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Abstract

By mixing ferric iron with serum protein, 20 cc of serum and 1 cc of ferric chloride or ferric ammonium sulfate (l0 mg ferric iron/cc in each), in the range of pH 5.4 to 8.2, a transparent brownish red colored solution can be obtained. Paperelectrochromatography proved the iron can mainly be detected in β-globulin fraction in bovine serum and in &#945-globulin and albumin fractions in human and rabbit sera. But the absorption spectrum proved that there is no formation of any new compound, giving almost the same absorption curve as in the serum protein itself. And by lowering the pH of media below 5.4, the solution gives immediately the positive reaction of ferric iron. From these rerults it is suggested that iron will be maintained in a colloidal state keeping the stability of this state in the presence of protein molecules. Freezing and drying are the procedures quite useful for keeping this material for a long period of time without changing the chemical characteristics.

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PATHOPHYSIOLOGICAL STUDIES ON FERRIC IRON
PART 1. CHEMICAL REACTION BETWEEN FERRIC IRON AND SERUM

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One of the specificities of iron metabolism lies in the difficulties of its absorption and excretion. The quantity of iron absorbed per day is only 15mg which is supplied from the foods and absorbed mainly from the stomach and upper part of duodenum. These are oxidized in serum and combined with β_1-metal-combining protein of SCHADE[1,2] $^{2a,\ 2c,}_{2b,}$ and utilized for hemoglobin synthesis or deposited as ferritin in the liver, spleen and other organs, combining with appoferritin[3,4]. This fact shows that the quantity of iron absorbed from the digestive organs is limited to a certain small amount depending upon the unsaturated β_1-metal-combining protein. This is one narrow path of iron to be absorbed as there is no other way to be absorbed without combining that protein. This narrow path for iron may be understood from the cyclic metabolism of iron extremely limiting its excretion. However, this will mean that the organism will be easily led to an iron deficient state, hypochromic anemia, by the unphysiologic loss of a certain amount of blood. Iron has been introduced to the anemic patients from various routes or by various sorts of chemcial compounds and in many cases the iron administrations proved to have an excellent curative effect, but oral administration often caused a severe stomach collic or diarrhea. These side effects are more dominant in ferric compounds than in ferrous compounds or more severe in solution than in amorphous powder[5], and yet the administration often showed not so marked therapeutic effect which may be reasonably considered from the above mentioned β_1-protein threshold and the limited area of absorption, stomach and the upper part of the duodenum where iron compounds could not be kept for a period long enough for the sufficient absorption of iron. However, it must be noticed that the oral administration of iron in a large quantity, say 3 to 5 grams daily, gives a good result. According to SENO[6] and others the introduction of iron in a large amount into the stomach results in severe damage of the mucous membrane, edema, bleeding and siderosis, causing

the physico-chemical invasion of iron into the interstitial tissues far from the physiologic absorption. The fact that the iron introduced in such a way is effective for the recovery of anemia may suggest the iron needs not to be combined with β_1-protein prior to be incorporated into hemoglobin.

The iron introduced intravenously as some organic iron compounds, vitamin C ferrous iron (HEILMEYER[7]), saccharated oxide of iron (KUHNS[8]), ferric asparagin (SENO[9]), etc., have been proved to be utilized for hemoglobin synthesis showing the marked therapeutic effects in anemic patients. This also may give another evidence that the iron combining with β_1-protein is not necessarily a mere iron source for the iron compounds in organisms such as hemoglobin, ferritin and so on. But the intravenous injection of iron showed some side effects of heavy metal intoxication[10], or some symptoms like colloid shock. Asparagin iron seems to be an ideal one but it decomposes easily, by which it can not be actually used for the anemic patients. And yet from the observations of SENO, KUHNS, and UNDRITZ, it is clear that the iron introduced intravenously shows a marked biological effect comparing to that given orally. From these reasons the author carried out pathophysiologic observations on iron metabolism and succeeded in obtaining the ferric iron combined with serum protein which can be introduced intravenously in a large quantity, 10 mg/kg, without showing any adverse symptoms, and accelerate markedly the hemoglobin synthesis. When the ferric iron is added to the serum, the so-called iron albuminate is produced forming insoluble gel. This can be dissolved forming a brownish red solution when the pH of the medium is elevated by adding alkali. By this method it is possible to combine serum with ferric iron in a fairly large quantity.

In this report the author describes qualitative investigation on the reaction between ferric iron and serum protein. Quantitative investigation, electronmicroscopic studies of ferric colloid in serum, and biological investigations will be reported in the following papers.

MATERIALS AND METHODS

Bovine serum, horse serum, and rabbit serum served mainly as materials. As the source of ferric iron ferric ammonium sulfate and ferric chloride were used, and for the regulation of pH of media, 3% Na_2CO_3 and n HCl were used. The binding between iron and serum protein has been observed by paper electrochromatography with Grassmann's apparatus, extended at the strength of 110V with the paper of

Karl Schleicher No. 2043a Mgl, and veronal, veronal-Na buffer, pH 8.6, μ 0.1, for 8 hrs. The pH of media was estimated by glass-electrode, Shimazu pH-meter, GU-3. The distribution of the iron and protein was determined by observing the electrophoretic pattern with densitometer of Grassmann Type.

Exactly 0.02 cc of the material was taken by the pipette for chromatography and lined on the base line on the paper, and extended by the method described above. After 8 hours the paper was dried at 100°C for 10 minutes fixing the protein, then the paper was divided into two parts cutting along the longitudinal midline; one for protein detection by brom phenol-blue staining and the other one for the detection of iron by potassium ferro-cyanide. For the determination of iron, the paper was treated with 1 *n* HCl solution for 5 minutes after the immersing in 5% potassium ferrocyanide for 5 minutes, washed with 0.5 *n* HCl solution and dried. A pair of these papers were made transparent immersing in liquid paraffin, and the density of protein and iron have been traced by Grassmann's densitometer respectively. Comparing these two data, the position of the iron appearing on the protein fractions was actually decided.

OBSERVATIONS AND RESULTS

When 20 cc of bovine serum is added with 1 cc of ferric chloride solution, whose iron content is 10 mg/cc, the serum is coagulated promptly forming an insoluble brown iron albuminate gel. The pH of the medium is found to be lowered near to 5.4.

This gelatinous material is divided into two parts, 10 cc each, and observed separately. The one is added *n* hydrochloric acid solution where the albuminate is dissolved turning to a slightly yellow transparent solution at pH 4. Further acidification causes no change. If 3% Na_2CO_3 solution is added to this solution slowly, it is gelatinized again near pH 5.4. By adding Na_2CO_3 solution further, the gelatinized material is dissolved turning to a reddish brown colored solution near pH 7.5. This state is kept without change in solubility till pH 8.2 to 8.3 where the color becomes more reddish. This is extended by paperelectrochromatography by the method mentined above. Another one of the original gel is added 3% Na_2CO_3 solution drop by drop elevating the pH of the media gradually without acidification. In this case likewise the albuminate sol is liquefied near pH 7.5 showing almost the same color and solubility as in the former case. At pH 8.2 to 8.3 a part of the solution is extended on the paper electrophoretically, the residue of the solution is added

Na_2CO_3 solution further and observed extending at pH 9.0, and pH 9.5 respectively. After extending, drying and staining by the above-mentioned method, the sites of the protein and iron are determined. As indicated in Figs. 1 and 2, in the case where the pH is lowered to 2 and then elevated to 8.2, though a small quantity of iron is detected moving to the positive pole till the fraction of the γ-globulin, most of iron is found to be remaining near the starting line. When the lowering of pH is stopped near 3, the quantity of iron moving to the positive pole increased but most of the iron is not moving the same as that in the former case.

In the sample whose pH was elevated directly to 8.2 by adding Na_2CO_3 solution without prior liquefaction by adding HCl, most of iron was detected at the site of β-globulin fraction showing the movement to the positive pole with protein. An elevation of pH of media higher than 8.2 resulted in the retardation of iron at the starting line, while serum protein was found to be extended, showing almost the normal pattern. These results show that the liquefying of iron albuminate in acidic media results in the separation of iron and the protein, this can be seen also in alkaline media above pH 9.0. The tests on rabbit and human sera in the range of pH 5.4 to pH 8.6 gave almost the same results as that of bovine serum though in these cases some quantity of iron was detected in the fraction of α-globulin and albumin (Fig. 3 a, b,). The absorption test of the solution prepared in the range of pH 5.4 to 8.2 by Beckman DK proved that it had no specific absorption maximum for the formation of new organic compound only leaving the curve of serum protein (Fig. 4).

The test on the separation of ferric iron of this solution in test tube proved that the Berlin blue reaction became positive when the pH of the media was lowered than 5.4. This was almost the same as that of ferric chloride solution (Table I a, b). All those tests that have been performed on the material frozen and dried and kept more than one year at 5°C gave entirely the same results. This indicates that the solution can be kept in a dehydrated state for a long period of time without changing its chemical properties.

COMMENTS

From the results described above it is supposed that the iron can be bound with serum protein in a certain manner, when ferric iron is added in the media of pH 5.4 to 8.3, forming a solution of reddish brown color. But the absorption spectrum of this solution proved to have no specific absorption showing no firm combination between iron and protein. This

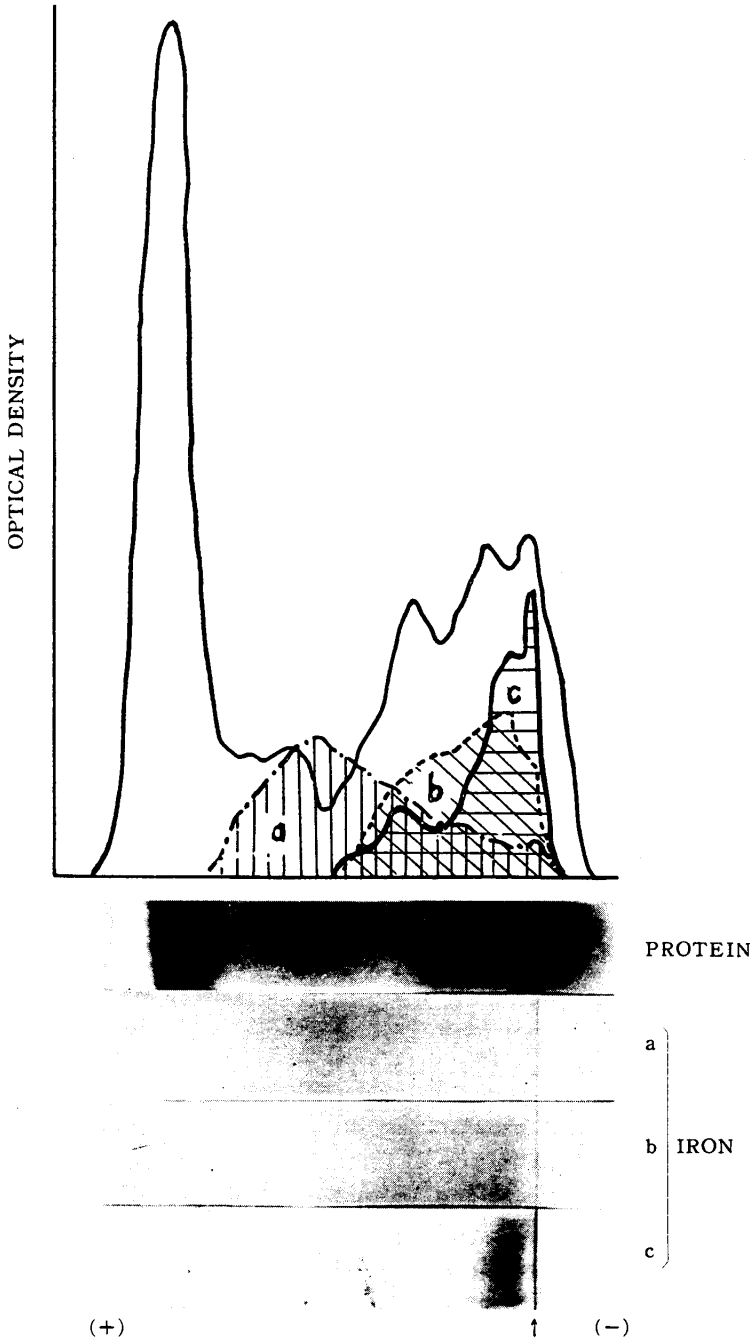

Fig. 1. Pictures of paperelectrochromatography of serum iron colloid (S. I. C.) at 110V for 8hrs. in veronalbuffer ($\mu = 0.1$), pH 8.6. Change in distribution of iron in the serum protein fraction can be seen by changing the pH of media from a) 5.6 to 8.2, b) 3.0 to 8.2, and c) 2.2 to 8.2 during the preparation.

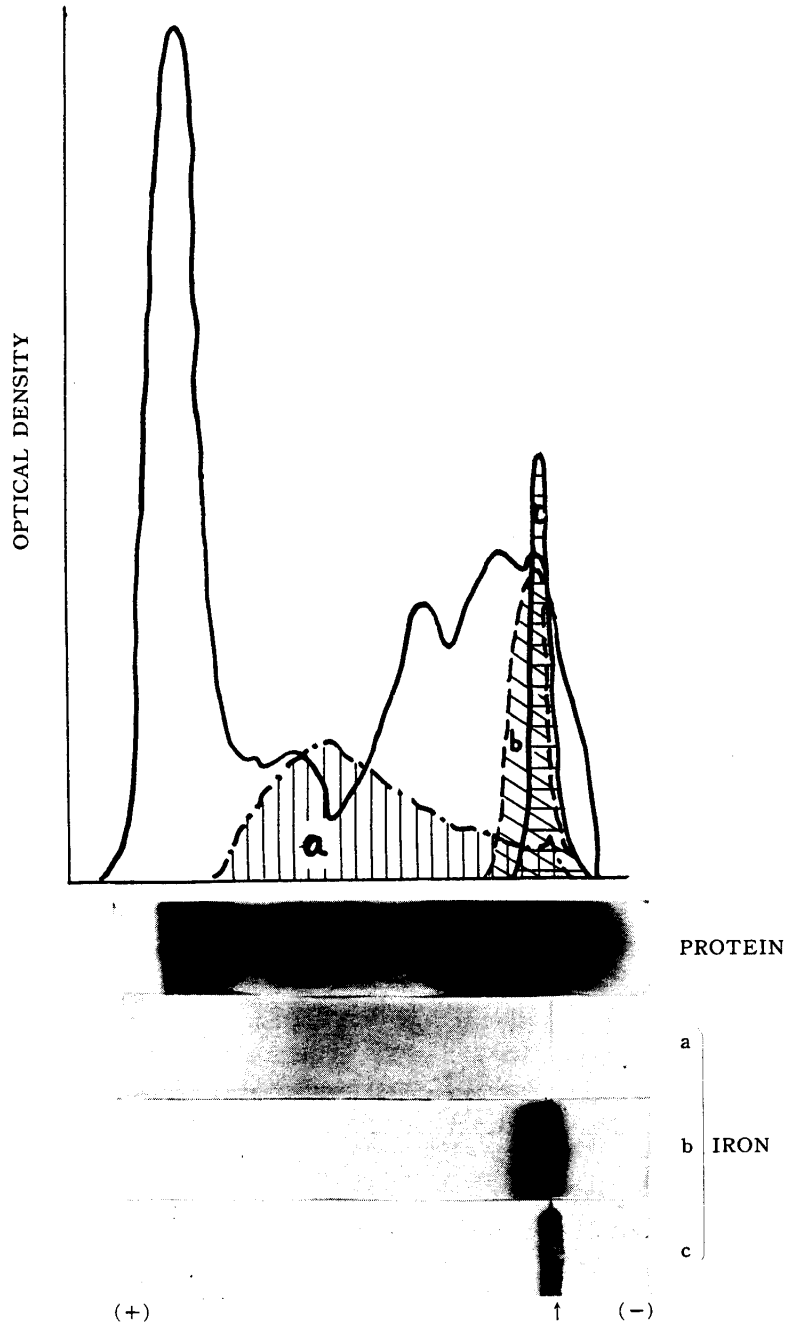

Fig. 2. Pictures of paperelectrochromatography of S.I.C. at 110V, for 8hrs. in veronalbuffer ($\mu = 0.1$) pH 8.6. Change in distribution of iron in the serum protein fraction can be seen by changing the pH of media from a) 5.6 to 8.2, b) 5.6 to 9.0 and c) 5.6 to 9.5 during the preparation.

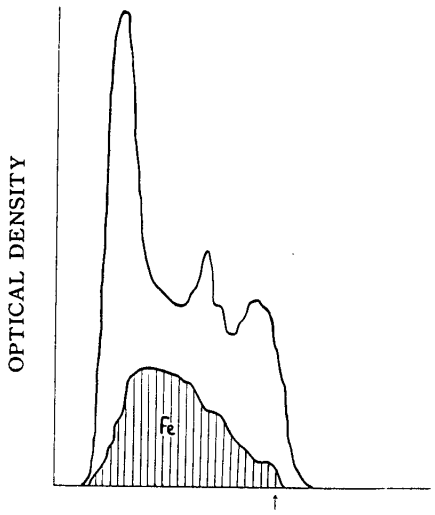

Fig. 3 a. Distribution curves of protein and iron in S. I. C. (rabbit serum iron colloid) obtained from a paperelectrochromatography extended at 150V for 4 hrs. in veronal-veronal-Na buffer of pH 8.6. The picture indicate that iron is mainly found in the fraction of α and β-globulin.

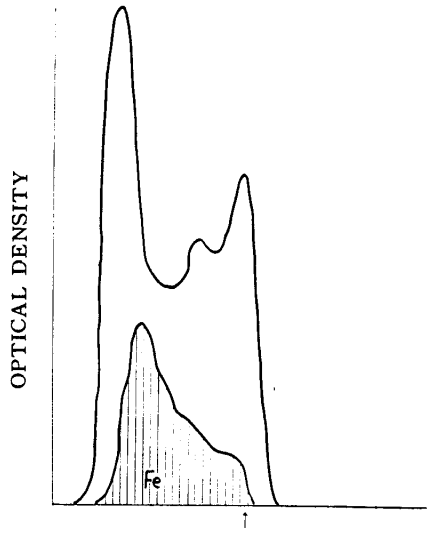

Fig. 3 b. This shows the curves of S. I. C. prepared with human serum and drown nnder the same condition as in Fig. 3 a.

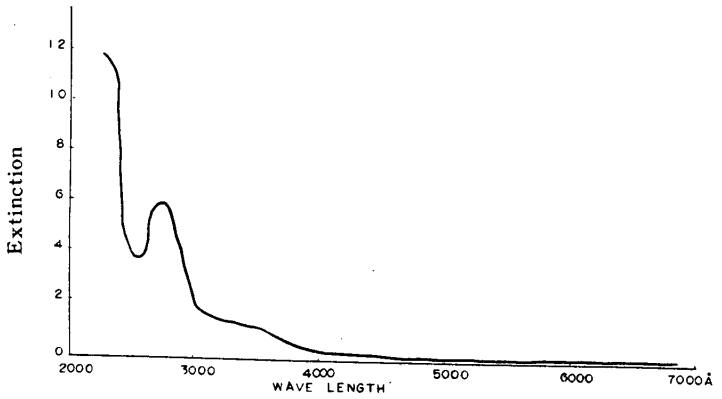

Fig. 4. Absorption curve of serum iron colloid prepared by using human serum.

suggests the formation of iron hydroxide colloid which can be kept in a fairly stable solution by the action of serum protein as the protective colloid. Paper electrochromatography proved the albumin or globulin molecules are mainly concerned with the stability of this colloid solution. The lowering of the pH of media below 5.4 seems to destroy this colloid system

Table 1 a. Range of pH of Fe^{+++}-Separation of S. I. C. prepared with Human Serum.

No. of reagent glass tubes	1	2	3	4	5	6
Serum colloid Fe (drops) in 10cc dist. water	3	3	3	3	3	3
1/10 N. HCl drops	0	1/20 N. 1	1	2	3	8
pH (colorimetric)	6.4	5.6	5.4	5.1	4.8	3.5
Berlin-blue reaction immediately	−	−	±	+	++	+++
Berlin-blue reaction after 15 minutes	−	−	+	++	+++	+++

Table 1 b. Range of pH of Fe^{+++}-Separation of S. I. C. prepared with Bovine Serum.

No. of reagent glass tubes	1	2	3	4	5	6
Serum colloid-Fe (drops) in 10cc dist. water	3	3	3	3	3	3
1/10 N. HCl (drops)	0	1	2	3	5	8
pH (colorimetric)	6.4	5.6	5.4	5.1	4.8	3.8
Berlin-blue Reaction immediately	−	−	±	+	++	+++
Berlin-blue Reaction after 15 minutes	−	−	+	++	+++	+++

separating the iron as ferric iron though this will be reversible reaction in a fairly high range of pH, a little above 5.0. This fact may mean the formation of some loose-binding between iron colloid and serum protein like absorption. The color changing to red in an alkaline medium suggests the growth of the colloid particles to larger ones[11]. At present the binding mode between iron and protein is still obscure, but it is reasonable to consider that such formation will be formed *in vivo* when a large quantity of iron introduced orally as pointed out previously where iron invaded into organism by physicochemical diffusion without passing the normal physiological absorption process. As it is proved this compound is effective for the therapy of anemic patients, there is a possibility that iron in such a compound as described here is capable of being utilized as a good iron source for hemoglobin synthesis when it is introduced directly into vein. But prior to the clinical application precise and careful observations on the biological properties of this new iron compound need to be made because the intravenous injection of iron is always accompanied by side-effects of a greater or less extent such as heavy metal intoxication or colloidal shock, as has been reported by many predecessors.

SUMMARY

By mixing ferric iron with serum protein, 20 cc of serum and 1 cc of ferric chloride or ferric ammonium sulfate (10 mg ferric iron/cc in each), in the range of pH 5.4 to 8.2, a transparent brownish red colored solution can be obtained. Paperelectrochromatography proved the iron can mainly be detected in β-globulin fraction in bovine serum and in α-globulin and albumin fractions in human and rabbit sera. But the absorption spectrum proved that there is no formation of any new compound, giving almost the same absorption curve as in the serum protein itself. And by lowering the pH of media below 5.4, the solution gives immediately the positive reaction of ferric iron. From these rerults it is suggested that iron will be maintained in a colloidal state keeping the stability of this state in the presence of protein molecules. Freezing and drying are the procedures quite useful for keeping this material for a long period of time without changing the chemical characteristics.

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