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Abstract

According to the method presented in the first report the author mixed ferric iron solution and serum with the various proportions of serum and iron, limiting the pH level of the media wihin 5.4 to 8.3. It was found there was a certain level exceeding which the iron could no longer move with protein on the paperelectrochromatography. The maximum level was found to be81, 500γ% in the case of ferric chloride and 77, 200γ% in ferric ammonium sulfate, when the bovine serum was used as a protecting colloid. The iron added in exce of this level was found retarding at the starting line suggesting the formation of gro iron hydroxide colloid.

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PATHOPHYSIOLOGICAL STUDIES ON FERRIC IRON
PART 2. QUANTITATIVE OBSERVATIONS ON THE REACTION BETWEEN FERRIC IRON AND THE SERUM PROTEIN*

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In the first report the author showed that there developed some chemical or physicochemical reactions between ferric iron and serum protein when these were mixed in some ratio and in the limited range of pH of media, and further suggested that the iron in this compound might be useful for hemoglobin synthesis when it was introduced intravenously. For the purpose of observing the biological effects of this iron the author observed the capacity and the qualification of the iron included in the serum, in some mode of combination with serum protein as indicated on paperelectrochromatography.

MATERIALS AND METHODS

In these experiments only bovine serum served as serum protein. The ferric compounds to be added were ferric chloride and ferric ammonium sulfate, 10 mg ferric iron per cc in content. Each 20 cc of serum was taken into 10 small beakers of 50 cc and 1, 5, 10, 15, 25, 30, 40, 60, and 80 mg of iron was added respectively leaving one without addition of iron sulfate. Another series was prepared in the same manner for ferric chloride. For the adjustment of pH in media a 3% Na_2CO_3 solution was used. The pH of the media was determined by using a pH-meter of glass electrode of Shimazu Company, Type GU-3.

For the paperelectrochromatography two apparatuses were used, the one was of Grassmann Type under the condition described in the first report, and the other was one designed in our laboratotry, 300 V in electric strength, 27 cm apart from pole to pole, 10 m amp/3.9 cm. The papers used were all of Carl Schleicher No. 2043a Mgl. For the extending media 1/10 n veronal-veronal-Na buffer, pH 8.6, μ 0.1, was used, and extended for 8 hours, by Grassmann Type and 2 hours by our own type. The

* The outline of this report was adressed at Japan Hematological Society, 1957.

materials were taken by pipette for paper chromatography, 0.02 cc exactly, and lined on the starting line respectively. After extending, the paper was dried at 100°C, cut into two parts and iron and protein were estimated separately by the same method as described in the first report.

Quantitative estimations of protein were carried out in each material by using the method of "Halbmikrokjeldahl" and refractometry by Pulfrich. The iron contained in serum was estimated quantitatively on paperelectrochromatography by Berlin blue reaction. For the use of Berlin-blue reaction for the quantitative estimation of iron, S. I. C. having various but known iron contents were taken by pippette exactly 0.02 cc in each solution, and these were lined serially on a paper of Carl Schleicher. After drying, the paper was treated with 5% potassium ferrocyanide and 1*n* HCl solutions, and the density was measured in each point by the method described in the first report. In this instance, observations were carried out to see whether or not proportional changes might have occurred between the quantity of the iron and the density corresponding to each sample.

OBSERVATIONS AND RESULTS

On the papers extended bovine serum at 300 volts for 2 hours, the peaks of each globulin fractions were found to be condensed in one peak which appeared following shortly after the albumin peak as indicated in Fig. 1 a and b. In the material containing 10 mg of ferric iron a fairly sharp peak of iron appeared between the peaks of albumin and globulin by extending at 300 volts, while a peak of iron having a large base with the appex between the peak of α and β-globulin appeared by extending at 110 volts (Fig. 2, a, b). As can be seen in these results, by extending at 300 volts the peak of iron appeared sharply but the exact relationship between the apex of iron and that of protein was rather ambiguous. At present for the author's study it is sufficient to make it clear whether iron moves with proteins or stands at the starting point and the author carried out all the experiments at the electric strength of 300 volts. The increasing of iron contents in serum till 25 mg Fe/20 cc of serum proved that the iron moved with serum protein raising the density at the same point observed in the case of 10 mg Fe was added to 20cc of serum (Fig. 2, a).

Exceeding 30 mg in the amount of Fe per 20 cc of serum, some retardation of iron occurred increasing the density of iron in the whole area of globulin fractions with the increase in the amount of iron remaining at the starting point.

For the quantitative estimation of the maximum amount of iron

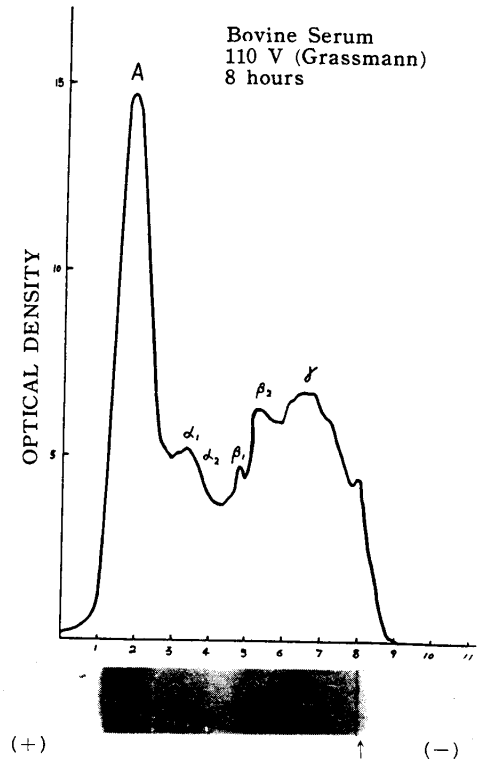

Fig. 1 a. The paperelectrophoretic patterns of bovine serum on Grassmann's apparatus at 110V for 8 hours in veronal buffer ($\mu = 0.1$), pH 8.6.

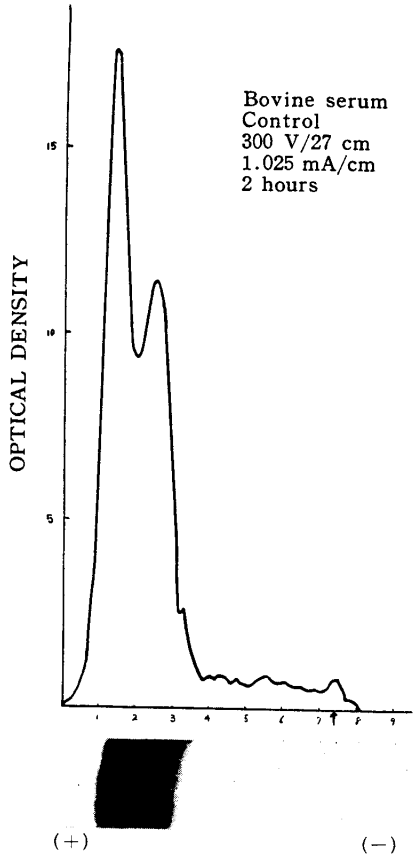

Fig. 1 b. The paperelectrophoretic patterns of bovine serum on the apparatus designed in our laboratory at 300 V for 2 hours in veronal buffer ($\mu = 0.1$), pH 8.6. The peaks of each globulin fractions have been condensed in one peak which appeared shortly after the albumin peak.

which can be moved electrophoretically with protein, the density of Berlin-blue reaction on the paper appeared after electrophoresis may be useful. But in this case it is necessary to confirm whether or not there is the exact proportional correlation between the quantity of iron and the color intensity of the Berlin-blue reaction. The observation on the Berlin-blue reaction given by the known quantity of iron lined on the paper of Carl Schliecher proved that the intensity of the reaction increased parallel with the increased concentration of iron satisfying the Lambert-Beer's law as is indicated in Fig. 3. This shows that the Berlin-blue reaction can be useful

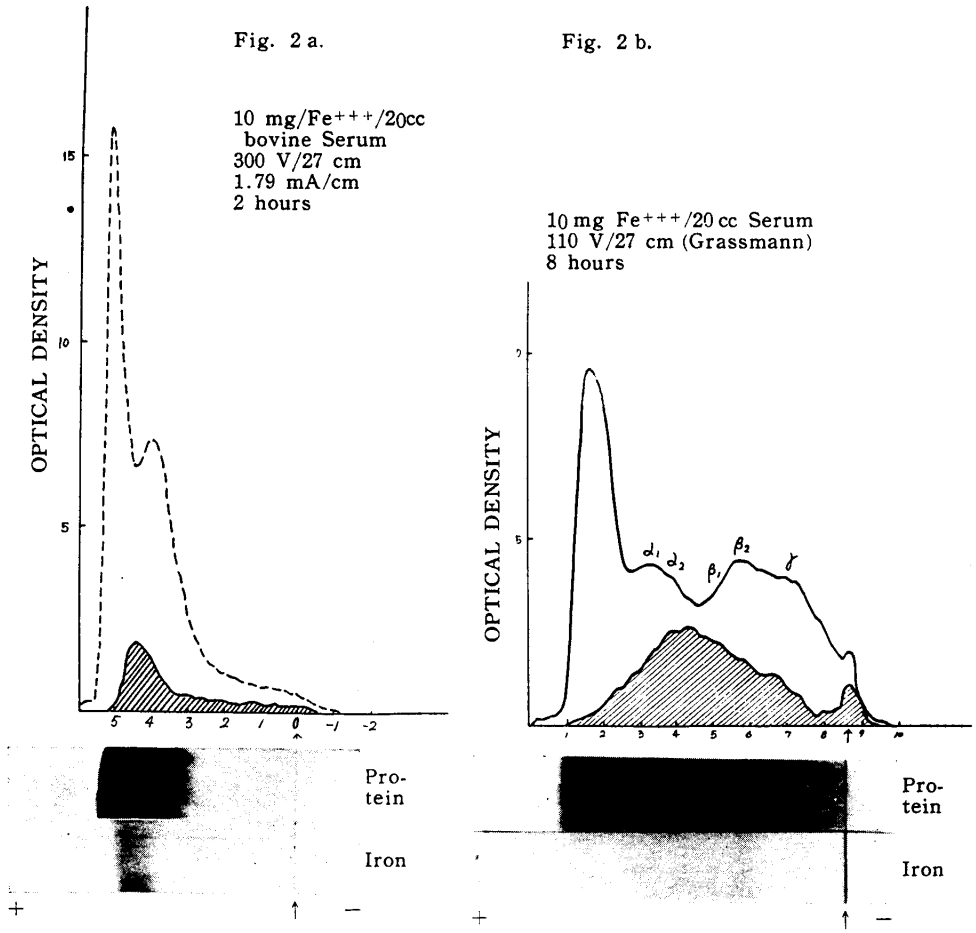

Fig. 2 a, b. The paperelectrophoretic patterns of bovine serum iron colloid, containing 10 mg iron in 20 cc of bovine serum. Showing the fairly sharp peak of iron appearing between the peaks of albumin and globulin, in the case extended by our oun apparatus 300 V for two hours (Fig. 2 a.) while in the case with grassmann's apparatus a relatively wide distribution of iron can be seen (Fig. 2 b).

for the qnantitative estimation of iron. Thus the quantity of iron moved with protein on the paper was estimated from the area surrounded by the curves obtained from the densitometry and the line perpendicular to the abscissa passing the middle point of the straight line connecting the apexes of globulin peak and the base line as indicated in Fig. 4. From the area described above and the whole area of iron as determined on the paper, the actual amount of iron moved with protein was calculated in each sample by the formula in the following, $Fe = C \times \frac{s}{S}$ where C, is the iron concen-

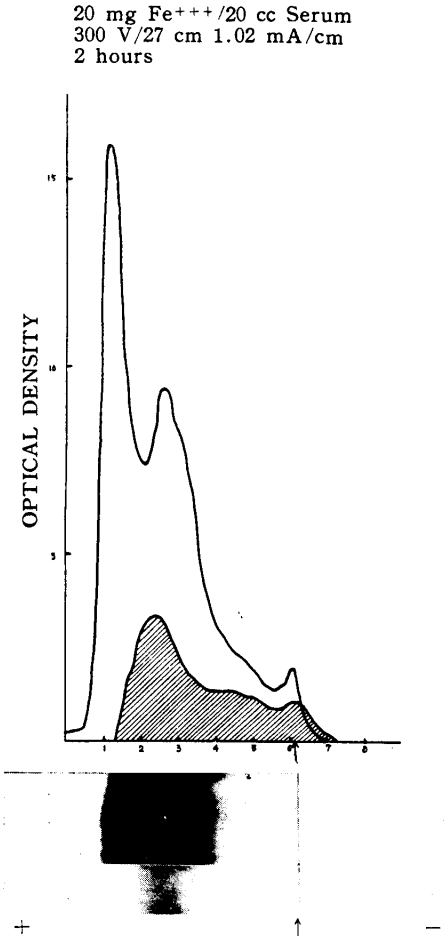

Fig. 2c. The paperelectrophoretic patterns of serum iron colloid containing 20 mg iron in 20 cc of bovine serum.

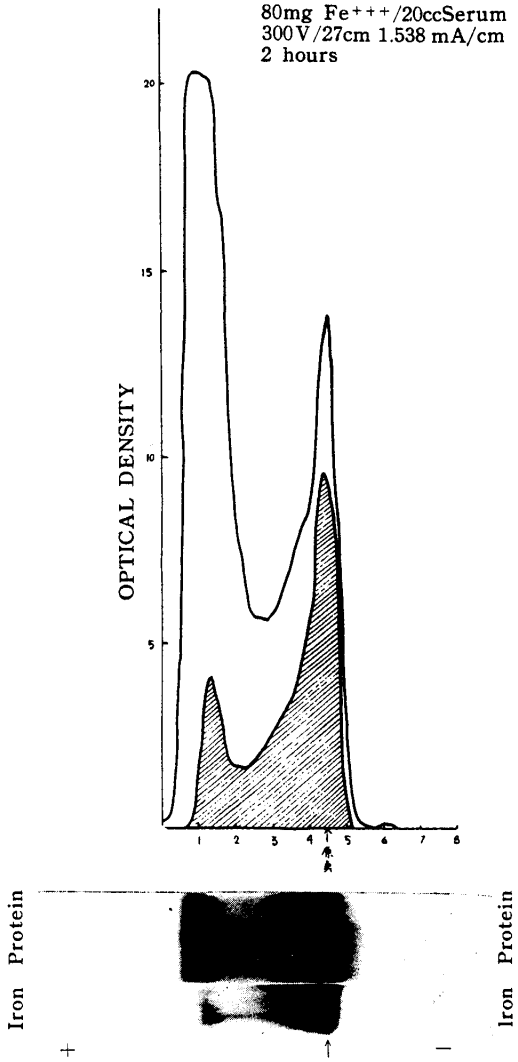

Fig. 2d. The paperelectrophoretic patterns of serum iron colloid, containing 80 mg iron in 20 cc of bovine serum, showing the marked retardation of iron at base line.

tration of the material; s, is the area representing the hatched part in Fig. 4, obtained by densitometry on the iron moved with protein; S, is the whole area given by densitometry on Berlin-blue reaction. As shown in Fig. 5, the data proves there is a certain maximum amount of iron

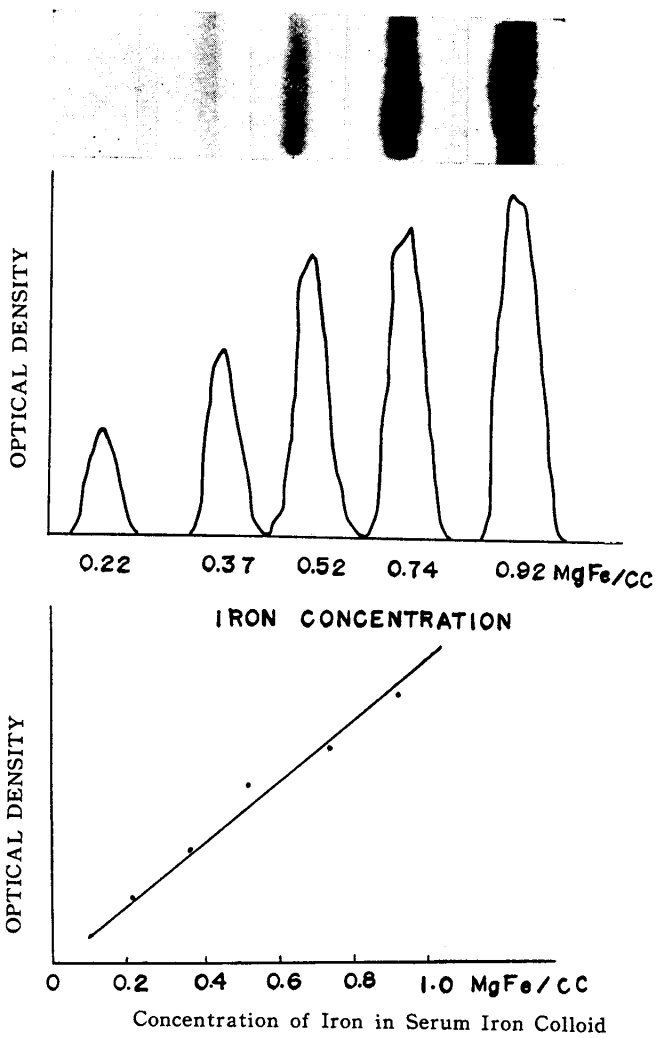

Fig. 3. The relation between the concentration of iron in S.I.C. and the optical density of iron on the paper by the method of Berlin blue reaction, presenting Lambert-Beer's law.

which can move with protein on the paper, 81,500γ% in the case of $FeCl_3$, and about 77,400γ% in the case of ferric ammonnium sulfate, showing almost 250 fold of the binding capacity of normal β_1-metal combining protein.

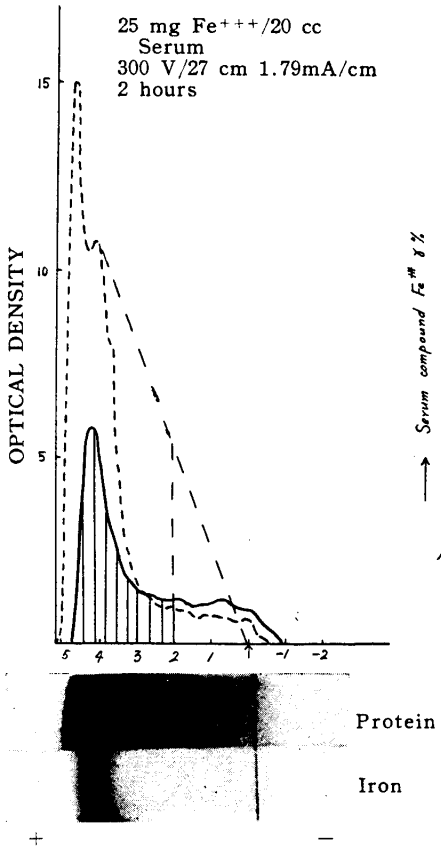

Fig. 4. The curves of protein and iron obtained by electropaperchromatography. The quantity of iron moved with protein on the paper was estimated from the area surrounded by the curves obtained from the densitometry and line perpendicular to the abscissa passing the midde point of the straight line connecting the apex of globulin peak and the base line.

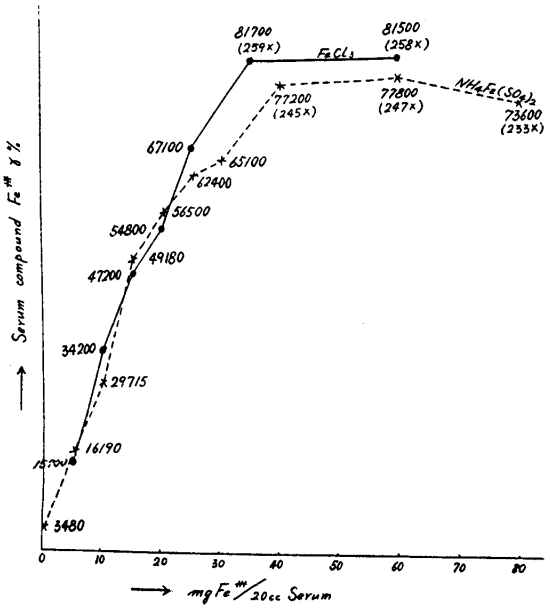

Fig. 5. The curves of amount of iron combined with serum protein, obtained by electropaperchromatography. The relation betwen the amount of iron combining with the serum protein and the concentration of iron in the serum iron colloid, can be seen showing the maximum value around 75,000 γ %.

COMMENT

As indicated in the above described results when the iron is added to serum as ferric iron, there is a maximum point beyond which the iron can not be combined with protein causing the retardation at the starting line on paperelectrochromatography. The maximum amount of iron combined with serum protein in the mode presented in the first report proved to be 77,400—81,500 γ % showing the capacity to be about 250 times as that of

β_1-metal combining protein.[1,2] Concerning the quantitative determination of iron it has been reported by several authors that the Berlin blue reaction can be used for the quantitative estimations of iron in the case of ferritin[3] and it has been also shown that pretreatment with 6 normal HCl at 100°C before the Berlin-blue reaction is required[4]. But in the case of serum iron colloid the iron quantity can be estimated directly by Berlin-blue reaction without any pretreatment. This can be easily understood from the fact that this compound can easily be separated ferric iron in acidic media lower than pH 5.4.

The phenomenon of the iron-retardation near the starting line which is observable when the iron is added beyond the level of the maximum binding capacity will be due to the formation of gross iron hydroxide particles similarly as in the case where the pH level of media is elevated exceeding 9.0.

SUMMARY

According to the method presented in the first report the author mixed ferric iron solution and serum with the various proportions of serum and iron, limiting the pH level of the media wihin 5.4 to 8.3. It was found there was a certain level exceeding which the iron could no longer move with protein on the paperelectrochromatography. The maximum level was found to be $81,500\gamma\,\%$ in the case of ferric chloride and $77,200\gamma\,\%$ in ferric ammonium sulfate, when the bovine serum was used as a protecting colloid. The iron added in excess of this level was found retarding at the starting line suggesting the formation of gross iron hydroxide colloid.

ACKNOWLEDGMENT

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