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Investigations in Serum Copper

II. Isolation of the Copper Containing Protein, and a Description of some of its Properties

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When pigs serum is precipitated with ammonium sulphate, the fraction obtained between 40 and 50 % saturation, has a distinctly bluish-green color and contains most of the copper present in this serum.

We have attempted to purify the blue protein contained in this fraction by alcohol precipitation in the cold. In this way it is possible to get rid of a considerable amount of colorless or yellow proteins, and to obtain a preparation, giving solutions of a deep blue color. The copper content of the best preparations thus obtained is about 0.03 %. When these preparations have been investigated with electrophoresis they show a big main peak with the mobility of an α_1-globulin and two smaller peaks, one on each side. It has, however, not been possible with this technique to obtain an electrophoretically homogeneous preparation. Some of the best preparations have been subjected to ultracentrifugation by Dr. K. Pedersen in Upsala. He found that the main component had a sedimentation constant, $S_{20} = 17.8$, two other smaller components had sedimentation constants $S_{20} = 7.2$ and 4.2 respectively. Preparative centrifugation at high speed revealed that the main component was colorless. It seemed obvious that the difficulties in preparing the blue protein originated from its tendency to be adsorbed on other proteins when these were precipitated.

As it seemed very probable to us that the hemocuprein of Mann and Keilin [1] had some connection with this blue protein we tried to treat our preparations with alcohol and chloroform at pH 6.5 expecting to obtain in this manner a preparation of pure hemocuprein. With such treatment one obtains a bulky precipitate mainly consisting of denaturated protein and a practically colorless

supernatant. By washing the precipitate several times with 0.9 % NaCl a blue substance is brought in solution. A solution of the blue substance is dialyzed and precipitated with ammonium sulphate.

When the blue protein thus obtained was further investigated it was at once clear that it differed in at least one property from hemocuprein. Its reduction by hydrosulphite ($Na_2S_2O_4$) was completely reversible, whereas Keilin and Mann state that hemocuprein, which has been decolorized with hydrosulphite, has definitely lost its blue color. It also retained the property which Holmberg [2] found for the impure preparation of catalyzing the oxidation of paraphenylenediamine.

We have now worked out a method which permits a preparation of this blue protein in a fairly pure state. The principles of this method are as follows:

PREPARATION

Principles of preparation

I. Salt precipitation of the blue protein together with the bulk of the globulins.

II. Precipitations of some euglobulins at pH 6.2 and 5.5, followed by precipitation of the blue component together with the main part of the globulins at pH 5.5 with alcohol (15 %) at low ionic strength. The hemoglobin and the main part of the albumins remain in the supernatant.

III. Denaturation of all proteins except the blue component in two steps with chloroform-alcohol at pH 6.5 and 5.5 respectively at low ionic strength and room temperature.

Preparation from pigs serum

Pigs serum is precipitated with ammonium sulphate. The fraction obtained between 36 % and 55 % saturation is dissolved in water and dialyzed for at least 24 hours against running tap water.

After dialyzing, the pH is adjusted with dilute acetic acid to 6.2. A yellow precipitate is centrifuged off and discarded. Now the pH is adjusted to 5.5. The solution is cooled down to 0°. The precipitate which is formed is centrifuged off and discarded. Alcohol is now added to a concentration of 15 % at a temperature between 0 and — 5°. The strongly blue precipitate is centrifuged down at low temperature and dissolved in water containing some NaCl. The solution is dialyzed over night.

After the pH has been adjusted to 6.5 this solution is precipitated at 0° with an equal volume of chloroform-alcohol (9 parts 90 % alcohol, and 1 part

chloroform). The temperature of the solution is allowed to rise, and the precipitate is centrifuged down at room temperature. The colorless supernatant is discarded *.

The faintly blue precipitate is extracted on the centrifuge with about equal volume of 0.9 % NaCl, until no more blue color is obtained in the supernatant. The pooled extracts are dialyzed over night. Next day the dialyzed solution is adjusted to pH 5.5, and again treated with chloroform-alcohol in exactly the same way as the day before. If the supernatant, after the treatment with chloroform-alcohol, has some blue color this solution is cooled down to — 15° and filtered at this temperature. The precipitate is dissolved in water and added to the pooled NaCl-extracts and the whole quantity dialyzed first against running tap water and after that against distilled water. A small precipitate, which appears during the dialysis, is centrifuged down and discarded. If the solution, which now contains practically pure blue protein, is too dilute for further work, it can easily be concentrated by precipitating with ammonium sulphate to 65 % saturation, redissolving the precipitate in a small volume of distilled water, and again dialyzing.

Preparation from human serum

The corresponding blue protein can easily be prepared from human pregnancy serum by applying the same method as previously described. Here, however, the blue protein begins to precipitate when the pH is adjusted to 5.5 before adding alcohol. Therefore, this precipitate is not discarded. Alcohol is added to a concentration of 10 % instead of 15 % used with pigs serum. Otherwise the procedure is exactly the same.

CHEMICAL PROPERTIES OF THE COPPER CONTAINING PROTEIN

Solubility

Impure preparations precipitate at pH 5.4 after prolonged dialysis. Pure preparations do not precipitate even *after prolonged* dialysis against distilled water, but *the protein comes down on acidification to the isoelectric point* (4.4). Even very small impurities greatly influence all solubility tests, as the *blue protein is adsorbed* on *most precipitating proteins in neutral and slightly acid solutions*, which might depend upon the very acid i. p. of the blue protein.

* The supernatant is colorless, if the ionic strength has been low enough (specific resistance $> 1000\ \Omega$).

On salt precipitation it comes down, when the ammonium sulphate concentration is raised *over 48* % saturation. From the preparation method it is evident that the blue protein has an alcohol solubility like most albumins but *is less sensitive to room temperature in alcohol-water mixtures than most albumins.*

Electrophoretic mobility

On electrophoresis in Tiselius-Svensson apparatus the blue protein moves as a practically homogeneous substance over a wide pH range (8—3).

Table 1. Electrophoretic measurements on blue protein from pig.

pH	Mobility u. 10^5 cm^2/volt sec		
	Anode	Cathode	
7.40	ascending 2.7	descending 2.7	
6.32	» 2.1	» 2.0	Phosphate
5.75	» 1.7	» 1.6	buffers
5.10	» 1.4	» 1.3	
4.87	» 0.76	» 0.73	
4.48	» 0.27	» 0.19	Acetate
4.33	descending 0.16	ascending 0.16	buffers
3.70	» 1.4	» 1.6	

We have determined its mobility in phosphate and acetate buffers. The total ionic strength was maintained at 0.2 with 0.18 derived from NaCl and 0.02 from the buffer. The protein concentration was about 0.2 %. It is evident from Fig. 1. that *the isoelectric point* when determined *in the presence of acetate ions is about 4.4.* At this pH the protein is partially decolorized.

Blue protein from pig and man have the same isoelectric points.

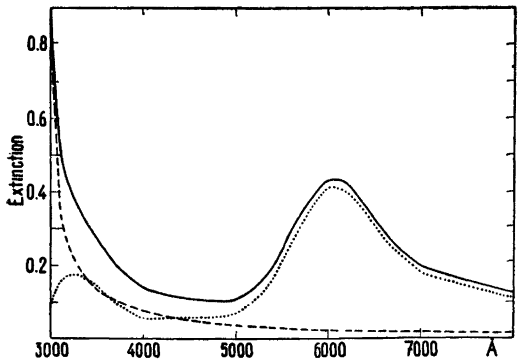

Fig. 1. Absorption spectrum.
——— *Blue protein (pH 6.5).*
- - - - *Blue protein decolorized with ascorbic acid.*
. . . . . *Spectrum of chromatic groups by subtracting 2 from 1.*

Molecular weight

Dr. Pedersen in Upsala has made an investigation into some of the physical constants of the blue protein. His results will be published separately in a special paper in this series. His value for the *molecular weight is about 151 000.* Identical for pig and man.

Copper content

The copper content has been determined in 6 different preparations, 3 from pig and 3 from man. The results are to be found in Table 2. Assuming the molecular weight of Pedersen to be correct, and taking into consideration the small amount of impurities present, we find *a copper content of eight atoms per molecule.* The hemocuprein of Keilin and Mann contains two atoms of copper in a molecule weighing about 35000. Therefore, it seems possible that our protein might contain four units of hemocuprein.

Absorption spectrum

The absorption spectrum at neutral pH is shown in curve 1, in Fig. 1. It shows *a broad absorption band* with a maximum of about *6050* Å. In the ultraviolet there is a maximum at about 2800 Å corresponding to the ordinary protein absorption maximum in this region.

When the blue protein is reduced with ascorbic acid in an inert gas atmosphere (curve 2) the *maximum at 6050 Å disappears and a progressive absorption between 3000 and 4000 Å is decreased. Upon reoxidation with oxygen the spectrum reverts* to the one pictured in curve 1.

Table 2. Light absorption, copper and protein contents in some preparations from pig and man.*

	Protein %	*E* (6050 Å 1 cm cuvette)	Cu mg/100 ml	Cu content of protein in %	Atoms Cu per protein molecule
Pig I.	0.37	0.238	1.26	0.34	8.1
II.	0.59	0.395	1.92	0.33	7.9
III.	1.28	0.778	4.14	0.32	7.6
Man I.	0.96	0.570	2.99	0.31	7.4
II.	1.2	0.750	3.85	0.32	7.6
III.	0.62	0.428	1.96	0.32	7.6

The spectrum of the chromatic group (curve 3) is obtained by subtracting curve 2 from curve 1. This spectrum contains two maxima, one at 6050 Å, and another at 3250 Å.

The *spectrum given for the blue protein is the same in a pH interval between 5.5 and 8.*

On *acidification to pH 3 the same spectrum is obtained as after reduction.* At this pH the copper becomes completely dialyzable. *At pH 12.5 the maximum at 6050 Å also disappears.* What happens to the maximum at 3250 Å is not so easy to determine as the protein maximum is shifted to a longer wavelength.

There exists a direct proportionality between copper content and extinction at 6050 Å.

$E \cdot 5 =$ Cu (mg/100 ml) when the extinction is measured in an 1 cm cuvette.

DISCUSSION

A blue protein has been isolated from serum of pig and man. It has a great similarity to the hemocuprein of Mann and Keilin but is not identical with this substance. The molecular weight is about 151000 and it contains eight atoms

* Biuret method.

of copper. It can be reduced to a colorless compound with reducing agencies such as sodium hydrosulphite, ascorbic acid, hydroxylamine and thioglycolic acid. It should be emphasized, however, that we have tried reduction with purified hydrogen gas in the presence of catalysts, such as palladium black and Raney nickel, without success. The reduction is completely reversible in the presence of oxygen, but attempts to oxidize with potassium ferricyanide were not successful. The absorption spectrum is similar to that of the hemocyanines. The question, therefore, seems pertinent whether we have here to do with a real oxidation and reduction, or with a reversible oxigenation. All that can be said at the present moment is that attempts to decolorize solutions of this protein by evacuation or saturation with an inert gas have been unsuccessful. The problem is being further investigated.

As has been pointed in the first paper of this series[3], it is probable that most if not all serum copper is present in the form of this blue compound. We propose that the new protein is named *cæruloplasmin.*

SUMMARY

A new copper containing protein has been isolated from mammalian plasma. A description is given of the method of the preparation and of some of the properties of this protein.

Our thanks are due to Professor Alf Sjövall, chief of the Obstetrical Clinic in Lund, and to Dr. Olle Dahl at Scan's cooperative butchery for providing us with material.

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