The Journal of Clinical Investigation

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J Clin Invest. 1949;28(1):73-78. https://doi.org/10.1172/JCI102056.

Research Article

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CHEMICAL, CLINICAL, AND IMMUNOLOGICAL STUDIES ON THE PRODUCTS OF HUMAN PLASMA FRACTIONATION. XXXVII. THE METAL-COMBINING GLOBULIN OF HUMAN PLASMA [1]

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(Received for publication June 14, 1948)

The separation of the proteins of human plasma into a series of fractions and subfractions, uniform with respect to certain physical properties, has generally led also to the separation of proteins with respect to their chemical and biological properties. Among the properties that have long been recognized has been the binding of metals by the plasma so that the metals were no longer freely dialyzable (1). When plasma fractionation was undertaken, early in the war, the Committee on Blood Substitutes of the National Research Council were informed that "investigations of the distribution of . . . metals known to be combined with proteins in the blood, such as copper, zinc, and iron, are planned and will be subsequently reported, as will studies upon the hormones and other physiologically important components of human plasma for which methods of bio-assay are available. We feel keenly that these should now be studied in the large amounts of human plasma fractions becoming available as by-products of the preparation of human albumin for transfusion in shock" (2).

Clinical studies had suggested not only that there was a binding of iron and copper, but that there might be a relation between iron and copper binding by the plasma proteins in the sense that an increase in the concentration of the one metal in the plasma was often associated with a decrease in the other (3–5).

The next advance in our knowledge of the binding of metals by proteins followed bacteriological investigations upon egg white. Schade (6) reported that the growth of the bacterium *Shigella dysenteriae,* which requires iron for its metabolism, was readily inhibited by egg white. The same phenomenon of inhibiting bacterial growth by the binding of iron by protein was next noted as a result of studies upon human plasma (7). Fractions of human plasma were made available to Schade, at his request, and the property of bacterial growth inhibition found to be completely concentrated in the fraction that had been designated IV–4(8).

The methods of detecting this protein component colorimetrically that were being developed by Schade were adopted, with slight modifications, for our studies, and led to the concentration of the iron-binding protein component of plasma into a new fraction (IV–7) (9), and finally to its crystallization (10).

This component of plasma has been designated the β_1-metal-combining globulin because of *in vitro* evidence, obtained with the crystallized protein, that it is capable of binding copper and zinc, as well as iron. The clinical and physiological studies reported in this series of communications have been carried out with Fraction IV–7, which is readily prepared in large amounts as a by-product of the fractionation of human plasma to yield serum albumin, γ-globulin, fibrinogen, thrombin and other stable blood derivatives, rather than with the more highly purified crystallized protein.

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[1] This paper is No. 72 in the series "Studies on Plasma Proteins" from the Harvard Medical School, Boston, Mass., on products developed by the Department of Physical Chemistry from blood collected by the American Red Cross. This work was originally supported by grants from the Rockefeller Foundation and from funds of Harvard University. It was aided early in 1941 by grants from the Committee on Medicine of the National Research Council, which included a grant from the American College of Physicians. From August 1941 to July 1946 it was carried out under contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University. Since then it has been aided by a grant recommended by the Panel on Hematology of the National Institute of Health.

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Fraction IV–7 consists to the extent of 76 per cent of the metal-combining protein; the inert components being mainly albumin and an α_2-globulin.

MATERIALS AND METHODS

The Fraction IV–7 and crystallized β_1-metal-combining globulin used in these studies were prepared from Fraction IV–4 from blood collected by the American Red Cross by methods to be described elsewhere (8, 9).[4]

Carbohydrate analyses were performed by a modification of the method of Sørensen and Haugaard (11), using orcinol. An equimolar solution of mannose and galactose was used as the standard.[5] Electrophoretic analyses were made in 0.1 ionic strength sodium diethyl barbiturate buffer at pH 8.6.[6]

Assays for metal-combining activity were made with a modification of the method of Schade and Caroline (7) by spectrophotometrically determining the colored iron-protein complex. To aliquots of a solution of the protein in pH 7.4, ionic strength 0.1 barbiturate buffer were added different amounts of a standard solution of ferrous ammonium sulfate. After standing 60 minutes at room temperature the optical densities of the solutions were read in the Beckman spectrophotometer at a wavelength of 465 mμ. On plotting optical density as a function of iron added, it was found that the extinction increased linearly to a maximum after which it remained substantially constant. The intersection of the two straight lines plotted through the points was taken as the most accurate estimate of the combining capacity. We are greatly indebted to Dr. Schade for his continuing collaboration as well as for periodic microbiological assays which confirmed the results of the spectrophotometric determinations.

RESULTS

A. Studies with the crystallized β_1-metal-combining globulin

The isolation of a pure crystallized protein with all the chemical and physiological properties of the metal-combining globulin proved the identity of the protein. We have therefore based our studies on the properties of the crystallized globulin. The behavior of less pure concentrates of the globulin, such as Fraction IV–7, should differ only quantitatively with respect to properties attributable to the specific component interacting with iron or other metals. Those properties observed with a concentrate, not attributable to the pure globulin, should therefore be attributed to impurities in the concentrate removed during the further purifications and crystallization.

1. Physical and chemical properties

Electrophoretically, the metal-combining globulin is characterized as a β_1-globulin. The protein of plasma has been estimated to contain 11 per cent of β_1-globulins (12) of which approximately 3 per cent is the metal-combining protein. The latter differs in size, shape, solubility, and composition, from the other β_1-globulins of the plasma which are largely separated into Fraction II + III (13). The metal-combining globulin resembles the albumins more than most of the other globulins in its solubility behavior and its stability under a variety of conditions of pH, ethanol concentration and temperature. Unlike the albumins, it is in an isoelectric condition near pH 5.9. The sedimentation constant, $S_{20,w} = 5.0$, and molecular weight of 90,000 indicates a molecule of somewhat larger dimensions than the albumins (10, 14). The metal-combining globulin contains but a negligible amount, if any, of lipid. Its carbohydrate content has been estimated to be 1.8 per cent. The nitrogen content was 14.7 per cent, corresponding to a nitrogen factor (gm. protein/gm. nitrogen) of 6.82. The latter is somewhat higher than that of the albumins and many other proteins, and undoubtedly reflects the presence of carbohydrate in the molecule.

2. Interactions with iron

Solutions of the complex formed between the crystallized globulin and iron exhibited the salmon red color which Schade (6, 7) had observed to be characteristic of fractions of plasma active with respect to the microbiological test, and indeed, of the iron-binding protein of egg white. The visible absorption spectrum of the iron-protein complex, measured against a protein blank, is represented

[4] Certain of the preparations of Fraction IV–4 were derived from out-dated dried plasma, being fractionated for the American Red Cross by E. R. Squibb and Sons, New Brunswick, New Jersey, and were released by the American Red Cross for this investigation. For other preparations of Fraction IV–4, fractionated from fresh plasma by the Division of Biologic Laboratories, Massachusetts Department of Public Health, we are indebted to Drs. V. A. Getting, G. Edsall, and D. J. Mulford.

[5] We are grateful to Dr. D. M. Gibson and Mr. Robert S. Gordon, Jr., for the carbohydrate analyses.

[6] We are indebted to Mr. M. J. E. Budka and Miss M. M. Hasson for these measurements.

in Figure 1.[7] The broad absorption band had its maximum at 465 mμ, with $E^{1\%}_{1\,cm.} = 0.570$.[8] This band was the basis of the spectrophotometric determination of iron-binding capacity, described previously. The width of the band allowed use of a number of wavelengths other than the maximum for the determination. Thus, Rath and Finch (15) have used the absorption at 525 mμ in their titrations of serum to avoid interference by other substances present which absorbed at shorter wavelengths.

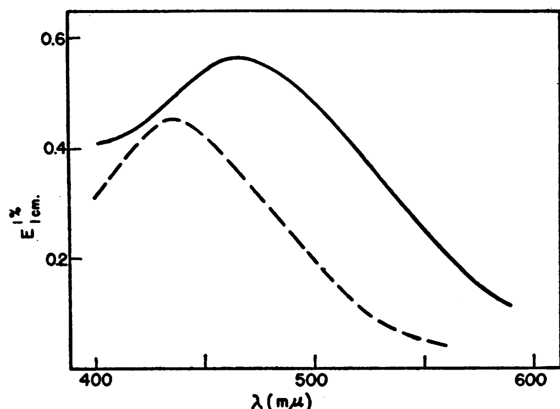

FIG. 1. ABSORPTION SPECTRA OF METAL-COMBINING GLOBULIN IN PRESENCE OF IRON AND COPPER, MEASURED AGAINST A PROTEIN BLANK: *Solid line*—COMPLEX WITH IRON, pH 7.4, Γ/2 0.1 BARBITURATE; *dashed line*—COMPLEX WITH COPPER, pH 8.5, Γ/2 0.05 BARBITURATE

Neither the iron-free globulin nor iron alone absorbed appreciable amounts of light at these wavelengths and in the concentration range used. Indeed, the formation of a colored complex with iron was a unique property of the metal-combining globulin, to the exclusion of all the other plasma proteins. This was confirmed by observation of the migration of a solution of the complex in the Tiselius apparatus at pH 8.6. The color was found to move in sharp coincidence with the peak of the protein Schlieren diagram throughout the duration of the experiment.

The reaction between iron and the metal-combining protein was dependent on pH. At neutral pH, above pH 6.5, the color and interaction were maximal. On acidification the color diminished, and below pH 5 the iron could be dialyzed from the solution. The dissociation of the complex and the loss of color were readily reversed by readjusting the system to pH 7. This dependence of the metal-protein interaction on pH is represented in Figure 2.

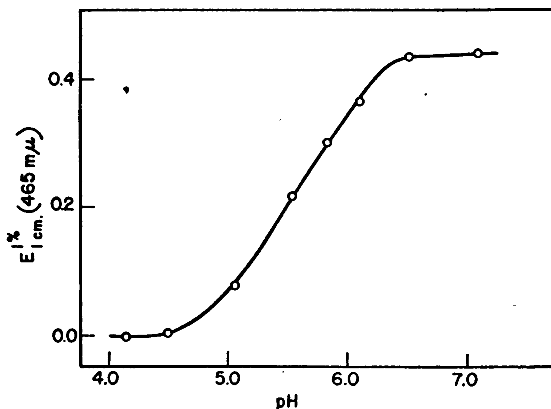

FIG. 2. SPECTROPHOTOMETRIC BEHAVIOR OF METAL-COMBINING GLOBULIN IN PRESENCE OF EXCESS IRON AS A FUNCTION OF pH

Iron (as $FeSO_4 \cdot [NH_4]_2SO_4 \cdot 6H_2O$) 2.8 γ/mg. Fraction IV–7. Measured against protein blank.

The experimental points were obtained by titrating aliquots of an acidified solution of Fraction IV–7, containing ferrous ammonium sulfate, with alkali in the absence of buffer. The amount of iron in each aliquot was the same and corresponded to approximately two-fold excess over that which could be bound to the protein at the pH of maximum binding. The optical density and pH of each aliquot were measured after standing overnight at 5° C.[9]

[7] Preliminary studies suggest that in the ultraviolet region, iron produces a non-specific enhancement of the usual protein absorption near 280 mμ. This has been observed also with bovine serum albumin.

[8] The extinction coefficient, $E^{1\%}_{1\,cm.}$, is a constant at any given wavelength, and is defined by the expression: $\log I_0/I = E^{1\%}_{1\,cm.} \cdot c \cdot 1$, where $\log I_0/I$ is the optical density, *c*, the concentration in gm./100 ml., and 1, the length of the path of light through the solution in the cuvette, measured in cm. This is the familiar Beer-Lambert equation.

[9] We have not attempted to evaluate the mass law equilibrium constant for the interaction because of the number of variables involved. However, at neutral pH, under conditions of maximum combining capacity, the interaction of the protein with iron was practically independent of the concentration of free iron in solution. Were this not so, the titration curve of the protein with iron would not have shown the sharp "break" at the point of saturation. At acid pH, this was not so, and the titration curves were of sigmoid shape.

The crystallized globulin was capable of binding 1.25 γ of iron per milligram of protein. This corresponded to two atoms of iron per molecule of protein of molecular weight 90,000. Although we have preferred to use ferrous salts for studying the interaction, iron in the form of ferric salts also interacted with the protein. The complex so formed was identical in its spectral absorption to that formed by the use of ferrous salts. The interaction with ferric salts was much slower, however, possibly because of their lower solubility and more rapid hydrolysis at neutral pH. Further evidence of the identity of the complexes formed with ferrous and ferric iron was obtained by Dr. Michaelis (16), who measured the magnetic susceptibility of the complex formed between the crystallized metal-binding globulin and ferrous ammonium sulfate. The results indicated that the iron in the complex is in the ferric state and that the complex is ionic, not covalent. It is therefore evident that the ferrous iron was oxidized prior to or during the formation of the complex with the protein.[10]

The iron-binding capacity of Fraction IV–4, or any of its subfractions which contained metal-binding globulin, has always been found equal, within the limit of error achieved, to the expected iron-binding capacity of the crystallized material on the assumption that all of the electrophoretically determined β_1-globulin in the fraction was the metal-combining globulin. Since no other fraction of plasma contained measurable quantities of the globulin, *we estimate that normal plasma contains 2.4 gm. per liter of this component, capable of binding a total of approximately 3 mg. of iron per liter.* This is in good agreement with the value of 315 γ per 100 ml. found by Rath and Finch (15).

3. Interaction with copper

The crystallized metal-combining globulin also interacted with copper in a manner similar, in most respects, to the reaction of the globulin with iron. The copper-protein complex had a green color whose absorption spectrum has been represented in Figure 1. The maximum was near 435 mμ with $E^{1\%}_{1\,cm.} = 0.45$. Copper-binding capacity was determined by titration with a standard solution of copper sulfate. A pH 8.4 barbiturate buffer of ionic strength 0.05 was used. Under these conditions each milligram of protein bound 1.4 γ of copper, corresponding to two atoms of copper per molecule of protein.

The interaction of copper with the crystallized globulin differed from that of iron in that the pH-binding curve for the copper was displaced approximately 1 pH unit toward alkaline reactions relative to that of iron. Maximum binding occurred near pH 8.5; at pH 7, where the protein had its maximum binding capacity for iron, its capacity to bind copper was only approximately half maximum.

It was of interest to study the relative affinity of the metal-combining globulin for copper and iron. Using the absorption spectra as an indication, no conditions have been observed under which copper displaced iron from the iron-protein complex. Further, below pH 8, iron displaced copper from the copper-protein complex. Copper and iron are therefore probably bound to the same groups on the protein molecule, the affinity for iron being higher at neutral pH.

Because of these results of *in vitro* studies we have designated this component of plasma the metal-combining globulin.[11] While the evidence from clinical studies (15, 18) has suggested that this protein is indeed responsible for the physiological transport of the iron in plasma, and the chemical studies have shown that this component was present in sufficient concentration to satisfy the iron-binding capacity of plasma, the evidence for the *in vivo* transport of copper is less clear. Thus, while chemical studies have shown that this component was capable of binding copper to a certain extent at physiological pH, they do not preclude the existence of a separate *in vivo* mechanism for the transport of copper.[12] Indeed, the binding of copper by albumins has been shown by Klotz (19).

[10] Studies on the time necessary for full development of color suggested that autoxidation of ferrous iron was a prerequisite to binding (10). Thus, at pH 8, full color developed within a few minutes, whereas at pH 6.5, several hours were necessary. Schade, Reinhart and Levy (17) have found that carbon dioxide has a role in the formation of the complex. Other factors may be involved as well (22).

[11] This component has been called "siderophyllin" by Schade, Reinhart and Levy (17) and "transferrin" by Holmberg and Laurell (23).

[12] Since this manuscript was submitted, the work of Holmberg and Laurell (23) has come to our attention.

4. Interaction with other metals

Of a series of metals investigated, including cobalt, nickel, zinc, manganese, as well as copper and iron, only the latter two formed a complex with the protein, as characterized by an absorption of light. However, failure of formation of color cannot be considered as evidence of failure of complex formation. Thus zinc, which failed to produce a color when added to solutions of the metal-combining globulin, was nevertheless bound to the protein at neutral pH as shown by direct analysis for zinc.[18] Studies on the interactions of the globulin with zinc and other metals will be the subject of later communications.

B. Properties of Fraction IV–7 of human plasma

In the subfractionation of Fraction IV–4, the metal-combining globulin was further purified and concentrated in Fraction IV–7, the fraction recommended for use in clinical studies on this component of plasma. The preparations which were fractionated at the Harvard Pilot Plant contained an average of 76 per cent of the metal-combining globulin. They differed with respect to this component only quantitatively from the crystallized material. Thus, Fraction IV–7 bound 0.8 to 0.9 γ of iron per milligram of protein.

The impurities in Fraction IV–7 were mainly albumin and α_2-globulin. Certain properties of this fraction were due to these impurities and were absent in the crystallized metal-combining globulin. Among these were an ability to bind bilirubin (20) and hematin (21). The binding of bilirubin was higher than could be attributed to the albumin present, and was found to be a characteristic of the α_2-globulin in the fraction. This latter component of Fraction IV–7 was thus responsible for the occasional presence of colored impurities in preparations of Fraction IV–7 derived from hemolyzed plasma. Highly colored samples from this source have, however, had unimpaired metal-binding capacities.

They confirm many of our findings and suggest further that the main part of the serum copper is normally associated with an α-globulin which is insoluble in 50% ammonium sulfate.

[18] We are grateful to Dr. B. Vallee for the zinc analyses.

Solutions of Fraction IV–7 for clinical use have been made by dissolving the dried protein in isotonic saline at pH 6.8 in a concentration of 25 gm. of protein per 100 ml. of solution. Neither acetyl tryptophane, which has been used to stabilize solutions of albumin, nor glycine, which stabilizes γ-globulin solutions, were as effective as sodium chloride in stabilizing solutions of Fraction IV–7. Such solutions were capable of binding approximately 22.5 mg. of iron per 100 ml. and were therefore concentrated approximately seventy-fold over plasma with respect to the metal-combining globulin.

SUMMARY

1. A component of normal human plasma, the metal-combining globulin was concentrated in Fraction IV–7 and crystallized therefrom.

2. The metal-combining globulin is a β_1-globulin and is distinct in its behavior and properties from other β_1-globulins of plasma.

3. The capacity of this component of plasma to bind metal ions, particularly iron, copper and zinc, has been discussed. These interactions with metals were readily reversed by change in pH and, in the case of copper and iron, were characterized by the formation of colored metal-protein complexes with characteristic absorption spectra.

4. The protein had a higher affinity for iron than for copper in solution as judged by the capacity of the former to displace copper from the complex.

5. Fraction IV–7 derived from human plasma has been recommended as a concentrated source of the metal-combining globulin. Its safety for injection in man has been investigated and its properties reported.

BIBLIOGRAPHY

1. Schmidt, C. L. A., The Chemistry of the Amino Acids and Proteins. Charles C. Thomas, Baltimore, 1938, pp. 746–772.
2. Report to the Subcommittee on Blood Substitutes, Division of Medical Sciences, National Research Council, July 18, 1941. Bulletin of Blood Substitutes, pp. 93, 98–101.
3. Barkan, G., Eisenstudien Die Verteilung des leicht abspaltbaren Eisens zwischen Blutkörperchen und Plasma und sein Verhalten unter experimentellen Bedingungen. Ztschr. f. physiol. Chem., 1927, **171**, 194.

4. Heilmeyer, L., Keiderling, W., and Stüwe, G., Kupfer und Eisen als körpereigene Wirkstoffe. Gustav Fischer, Jena, 1941.
5. Laurell, C. B., Studies on the transportation and metabolism of iron in the body with special reference to the iron-binding component in human plasma. Acta Physiol. Scand., 1947, **14**, Suppl. 46.
6. Schade, A. L., and Caroline, L., Raw hen egg white and the role of iron in growth inhibition of Shigella dysenteriae, Staphylococcus aureus, Escherichia coli and Saccharomyces cerevisiae. Science, 1944, **100**, 14.
7. Schade, A. L., and Caroline, L., An iron-binding component in human blood plasma. Science, 1946, **104**, 340.
8. Cohn, E. J., Strong, L. E., Hughes, W. L., Jr., Mulford, D. J., Ashworth, J. N., Melin, M., and Taylor, H. L., Preparation and properties of serum and plasma proteins. IV. A system for the separation into fractions of the protein and lipoprotein components of biological tissues and fluids. J. Am. Chem. Soc., 1946, **68**, 459.
9. Surgenor, D. M., Strong, L. E., Taylor, H. L., Gordon, R. S., Jr., and Gibson, D. M., Preparation and properties of serum and plasma proteins. XX. The separation of the choline esterase, mucoprotein, and metal-combining globulin into subfractions of human plasma. J. Am. Chem. Soc., in press.
10. Koechlin, B., In preparation.
11. Sørensen, M., and Haugaard, G., The application of the orcinol reaction to the estimation of the nature and amount of carbohydrate group in proteins. Compt. Rend. trav. lab. Carlsberg, 1933, **19**, No. 12, 1–45.
12. Cohn, E. J., Chemical, physiological, and immunological properties and clinical uses of blood derivatives. Experientia, 1947, 3, 125.
13. Oncley, J. L., Melin, M., Richert, D. A., Cameron, J. W., and Gross, P. M., Jr., Preparation and properties of serum and plasma proteins. XIX. The separation of the antibodies, isoagglutinins, prothrombin, plasminogen and X-protein into subfractions of human plasma. J. Am. Chem. Soc., in press.
14. Oncley, J. L., Scatchard, G., and Brown, A., Physical-chemical characteristics of certain of the proteins of normal human plasma. J. Physiol. & Coll. Chem., 1947, **51**, 184.
15. Rath, C. E., and Finch, C. A., Chemical, clinical, and immunological studies on the products of human plasma fractionation. XXXVIII. Serum iron transport: measurement of iron binding capacity of serum in man. J. Clin. Invest., 1949, **28**, 79.
16. Michaelis, L., Personal communication.
17. Shade, A. L., Reinhart, R. W., and Levy, H., Carbon dioxide and oxygen in complex formation with iron and siderophyllin, the iron-binding component of human plasma. Arch. Biochem., in press.
18. Cartwright, C. E., Black, P., and Wintrobe, M. M., Chemical, clinical, and immunological studies on the products of human plasma fractionation. XXXIX. The anemia of infection: studies on the iron-binding capacity of serum. J. Clin. Invest., 1949, **28**, 86.
19. Klotz, I. M., and Curme, H. G., The thermodynamics of metallo-protein combinations. Copper with bovine serum albumin. J. Am. Chem. Soc., 1948, **70**, 939.
20. Martin, N. H., Preparation and properties of serum and plasma proteins. XXI. Interactions with bilirubin. J. Am. Chem. Soc., in press.
21. Rosenfeld, M., In preparation.
22. Fialo, S., and Burk, D., On the mode of iron-binding by siderophyllin, conalbumin, hydroxylamine, aspergillic acid, and other hydroxamic acids. Arch. Biochem., in press.
23. Holmberg, C. G., and Laurell, C. B., Investigations in serum copper. I. Nature of serum copper to the iron-binding protein in human serum. Acta Chem. Scand., 1947, **1**, 944.