from horse liver in the form of discrete particles that appear to be crystalline, although their exact crystal habit cannot easily be discerned. The copper content of this protein was variable; therefore, it may have a storage function.

Isolation

The procedure for isolating the liver copper protein is described below. Except when stated otherwise, the isolation was carried out in a cold room maintained at 3–5°.

1. Two kilos of horse liver is minced and mixed with 6 liters of distilled H_2O, the pH adjusted to 7.2, and the mixture continuously stirred with an electric stirrer for 4

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to 6 hours at room temperature. The pH is then readjusted to 7.2 and the mixture left overnight in the cold room. Next morning the mixture is filtered through cheese cloth and the residue discarded.

2. To the filtrate is added ½ volume of cold acetone and the mixture left to stand overnight. The clear layer of supernatant fluid is then syphoned off and the remainder filtered with suction in the cold room. The precipitate is discarded.

3. To this filtrate is added 0.7 volume of cold acetone and the mixture again left to stand overnight. The clear layer of supernatant fluid is syphoned off and the rest centrifuged in a refrigerated centrifuge. The supernatant fluid is discarded.

4. The precipitate is dissolved in sufficient distilled H_2O to give a final volume of approximately ⅓ that obtained in step 1. The pH is adjusted to 7.0 and the mixture left to stand for 3 to 5 hours. The mixture is then centrifuged and the precipitate discarded.

5. The filtrate is heated in a large Erlenmeyer flask over an open flame with continuous stirring to 70°, cooled immediately under running cold water, and filtered with suction. To the filtrate from this is added solid lead acetate (0.5 to 0.75 mgPb^{++}/ml.) to the point of incipient precipitation. Care is taken to maintain the pH at 7.2 throughout (by adding NaOH). The mixture is left to stand in the cold room overnight and then centrifuged in the cold.

6. The clear supernatant fluid is mixed with ½ a volume of cold acetone, left overnight in the cold, and centrifuged.

7. The clear supernatant fluid is mixed with 0.7 volume of cold acetone, again left overnight in the cold and centrifuged.

8. The precipitate is dissolved in H_2O and the pH adjusted to 7.0 and centrifuged. This protein solution contains a number of proteins, two of which were subsequently separated and crystallized[1] in addition to the copper protein.

9. A saturated ammonium sulfate solution (70 gm./100 ml.) is added with stirring to the solution from step 8 to the point of 62 per cent saturation. The pH is adjusted to 6.8, the mixture left to stand at room temperature for 24 hours and then it is centrifuged. From this point on the solutions and precipitates obtained are colored blue green.

10. The supernatant fluid is dialyzed against H_2O to remove all the ammonium sulfate. The resulting solution is then mixed with 0.7 volume of cold acetone, centrifuged, and the precipitate discarded. The supernatant fluid is mixed with excess cold acetone and the resulting precipitate isolated by centrifugation.

11. The precipitate is taken up in distilled H_2O and mixed with saturated ammonium sulfate to give 50 per cent saturation. The mixture is allowed to stand 12 to 18 hours and any precipitate formed is centrifuged out and discarded. To the supernatant fluid more saturated ammonium is added to the point of 60 per cent saturation. The pH is adjusted to 6.6 and the mixture is allowed to stand at room temperature for several days to effect crystallization.

Recrystallization.—The crystalline blue-green precipitate is packed down by centrifugation, redissolved in distilled H_2O and to the resulting clear extract

[1] Ferritin and a yellowish colored protein. The function and cause of the color of the latter are unknown.

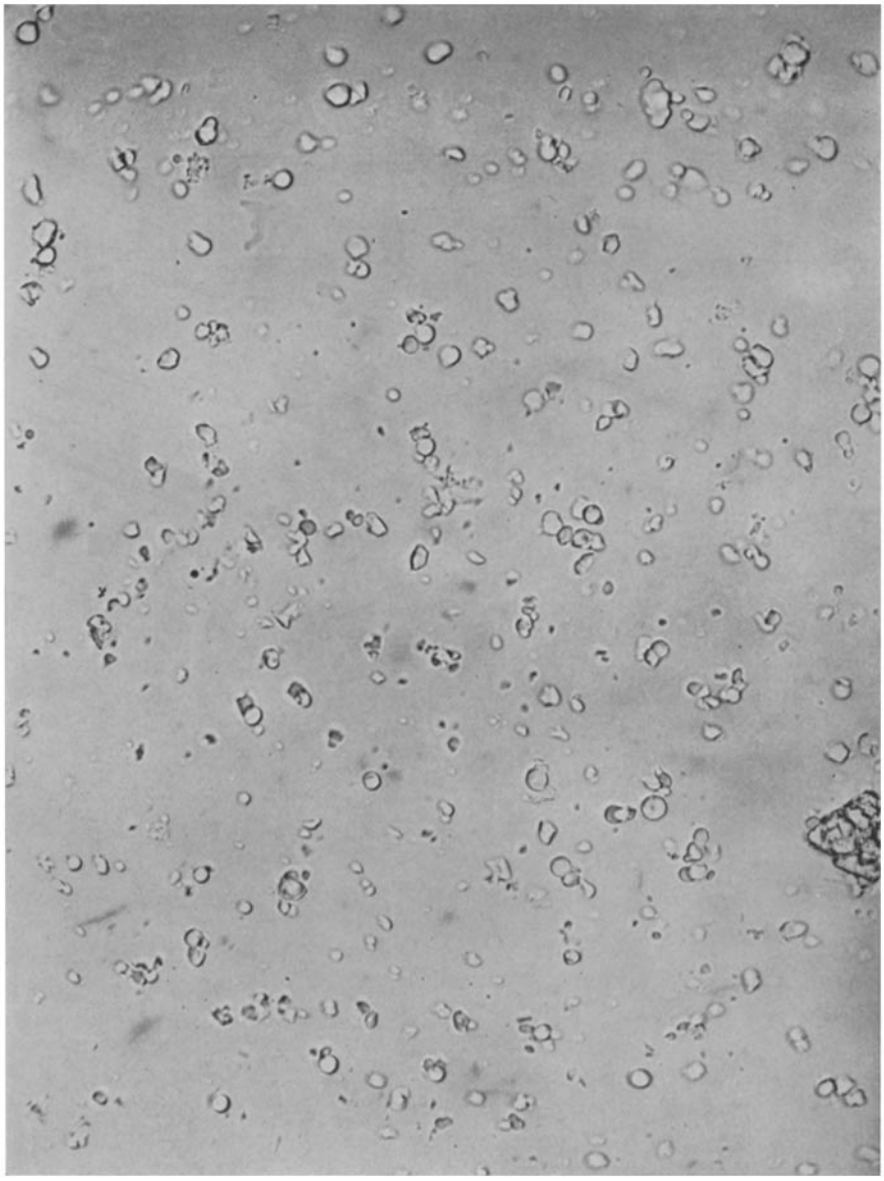

FIG. 1. Photomicrograph of blue-green copper protein. × 550.

saturated ammonium sulfate is added to give 60 per cent saturation. The pH is adjusted to 6.6 and the mixture allowed to stand at room temperature to crystallize. A photomicrograph of the protein is reproduced in Fig. 1. This shows the irregular shapes of the material. If they are not true crystals, the

evidence to be presented below is evidence of the homogeneity of the isolated copper protein.

Properties

Composition.—The copper content varied somewhat in different preparations, values of 0.340, 0.306, and 0.410 per cent being obtained. Nitrogen determination by the micro-Kjeldahl method gave values of 15 to 15.3 per cent.

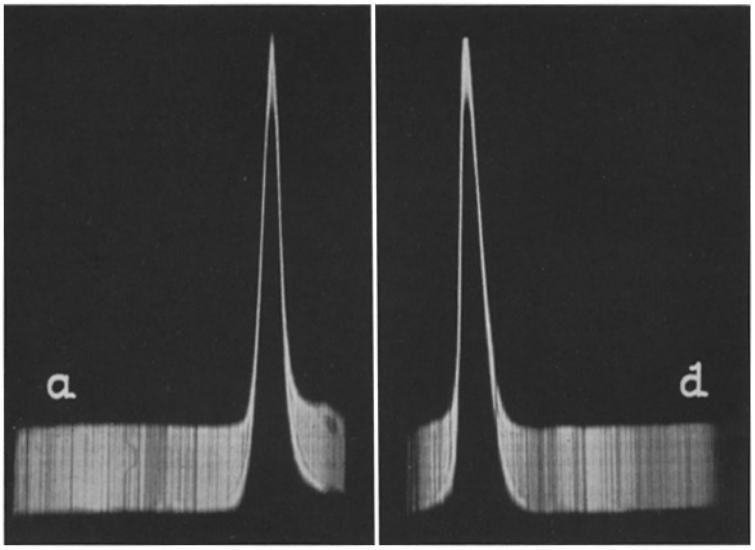

FIG. 2. Electrophoresis pattern of copper protein at pH 6.8 in veronal buffer, $\mu = 0.1$. Ascending and descending boundaries.

Homogeneity.—A number of electrophoresis runs were carried out on the protein at various pH values. A single boundary was obtained with no separation of compounds, which indicates its homogeneity. A diagram of the run made at pH 6.8 is shown in Fig. 2. In this run at ionic strength of 0.1, the calculated mobility for the descending limb was 2.4×10^{-5} and 2.3×10^{-5} cm./sec./volt cm. for the ascending limb. A determination in the Spinco analytical centrifuge also showed only a single sedimenting boundary. The sedimentation constant at 20°, calculated from this run, was 2.9 Svedberg units. This indicates the molecular weight is of the order of 30,000 to 40,000.

A colorless crystalline protein obtained from the colored copper protein as described below yielded a sedimentation constant of 3.4 Svedberg units in the ultracentrifuge and a mobility of 5.2 to 5.7×10^{-5} cm./sec./volt/cm. at pH 7.0 and $\mu = 0.1$.

Enzyme Activity.—Negative results were obtained when the protein was tested for oxidase activity with catechol, tyrosine, and ascorbic acid as sub-

strates, for rhodanese activity and for threonine dehydrase activity. The variable copper content and the occurrence of other copper proteins without phenol oxidase activity offers some reason to assume that this protein may have a copper storage rather than an enzyme function. However, an enzyme function is not ruled out by the few negative tests mentioned above.

Reversibility of Copper Binding.—The protein retains its copper throughout the prolonged and comparatively drastic procedures employed in its isolation. Experiments were undertaken to determine whether the copper could be dissociated from and reunited to the protein. In one experiment, the protein (1.2 per cent solution) was dialyzed against 0.1 M NaCN (at pH 7.5 for 24 hours) and then against running distilled H_2O for 48 hours. Part of the protein was then precipitated with acetone, dried, and analyzed for copper. Another portion of this solution was dialyzed against copper acetate for 24 hours followed by dialysis against running H_2O for 48 hours. This too was treated as above and analyzed for copper.

The results obtained were: original copper protein, 0.306 per cent Cu; CN^--dialyzed protein, 0.234 per cent; redialyzed against copper acetate, 0.415 per cent. The results indicate the reversible dissociation of the copper from the protein. Dialysis of the copper protein against 0.05 M sodium hydrosulfite solution resulted in the complete loss of the blue color in a few hours. The blue-green color of the resulting colorless protein was restored on redialysis against copper acetate. Holmberg and Laurell (8) found that the copper of coeruloplasmin could be completely removed by 48 hour dialysis against KCN (at pH 7.0). However, neither the blue color, nor its enzyme activity could be restored by the addition of copper.

The bound copper of serum has been found to be easily removed by lowering the pH (9, 10). Dialysis of coeruloplasmin at pH below 5.0 resulted in a proportional decrease in the blue color and copper content.

On the basis of the above observations, a solution of the copper protein in water (0.2 per cent protein) was made up to 60 per cent saturation with saturated ammonium sulfate solution. Dilute sulphuric acid was added to bring the pH down to 2.8, and additional ammonium sulfate added to raise the saturation to 65 per cent. Upon allowing the mixture to stand at room temperature, a white crystalline protein[2] slowly forms. A photomicrograph of this is shown in Fig. 3. No question can be raised regarding the crystallinity of this protein. The blue-green color, and presumably the copper, can be introduced again by dissolving the crystalline precipitate in water, dialyzing against running distilled H_2O to remove the ammonium sulfate, and then dialyzing against copper acetate for 24 hours. The protein returns to its original blue-green color, which persists on dialysis against water. The copper protein can now be crystallized once again by the ammonium sulfate procedure described above.

[2] Unfortunately, this preparation was broken in shipment when sent for copper analysis.

FIG. 3. Photomicrograph of colorless protein obtained from colored copper protein. × 550.

SUMMARY

1. A procedure is described for isolating a copper protein from horse liver in pure form, in a state that appears to be crystalline.

2. This copper protein is colored blue-green, and its copper content varied

between 0.3 and 0.4 per cent. No indication of any catalytic properties was obtained in the limited number of tests made. Because of its variable copper content, the protein may function for the storage of copper in the body.

3. The copper could be removed partly from the protein by dialysis against cyanide, and a colorless crystalline protein was obtained by lowering the pH to 5.0, followed by dialysis. The original blue-green color of the protein could be restored by dialysis against copper acetate solution.

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Note Added in Proof.—The oxidation of *p*-phenylenediamine also was not catalyzed by the copper protein.