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Investigations in Serum Copper

I. Nature of Serum Copper and its Relation to the Iron-binding Protein in Human Serum

CARL G. HOLMBERG and C.-B. LAURELL

Laboratory of Clinical Chemistry, University of Lund, Lund, Sweden

Since the finding of Warburg and Krebs [1] that blood serum has a fairly constant copper content of about 100 γ per 100 ml, the nature and functions of this serum copper have been subjects of many investigations. Our knowledge about serum copper is however still very incomplete.

Already Krebs [2] has shown that in man there is a physiological rise in serum copper during pregnancy; and mainly through the work of Heilmeyer and collaborators [3] it has been shown that also infections are associated with a rise. The latter pointed out that serum copper often increases under conditions which lead to a decrease in serum iron.

Abderhalden and Möller [4] have shown that serum copper is non-dialyzable. Warburg and Krebs [1], Locke *et al.*[5] and Boyden and Potter [6] have shown that the binding of serum copper is broken by lowering pH.

When serum proteins are precipitated with trichloroacetic acid the copper goes quantitatively with the filtrate.

Eisler, Rosdahl and Theorell [7] have studied the behavior of serum copper during electrophoresis and conclude from their experiments that it is bound to albumin.

Mann and Keilin [8] prepared from blood corpuscles a crystalline copper protein with a copper content of about 0.34 % which they named haemocuprein. A similar compound was also prepared from horse serum. Haemocuprein is a blue protein with a molecular weight of about 35,000. Cohn *et al.*[9] report that crystalline haemocuprein is precipitated from horse serum by increasing the ammonium sulphate concentration from 0.62 to 0.68 saturation.

Cohn *et al.*[10] found that the iron combining β_1-globulin in serum also combines with copper and that this globulin is responsible for the transport of

both iron and copper, perhaps of zinc too. They refer to the old finding of Heilmeyer regarding the interrelation of iron and copper in serum, and their idea seems to be that this interrelation should be explained as a competition between the two metals for the transport protein.

Holmberg [11] has shown that the assumption of Eisler, Rosdahl and Theorell that copper is bound to albumin cannot be quite correct. Part of the serum copper was found by him in Green's P_1-globulin.

The function of serum copper is still obscure. Probably at least a part of it is to be regarded as transport copper. Mann and Keilin were not able to show any catalytic activity of haemocuprein. When this compound is reduced by hydrosulphite, the blue color disappears and does not reappear when the solution is oxidized by shaking. Holmberg, however, found that the blue color of the copper-containing P_1-globulin, which also disappears on reduction, reappears when the solution is left in contact with the air. He also found that this copper protein catalyzes the oxidation of paraphenylendiamine.

EXPERIMENTS WITH SERUM AIMING TO EXPLAIN THE RELATION BETWEEN SERUM IRON AND COPPER

When, about three years ago, in this laboratory it was found that serum had a latent iron binding capacity of a fixed value, our interest in the finding of Heilmeyer [3] that a decrease in serum iron often runs parallel with an increase in serum copper was at once awakened. The hypothesis which immediately suggested itself to us was that, when the iron binding capacity remained unchanged, the variations in serum iron could be explained by assuming that copper and iron competed for the same protein.

This hypothesis, which has now been taken up by Cohn [10] was however proved to be erroreous by the following experiments.

1. a) *When an excess of copper was added to serum this did not effect the iron binding capacity of the serum.*

b) *If the iron binding capacity was saturated, addition of copper did not result in a breaking up of the iron complex.*

These experiments show that if copper enters into combination with the same protein as iron, the affinity of this protein for iron must be greater than the affinity for copper.

2. Experiments were made with addition of sodium diethyldithiocarbamate to serum (normal serum and pregnancy serum with high copper content).

a) By photometric analysis it could be shown that the serum copper in no instance reacted with the sodium diethyldithiocarbamate at physiological and slightly alkaline pH.

b) When small amounts of copper as copper sulphate was added to serum, it could be shown that this copper reacted quantitatively with sodium diethyldithiocarbamate. These results were independent of the primary concentration of copper in the serum employed. Even serum from newborns with a very low copper content was unable to bind any copper added, so that it did not react with sodium diethyldithiocarbamate.

c) Addition of excess iron could not expel copper from firm binding in serum so that it could react with sodium diethyldithiocarbamate.

These experiments prove that *the capacity of serum to bind copper contrary to its iron binding capacity is in all instances used up to a hundred per cent;* they also prove that *iron has no stronger affinity than copper for the copper binding protein* (it is to be emphasized that iron was allowed to react with serum for a considerable length of time).

From the experiments here reported we concluded that *the hypothesis about iron and copper competing for the same protein in serum cannot be correct.*

EXPERIMENTS CONCERNING THE NATURE OF SERUM COPPER

Up to present but very little has been published about the behavior of the copper protein in serum on salt precipitation. As pointed out already Cohn et al. state that haemocuprein in horse serum is precipitated between 0.62—0.68 ammonium sulphate saturation.

Wishing to see if the additional copper which appears in serum during pregnancy and infections behaves in the same way as the normal serum copper, we have performed some salting out experiments on different types of human sera.

For these experiments we used ammonium sulphate which had been freed from copper by treatment with sodium diethyldithiocarbamate. Each serum, diluted with an equal amount of glass-distilled water, was precipitated with an equal amount of neutralized saturated ammonium sulphate. The precipitates were washed twice with half-saturated ammonium sulphate. Copper determinations, after wet combustion, were performed on serum and globulin precipitate. In most cases double determinations were made.

The results of these experiments are recorded in Table 1, from which *it is evident that most of the copper in serum is precipitated by 50 % saturation with neutralized ammonium sulphate.* It is also evident *that the increase in serum copper during pregnancy and infections is due to an increase in a fraction which can be precipitated with 50 % ammonium sulphate saturation* (globulin copper). As a matter of fact, the amount of copper which cannot be precipitated under these conditions in normal sera is less than 10 %. It is quite possible that it

Table 1. Amount of copper precipitable by 50 % saturation with ammonium sulphate at different serum copper concentrations.

Serum number	Total Cu γ/100 ml	Globulin Cu γ/100 ml	Albumin Cu γ/100 ml
1.	157	138	19
2.	119	109	10
3.	109	112	—3
4.	94	84	10
5.	116	109	7
6.	282	256	26
7.	328	288	40
8.	288	214	74
9.	258	214	44
10.	300	242	58
11.	201	168	33
12.	226	173	53
13.	222	178	44

1— 5. Normal sera.
6—10. Pregnancy sera.
11—13. Infection sera.

might be attached to a globulin which cannot be completely separated from the albumin in this way. The proportional increase in albumin copper with increase in total copper supports this view. In this connection *it should be pointed out that the iron binding component in plasma is not precipitated by 50 % ammonium sulphate.*

Luetscher [12] and Pedersen [13] have described a blue globulin which was found in a fairly high concentration in pig serum, probably identical with the one described by Holmberg (1944) in human serum. Later Cohn *et al.* have found the same globulin, which they describe as an α-globulin. In this laboratory we have purified this blue globuline by repeated precipitations at different pH's and different alcohol concentrations, and the purified preparations we found to have a copper content of about 0.03 %. More recently we found that if this globulin is treated with alcohol and chloroform in the same concentrations as were used by Mann and Keilin in the preparation of haemocuprein, the main part of the protein is rendered insoluble and settles as a bulky colorless precipitate. The supernatant fluid is a clear blue solution with a very small protein content. By fractional precipitation with ammonium sulphate it was

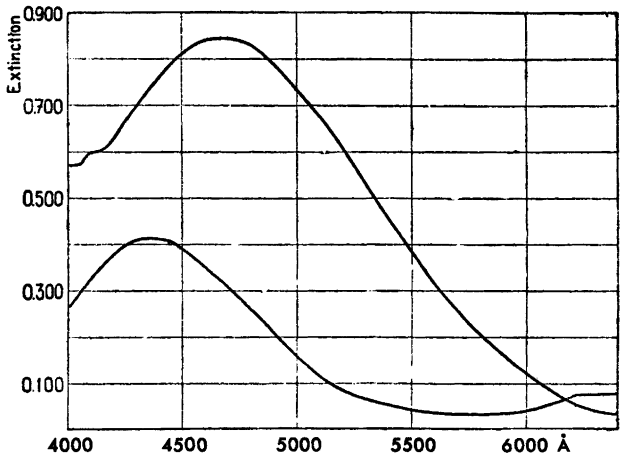

Fig. 1. Absorption curves for iron and copper complexes with metal combining protein. The curves have been obtained by reading a solution of metal free protein against a solution of protein saturated with metal at pH 7.5. Upper curve iron and lower curve copper. Binding of 100 γ % iron corresponds to an increase in extinction of 0.0435 at 4600—4700 Å (1 cm layer). For copper the corresponding extinction at 4300—4400 Å is 0.0202.

possible from this solution to prepare a copper protein with a copper content of 0.35 % (protein determination after Kjeldahl method). This protein is not precipitated by half saturation with ammonium sulphate, but comes down when the concentration is increased from 50 to 65 % saturation, and might be identical with the haemocuprein of Mann and Keilin.

On correlating these results with our finding that most of the serum copper is precipitated by 50 % ammonium sulphate, *it seems reasonable to assume that at least the main part of the serum copper is to be found in the α-globulin and that very little or no free haemocuprein exists in native serum.*

EXPERIMENTS WITH THE PURIFIED IRON-COMBINING PROTEIN

Using the pure iron-combining protein prepared by Laurell [14] we have been able to confirm the finding of Cohn *et al.* that this protein combines with copper. It gives a yellow complex which shows a broad absorption maximum at 4400 Å. A given amount of protein can bind an amount of copper equivalent to the amount of ferrous iron that can be taken up. Fig. 1 shows the absorption curves for the copper and iron complexes of this protein. The curves have been plotted by reading solutions of the metal-saturated protein against a solution of protein which has been freed from metals.

If a solution of this protein is saturated with copper, and iron then is added, this iron will quantitatively expel the copper, and an iron complex is formed, which can be shown by spectrographical analysis. If, on the other hand, the protein is first saturated with iron, and copper is then added, nothing happens. We have also tried if zinc interferes with the formation of copper and iron complexes by this protein. This is not the case. It seems therefore clear that the affinity of the protein for iron is greater than its affinity for copper, and that its affinity for both iron and copper is greater than for zinc. All these experiments were carried out at pH 7.5.

Even if the protein is only half-saturated with copper and sodium diethyldithiocarbamate is added the copper complex is broken up, and the copper combines rapidly with diethyldithiocarbamate.

These facts as well as the finding in the serum experiments go against the view of Cohn *et al.* that this protein should serve as a carrier of copper and zinc in serum.

If copper is added to serum with a high iron-binding capacity and a low iron content, it should be possible to show an increase in light absorption at 4400 Å. If Cohn's idea about copper being taken up by the same protein as iron were correct, the amount of this increase in absorption could be calculable from the experiments on the pure protein.

Such experiments were made, but no increased light absorption was found. The most probable explanation of this fact is that the copper complex with the iron-binding protein at the pH of serum is dissociated in at least the same degree as the complexes formed between copper and other serum proteins, and that therefore only a very small amount of the added copper forms a complex with the iron binding protein in serum. If this holds true for copper, it is evident from the competitional experiments with zinc and copper that it also applies to zinc.

The absorption at 4400 Å of a complex between iron combining protein and copper disappears upon addition of serum. This proves that other serum proteins have a greater affinity for copper than has the iron-binding protein.

SUMMARY

It has been shown that at least 90 % if not all of the serum copper is to be found in the globulin fraction (precipitated by 50 % saturation with ammonium sulphate) and that this also applies to the increase in copper during pregnancy and infections. It is probable that the haemocuprein of Mann and Keilin forms an integrating part of the blue α-globulin. The idea of Cohn *et al.* that the variations in serum iron and serum copper can be explained

by assuming the two metals to compete for the same protein is not tenable. Even though this protein combines with copper in vitro, it can be shown that in serum it is not combined with copper to any appreciable extent. The same must hold true for zinc.

In view of these facts we suggest that the new metal-combining protein (iron-binding component) in serum be called transferrin.

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