J. Fac. Fish. Anim. Husb.,
Hiroshima Univ. (1976), **15** : 151 ~ 160

Inhibitory Effect of Ascorbic Acid on the Hydrolytic Polymerization of Iron and a Consideration of the Effect in the Intestinal Absorption of Iron

Tsuneaki IMAMURA, Chitoshi HATANAKA and Kokichi TAKAHASHI

Department of Food Chemistry and Technology, Faculty of Fisheries and Animal Husbandry, Hiroshima University, Fukuyama

Recived September 6, 1976.

(Figs. 1 – 9)

We reported previously the preparation of Fe(III)-sugar complexes[1] and the partial purification of the complexes by gel-filtration chromatography.[2] These experiments were undertaken to apply these complexes to the development of some new food additives as Fe-fortifizer. As for the nutritional significance of Fe-sugar complexes, many workers have been studied. CHARLEY *et al.*[3] claimed that the rate of the intestinal absorption of an Fe(III)-fructose complex was higher than those of inorganic Fe-salts. However, we could not observe such an effect of fructose on the intestinal absorption of ferric ions.[1] Since ferrous ions have long been believed to be absorbed more easily than ferric ions through the intestinal membrane, their ionic states, i. e., the divalent and the trivalent states, may affect their intestinal absorptions. In the meantime, ferric ions are known to polymerize easily in neutral or alkaline media and to give an Fe(III)-hydroxo polymer complex of high molecular weight. It is quite reasonable, therefore, that the lower absorption of ferric ions as compared to that of ferrous ions due mainly to their great tendency toward hydrolytic polymerization.

In view of such properties of ferric ions, the inhibitory effects of glucose, fructose, sorbitol, and ascorbic acid (AsA) on the hydrolytic polymerization of Fe have been examined in this work. Among them AsA was the most effective agent to prevent the hydrolytic polymerization.

MATERIALS AND METHODS

1. Reagents

All the reagents used were of guaranteed grade and the water used for dissolving them was distilled-deionized water.

2. Analytical methods

Absorption spectra of Fe-compounds were measured with a Hitachi type 124 spectrophotometer.

The amount of ionic Fe (E_i) was determined by the *o*-phenanthroline method.[4] The total amount of Fe (E_t) was determined by the same method after disrupting polymerized Fe by heating the acidified sample solutions.

3. Calculation of the polymerization degree of Fe

The degree of the polymerization of Fe was given by the following equation.[5]

$$\text{The polymerization degree (\%)} = \left(1 - \frac{\text{Ei}}{\text{Et}}\right) \times 100$$

4. Paper electrophoresis

Paper electrophoresis of Fe-complexes was carried out with Toyo Roshi No. 50 paper strips (2 X 40 cm) in one of the following three buffer solutions, i. e., HCl-KCl (pH 2.0, $\mu = 0.1$), Tris-HCl (pH 7.5, $\mu = 0.1$), and $NaHCO_3$-Na_2CO_3 (pH 11.2, $\mu = 0.1$), and a voltage of 500 V was maintained. After electrophoresis for 2 hours at 20°C, the band of Fe was located by spraying with 5 % potassium ferrocyanide solution.

RESULTS

1. Hydrolytic polymerization of Fe in acidic media

Changes of the absorption spectrum of a 2 X 10^{-4} M $FeCl_3$ solution of pH 3.8 were examined in relation to time. As shown in Fig. 1, the spectrum of the fresh sample, immediately after preparation, showed a distinct absorption maximum at 295 nm. However, the absorption maximum disappeared rapidly and, within 20 minutes, the absorption curve came to indicate one of the "general absorption curves" which do not have any absorption maximum. The disappearance of the absorption maximum indicates the occurrence of the hydrolytic polymerization of Fe. The polymerization degree of Fe depends on the pH value of the medium, and the molecular weights of the resulting Fe-polymers vary over the range from 1,500 to 200,000. When the Fe-polymers with large molecular weights are formed in the small intestine, their transports through the intestinal membrane would become difficult. Oral-administrated Fe-salts pass through the esophagus into the stomach, where the

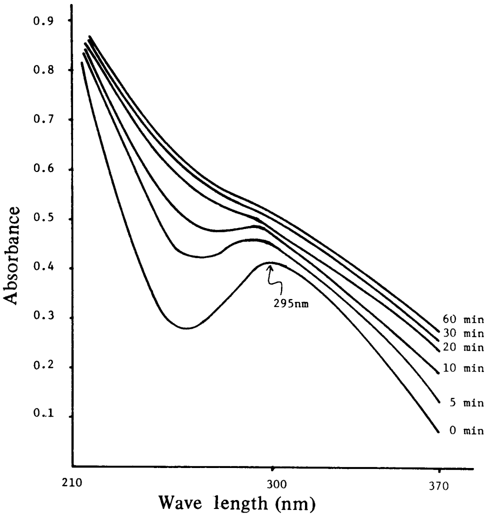


Fig. 1. Changes of the absorption spectrum of $2X10^{-4}M$ $FeCl_3$ (pH 3.8) with time.

strongly acidic gastric juice is secreted, and then they are sent to the small intestine. The intestinal juice is usually maintained fairly constant at a slightly acidic or a nearly neutral pH value. Such changes in the pH of the surrounding media during the passage of Fe-ions through the gastrointestinal tract may markedly affect the intestinal absorption of the Fe-ions. Accordingly, the effect of pH on the absorption spectra of Fe-ions was examined. The result is illustrated in Fig. 2. Aliquots of a $FeCl_3$ solution were brought to pH 2.06, 2.14, 2.20, 2.33, 2.40, 2.50, 2.64 2.85, and 4.55, and the final concentrations of the Fe-ion in the aliquots were adjusted to 1 X 10^{-2} M. Measurement of the absorption spectra was made immediately after preparation of the sample solutions.

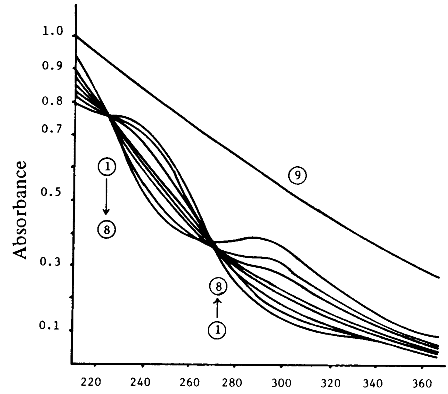


Fig. 2. Absorption curves of $FeCl_3$ solutions with different pH values.
Number in circles represents the pH value of individual system, i.e., ① ; 2.06, ② : 2.14, ③ : 2.20, ④ : 2.33, ⑤ : 2.40, ⑥ : 2.50, ⑦ : 2.64, ⑧ : 2.85, and ⑨ : 4.55.

The most acidic solution (pH 2.06) gave the absorption curve with one maximum peak at 240 nm. This peak, however, disappeared with increasing the pH and another peak appeared at 300 nm. The eight absorption curves given by the solutions with pH values below 2.85 made two isosbestic points, the first at 222 nm and the second at 270 nm. These two points suggest that two types of the Fe-ions exist in the solutions. When the pH of the solution increased to 4.55, the absorption curve was found to show one of the general absorption curves.

2. Effects of glucose, fructose, sorbitol, and AsA on the hydrolytic polymerization of Fe

The hydrolytic polymerization of Fe has been known to depress in the presence of sugars, sugar alcohols, hydroxycarboxylic acids, and AsA.5)-8) SHADE *et al.*9) demonstrated that the acids in the stomach appeared to facilitate the chelation of ferric ions with AsA.

Accordingly, the inhibitory effect of AsA on the hydrolytic polymerization of Fe was examined and was compared with those of glucose, fructose, and sorbitol. Each agent was added to a 2 X 10^{-2} M $FeCl_3$ solution in the molar ratio of Fe : agent as 1 : 20. After adjusting the pH to the desired value, the solution was kept standing at room temper-

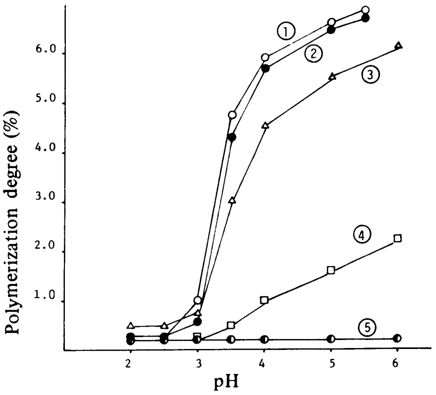


Fig. 3. Inhibitory effect of AsA on the hydrolytic polymerization of Fe(III).

① : $FeCl_3$, ② : $FeCl_3$+ Glucose, ③ : $FeCl_3$+ Fructose, ④ : $FeCl_3$+Sorbitol, ⑤: $FeCl_3$+AsA.

ature for 5 minutes, and the polymerization degree of the Fe was determined. The results are illustrated in Fig. 3. In the case of $FeCl_3$ alone, the polymerization degree was small in the pH range of 2 to 3. However, the degree increased rapidly above pH 3 and, over pH 4, gradually leveled off. The addition of glucose or fructose gave a slight inhibition, while that of sorbitol resulted in a considerable inhibition and the polymerization was depressed to about one-third. Among the agents examined, AsA was most effective.

3. Formation of the Fe-AsA complex

Examination of the absorption spectrum of the sample solution containing a metal and a ligand is usually applied to identify the formation of the metal chelate compound. When AsA is used as ligand, however, this way can not be applied to the identification of the chelate compound because the absorption maximum of AsA overlaps with that of an Fe-ion. According to LAWENDEL,10) the absorption maximum of AsA, $[AsA]^-$, and $[AsA]^{2-}$ are at 243, 265, and 300 nm, respectively. Accordingly, we tried other experiments for identifying the Fe-AsA complex.

First, an appropriate amount of AsA was added to a 1 X 10^{-2} M $FeCl_3$ solution and the observation was made whether the lowering of the pH of the solution would occur or not: The lowering of pH is an indication of the formation of a metal chelate compound because a chelation is generally accompanied by the protonation of at least one H-ion. As shown in Fig. 4, the pH of the $FeCl_3$ solution was lowered from 7.5 to 5.3 by the addition of AsA during the first 90 minutes.

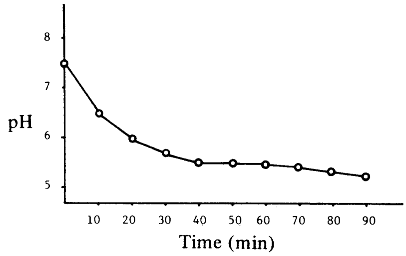


Fig. 4. Lowering of the pH of a $FeCl_3$ solution by adding AsA.

Then, the effect of AsA on the formation of insoluble Fe-polymers was examined. Ferric ions are known to be polymerized easily in aqueous media, especially in neutral or alkaline media, and a part of the resulted Fe-polymers precipitates as hydrous ferric oxide colloids. The rest of the Fe-polymers can be mostly separated by centrifugation at 30,000 x g for 60 minutes. On the other hand, such Fe-polymers are often solubilyzed by chelation with suitable ligands. Accordingly, the solutions which contained $FeCl_3$ and AsA in various ratios were prepared and were kept for 24 hours at room temperature. After centrifuging the solution at 30,000 x g for 60 minutes, the amount of Fe in the supernatant was determined and expressed as %. The amounts of the soluble Fe in the solutions at the various ratios of Fe/AsA were 0 % at 0.1, 69 % at 0.2, 78 % at 0.3, 82 % at 0.4, 82 % at 0.5, and 85 % at 1.0, respectively.

These results indicate that an Fe-AsA complex was formed by mixing the two solutions of $FeCl_3$ and AsA.

Solubility of an Fe-polymer is produced by the surface charge due to polar ligands surrounding the Fe atom. The type of the binding between the ligand and the Fe atom is a coordination bond. When a metal and a ligand constitute a chelate compound, they behave together in solution as one group. Accordingly, both chemical species migrate together and are detected at the same position on the paper electrophoretogram. Fig. 5 is the paper electrophoretograms obtained with • Fe-AsA mixtures at different pH values. Considering the difference between the charges in acidic and in alkaline media, the electrophoreses were carried out at pH 2.0, 7.5 and 11.2. A band of Fe was detected toward the side of the cathode at pH 2.0 and toward the reverse side at pH 7.5 and 11.2. Such differences may be explained on the basis of the different dissociations of AsA in acidic and in alkaline media.

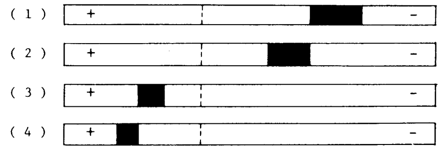


Fig. 5. Paper electrophoretograms of $FeCl_3$ and $FeCl_3$-AsA solutions

▬ : Spot of iron. (1) 10^{-2}M $FeCl_3$system (pH 2.0), (2) 10^{-2}M $FeCl_3$–10^{-2}M AsA system (pH 2.0), (3) 10^{-2}M $FeCl_3$–10^{-2}M AsA system (pH 7.5), (4) 10^{-2}M $FeCl_3$–10^{-2}M AsA system (pH 11.2).

DISCUSSION

1. Hydrolytic polymerization of Fe in acidic media

Ferric salts in aqueous media, especially in alkaline media, are known to dissociate as the following three stages:

$$Fe^{3+} + H_2O \rightleftharpoons [Fe(OH)]^{2+} + H^+ \quad \ldots\ldots (1)$$

$$[Fe(OH)]^{2+} + H_2O \rightleftharpoons [Fe(OH)_2]^+ + H^+ \quad \ldots\ldots (2)$$

$$[Fe(OH)_2]^+ + H_2O \rightleftharpoons [Fe(OH)_3]\downarrow + H^+ \quad \ldots\ldots (3)$$

According to Lamb *et al.*,[11] when the concentration of $FeCl_3$ is below 1 X 10^{-3} N, the dissociation of the salt reaches to the stage 2 as soon as the salt is dissolved. While, in the case of a 1 X 10^{-2}N $FeCl_3$ solution, the dissociation is maintained in the equilibrium of the stage 2 for about 50 minutes and then gradually proceeds to the stage 3. As shown in Fig. 1, the absorption spectrum of a 2 X 10^{-4}M $FeCl_3$ solution, immediately after preparation, showed an absorption maximum at 295 nm; this maximum peak suggests the existence of Fe-hydroxo ions in the solution. On the other hand, in the case of a 2 X 10^{-2} M $FeCl_3$ solution, the specific maximum peak by free Fe-ions was observed at 245 nm (Fig. 2). The peak at 245 nm was observed on the solutions

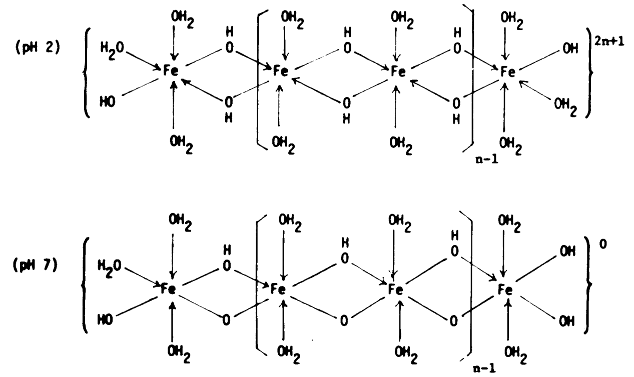


Fig. 6. Models for the structure of an iron polymer by Fujita.

of which pH values were below 2.20, while another peak at 300 nm was observed on the solutions with pH values above 2.64. When the pH of the solution was 4.55, both peaks disappeared and the absorption curve became one of the general absorption curves, by which the formation of Fe-polymers is suggested. Thus, the hydrolytic polymerization of Fe was identified in acidic media. A model for the structures of Fe-polymers was proposed by FUJITA[12] (Fig. 6).

As illustrated in Fig. 2, the eight absorption curves given by the solutions with pH values from 2.06 to 2.64 made two isosbestic points. According to SIDDAL and VOSBURG,[13] these isosbestic points suggest the existence of two types of Fe-ions in the solutions, i. e., Fe^{3+} and $[Fe(OH)]^{2+}$ ions. The types of Fe-hydroxo ions in aqueous media change with the pH of the media. Fig. 7, reported by PARK and BRUYN,[14] shows the distribution of each type of the Fe-ions at various pH.

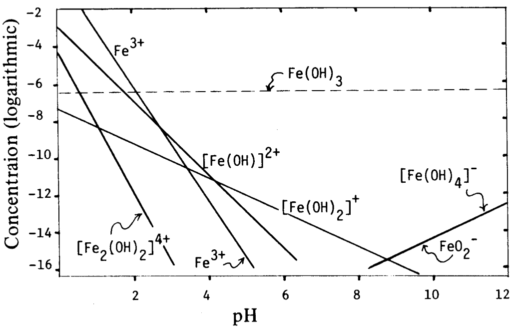


Fig. 7. Distribution of Fe-hydroxo ions in aqueous media with various pH values demonstrated by Park & Bruyn.

The above facts that the distribution of Fe-ions changes with the pH of the media and that the hydrolytic polymerization of Fe occurs even in acidic media are very important in view of the intestinal absorption of Fe, because the molecular weights of Fe-polymers are known to vary over the range from 1,500 to 200,000 and such high Fe-polymers would not be easily absorbed through the intestinal membrane.

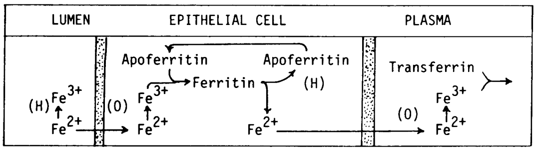
2. Inhibition of the hydrolytic polymerization of Fe by AsA and the significance of AsA in the intestinal absorption of Fe

Nutritional chemists have long been believed that ferrous salts are more easily absorbed than ferric ions in the small intestines. The reason for this belief was based on the following observations, i. e., ferrous ions can exist in aqueous media up to approximately 1 M at pH 7, while ferric ions are insoluble at a concentration higher than 1 X 10^{-7} M at the same pH of 7.[6] However, the hydrolytic polymerization of Fe occurs even in acidic media as shown in Figs. 1 and 2, and the molecular weights of the resulted polymers are probably too high to be absorbed through the intestinal membrane. For example, SPIRO *et al.*[15] obtained a preparation of an Fe-polymer with an average diameter of 7 nm from a $Fe(NO_3)_3$ solution at pH 2.5. FUJITA[12] also observed the formation of Fe(III)-polymers, 3 – 5 nm in width and 10 – 15 nm in length, in a $FeCl_3$ solution at pH 2 – 2.5. These observations suggest that another explanation is required for the understanding of the transport mechamism of Fe through the intestinal membrane.

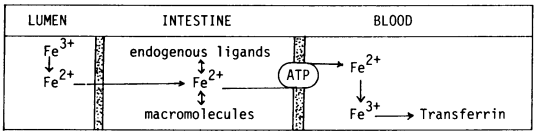
SALTMAN[16] summarized a number of the literature on the intestinal absorption of Fe and classified the transport mechanisms into three types, i. e., (a) the mucosal block mechanism, (b) the active transport mechanism, and (c) the passive transport mechanism. These types of mechanisms are illustrated in Fig. 8. The third theory was proposed by

Saltman, in which Fe-chelates play an important role in the Fe-transport.

a) Mucosal Block Mechanism



b) Active Transport Mechanism



c) Chelation Mechanism

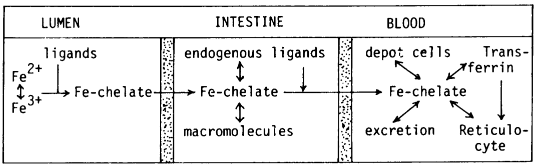


Fig. 8. Three models for the Fe-absorption proposed by Saltman.

According to Shade *et al.*,[9] the accelerating effect of AsA on the Fe-absorption is due to the chelating activity, by which, even in neutral media, the solubility of ferric ions is maintained. On the other hand, Terato *et al.*[6] said that the most important difference between the properties of ferrous and ferric ions was not their solubilities, but the degrees of the hydrolytic polymerization, and that the addition of carbohydrates brought about a considerable inhibition of the hydrolytic polymerization of Fe and resulted in the lowering of the molecular weights of Fe-polymers. Then, they explained the mechanism of the Fe-absorption as follows. (1) Fe, administrated or fed, is solubilized in the stomach and will bind with organic acids, amino acids, carbohydrates, or other exogenous ligands. (2) Fe will be hydrolyzed and polymerized in the doudenum and in the jejunum by increasing the pH of the medium, and then will be transformed into the high molecular weight polymers or the precipitates. The Fe bound with the ligands will be sustained in a soluble and permeable form. (3) Fe-chelates and low molecular weight Fe-polymers will be transferred from the intestinal lumen into the peripheral blood stream mainly in their original forms, which are largely kept unchanged in the intestinal cells. (4) The Fe-transport from the mucosa to the serosa depends on its concentration in the mucosal medium; the concentration being under the regulation by some saturation phenomena in the intestinal cells. (5) The Fe-transport through the intestinal membrane depends on the nature of Fe, especially on its molecular weight, while its valence is not an essential factor.

3. Formation of an Fe-AsA complex and its significance in the intestinal absorption of Fe

The addition of AsA to a $FeCl_3$ solution resulted in a considerable decrease of the pH of the solution (Fig. 4) and in an increase of the amount of the Fe in the supernatant; the supernatant was obtained by centrifuging the solution. These results indicate the formation of an Fe-AsA complex. The chelation of Fe with AsA inhibits the formation of insoluble Fe-polymers and makes them simultaneously to low molecular weight polymers. As a result, such polymers of low molecular weight become capable of being absorbed through the intestinal membrane. Terato *et al.*[5] examined the contents of an Fe(III)-citric acid system by gel-filtration chromatography and observed the existence of a high molecular weight fraction of 10 – 15 %. On the basis of the molecular shieve effects of Biogel P-6 and P-2, the molecular weight of the high molecular substance was assumed to be 1,500. In a similar manner, they obtained the value of 2,000 – 3,000 for the molecular weight of the high molecular substance separated from an Fe(III)-gluconic acid system. In the previous paper,[2] we also presumed that the high molecular substance obtained from an Fe(III)-lactose system had a molecular weight of about 5,000. From these results, it may be presumed that the Fe-polymer obtained from the Fe(III)-AsA system in this experiment has a molecular weight of about 5,000.

SPIRO *et al.*[15] demonstrated a model for the structure of a polymerized Fe(III)-sugar complex (Fig. 9). The model, in which the sugars coat the surface of the Fe-hydroxo polymer, is likely to be useful for explaining both the structure of the Fe-AsA complex and the distribution of its surface charge. The paper electrophoretograms of the Fe-AsA complex revealed that the surface charge of the complex was negative in alkaline or neutral media and was positive in acidic media. According to FUJITA,[17] certain ions are able to form a surface complex on the surface of a solid and constitute the surface charge of the solid. For example, citrate ions give a negative charge to a hydrous ferric oxide colloid through forming a surface complex on the surface of the hydrous ferric oxide colloid. Therefore, the difference in charge of the Fe-AsA complex in acidic or alkaline media is probably explained on the basis of the above concept. That is, AsA ions form a surface complex of negative charges in neutral or alkaline media. The effect of AsA on the acceleration of the Fe-absorption through the intestinal membrane depends mainly on its ability to prevent both the formation of Fe-polymers with high molecular weights and the precipitation of hydrous ferric oxide colloids. The detailed account of the nutritional significance of the Fe-AsA complex will be published elsewhere.

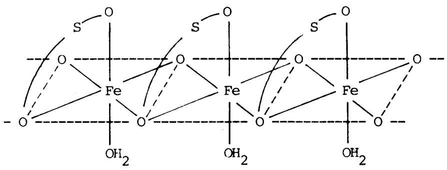


Fig. 9. A model for the structure of Fe-sugar complex proposed by Spiro *et al.*

S represents sugar coodinated to Fe.

SUMMARY

The Fe-sugar complexes prepared so far in this laboratory were found to be difficult in their intestinal absorptions. For the reason we considered the formation of high molecular weight Fe-polymers under the experimental conditions. Therefore, the inhibitory effects of glucose, fructose, sorbitol, and ascorbic acid (AsA) on the hydrolytic polymerization of Fe were examined in this work.

As the Fe-salt, $FeCl_3$ was used in the concentrations of $1 \times 10^{-2} - 1 \times 10^{-4}$M. The amounts of Fe were determined by the *o*-phenanthroline method and the polymerization degree of Fe was obtained from the difference between the amounts of the total

Fe and the ionic Fe in the sample solutions.

On the basis of the change in the absorption spectra of the $FeCl_3$ solutions at various pH values, we concluded that the hydrolytic polymerization of Fe occurs rapidly even in acidic media, especially in aqueous media of above pH 4.5. The polymerization of Fe was markedly inhibited by AsA. Sorbitol also showed a considerable inhibition and its addition to the $FeCl_3$ solution lowered the polymerization to about one-third. However, almost no effect was observed by the addition of glucose or fructose. When AsA was added to the $FeCl_3$ solution, an Fe-AsA complex was formed. The paper electrophoretograms of the Fe-AsA complex, of which the molecular weight was rather small and presumed to be about 5,000, revealed that the charge of the complex was negative in alkaline or neutral media and was positive in acidic media. The electric charge of the complex is considered to be produced by formation of the surface complex of AsA on the surface of Fe atoms, which constitute generally a polynuclear Fe-hydroxo complex in aqueous media; AsA probably coordinates to the Fe atoms just like a cover that coats the polynuclear polymer. The changes of the dissociation of the coated AsA with pH values of the media produce the negative or the positive charges.

Some nutritional considerations of the intestinal absorption of Fe were given in connection with the inhibitory effect of AsA on the hydrolytic polymerization of Fe.

ACKNOWLEDGEMENT

The material presented here has been taken from the thesis of Kokichi TAKAHASHI, submitted in partial fulfillment of the requirements for the degree of Master of Agriculture in the Graduate School of Hiroshima University. We are indebted to Dr. K. TERATO of the Eizai Research Laboratories for kindly providing us with a number of the literature on the Fe-complexes.

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アスコルビン酸による鉄の加水分解重合阻止とその鉄吸収に与える効果の考察

今村経明・畑中千歳・高橋厚吉

著者らはこれまで，鉄ー糖錯体の調製について報告してきた。その目的は新しい鉄強化剤の開発にあるが，得られた標品の腸管壁透過性は，諸外国の報文に反して，予想外に悪かった。この原因は，鉄の加水分解重合による高分子量多核錯体の形成にあると考え，若干の糖および糖誘導体によるポリマー化阻止を調べた。その結果，アスコルビン酸(AsA)が著しい阻止効果をもつことを確かめ，このことが鉄の腸管吸収に与える影響について考察した。

鉄は希薄 (10^{-2} ~ 10^{-4}M) な$FeCl_3$溶液として用い，吸収スペクトルの変化によってポリマー生成の定性的な確認をし，全鉄量とイオン性鉄量の測定値の差からポリマー化程度を調べた。このとき必要な鉄の定量は，*o*-フェナンスロリン法で行なった。

本研究に用いた$FeCl_3$を含めて，第一鉄塩がアルカリ性溶液中で加水分解重合し易いことはよく知られているが，われわれの実験結果は，酸性条件でもかなり速かにポリマー化することを示した (Fig. 2) 。このポリマー化は，等モル比のAsAを加えることによりほぼ完全に，ソルビットによって⅓程度に阻止されたが，グルコースとフルクトースにこの効果はなかった (Fig. 3) 。AsAは鉄と錯体を形成するが (Fig. 4) ，沪紙電気泳動の結果から，このものは，溶液のpHによって正もしくは負に荷電していることが分った (Fig. 5) 。この表面荷電に関する考察に付随して，多核鉄ポリマーの鉄にAsAが配位して表面錯体を形成している構造を推察した。

以上の結果，すなわち鉄ポリマーの低分子量化と荷電が，鉄の腸管腸収に良い影響を与えると考えた。