Purification of Iron(III)-Lactose Complex
by Gel-Chromatography

Tsuneaki IMAMURA, Chitoshi HATANAKA and Hidekazu KAWAMOTO

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

(Figs. 1−9)

The adult human body is supposed to have about 4.5 g of iron (Fe) as one of the constituent elements of porphyrins, such as hemoglobin, ferritin, myoglobin and cytochrome. An average new-born baby possesses only 0.3 g of Fe immediately after parturition and yet needs three times as much Fe as an adult during the first two years after birth. Human milk contains a sufficient amount of Fe (about 0.15 mg %) to answer this demand, whereas cow's milk provides only one-third (0.06 mg %) of the amount required.[1] Modified milk powder is manufactured in which the different nutrients are added in a quantity appropriate for three-month-old babies. Thus, Fe of 4 ~ 7 mg % is added to the milk powder in a salt form of Fe-citrate or Fe-lactate. The effective use of both these salts, however, has not been accounted for nutritionally. There is a possibility that more desirable Fe-salts may be found. Fe-sugar complexes perhaps may be the desirable ones as they have already been studied for therapeutic use in anemia. In this line of research the purification of the Fe-lactose complex was examined and the nutritional significance of the complex are discussed in this paper.

In 1959, BERSIN and SCHWARZ[2] examined the electrophoretic migration of three types of Fe(III)-sugar complexes and the dissociation rates of Fe(III) ions in acidic solutions. The first paper, dealing with the role of sugar complexes in the transport of Fe through the intestinal wall of a rabit, was published by SALTMAN and CHARLEY (1962).[3] They examined various metal-sugar complexes and concluded that all the complexes were stable in aqueous solutions over a wide range of pH, dialyzable through a visking sac, and nontoxic. In addition, they came to the confirmation that the rate of the intestinal absorption of Fe was higher in the case of Fe^{59}- fructose complexes than in case of inorganic Fe-salts. CHARLEY *et al.*[4][5] proposed the passive transport mechanism of Fe-chelates through the intestinal wall on the basis of the above mentioned facts. SALTMAN[6] also reported on the role of chelates in the Fe-metabolism *in vivo*.

In 1964, other researchers reported on the intestinal absorption of the Fe (III)-fructose complex in rats, on the stability of the complex in aqueous solutions with various pH values, and on the NMR.   HOPPING *et al.*[7] performed a similar examination of other Fe-chelates and said that the intestinal absorption of Fe was accerelated by chelating with citric acid.   Moreover, in 1969, the fact that gastoferrin was composed mainly of sugars and their derivatives,[8] and the results of an electron microscopic study on the structure of an Fe(III)-dextran were reported.   Recently, TERATO *et al.*[9] have published a noteworthy paper on the relation between molecular weight of Fe(III)-polymers and the intestinal absorption of Fe.

MATERIALS AND METHODS

1.   Reagents

A commercial lactose preparation was used after removal of nitrogen compounds in the same manner as described in the previous paper.[10]   The other reagents used were of a guaranteed grade and the water used for dissolving the reagents was distilled-
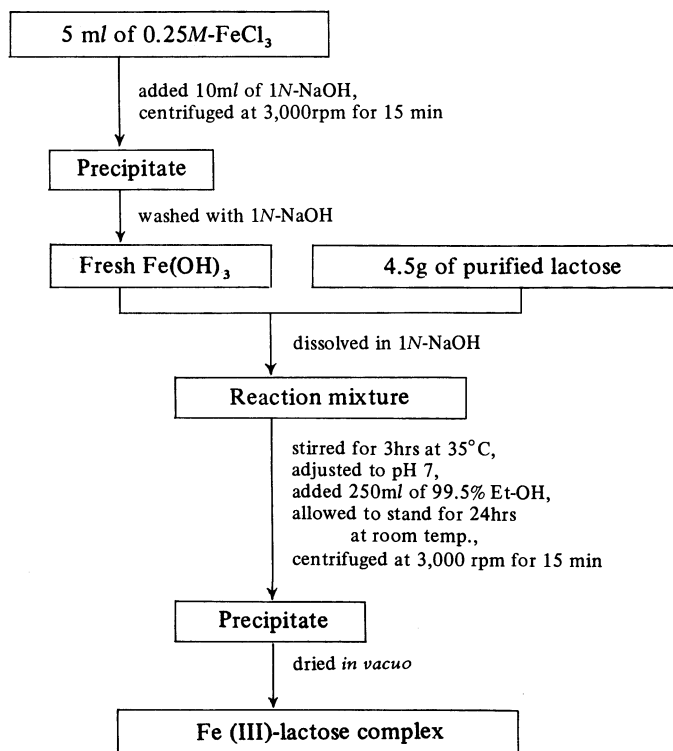

Fig.1.   Preparation of Fe-lactose complex

deionized water.

2.   Preparation of Fe(III)-lactose complex

The Fe(III)-lactose complex was prepared in the same way as described in the previous paper.[10] An outline of this method is shown in Fig. 1.

3.   Gel-filtration

The gel-filtration of Fe-complexes was performed with a column of Sephadex G-15 (2.5 x .40 cm) or Sephadex G-50 (2.5 x 50 cm). The eluent used was a BRITON-ROBINSON buffer solution ($\mu = 0.1$) and a bicarbonate buffer solution ($\mu = 0.1$, pH 9.5), V_0 was determined with Pharmacia's Blue-dextran as a marker.

4.   Determination of Fe and sugars

According to the previous paper, Fe was determined by an atomic absorption method and the sugars by the phenol-sulfuric acid method.

RESULTS

1.   Effects of pH of eluents

The Fe(III)-sugar complexes in the previous experiment were stable in the regions of pH 5 ~ 9 but decomposed below pH 2. This suggests that the Fe(III)-lactose complex also would decompose if treated with an acidic solution below pH 2. For this reason, the effects of pH on the elution pattern of the Fe(III)-lactose complex were examined with BRITTON-ROBINSON buffer solutions as the eluent. In these experiments, the sample (0.2 g) of the Fe(III)-lactose complex was put on the column of Sephadex G-15 and eluted with buffer solutions of various pH values. The flow rate of 60 ml/hr was maintained and 5 ml-fractions were collected. In Fig. 2, the amounts of Fe in each fraction is illustrated by solid lines and that of sugars by dotted lines. If any decomposition of the Fe-sugar complex did not occur during the gel-filtration, the lines from the two components of the complex should be superposed. The superpositions were observed at V_0 of the chromatograms except for that obtained with the buffer of pH 2. The molar ratios of Fe/sugar of the peaks at V_0 were almost 1 for the eluent of pH 12 and about 0.5 for the eluents of pH 8 and 6.

In this experiment, when the two components of the Fe-lactose complex were dissolved in the molar ratio of Fe : lactose as 1 : 10, peaks of free sugars were observed in all cases as shown in Fig. 2. With acidic eluents, a large peak of free sugars was observed at fraction No. 27 ~ 28. In the case of alkaline eluents, however, the peak became smaller and one or two other small peaks appeared on the side of V_0. The large peak observed at fraction No. 27 ~ 28 coincides with that of a hexose. Hexose is considered to be produced surely from lactose, but there remains one questionable point of whether the fission of the galactoside-bond of lactose occurs under the alkaline conditions or not. In order to elucidate this point, the following experiment was
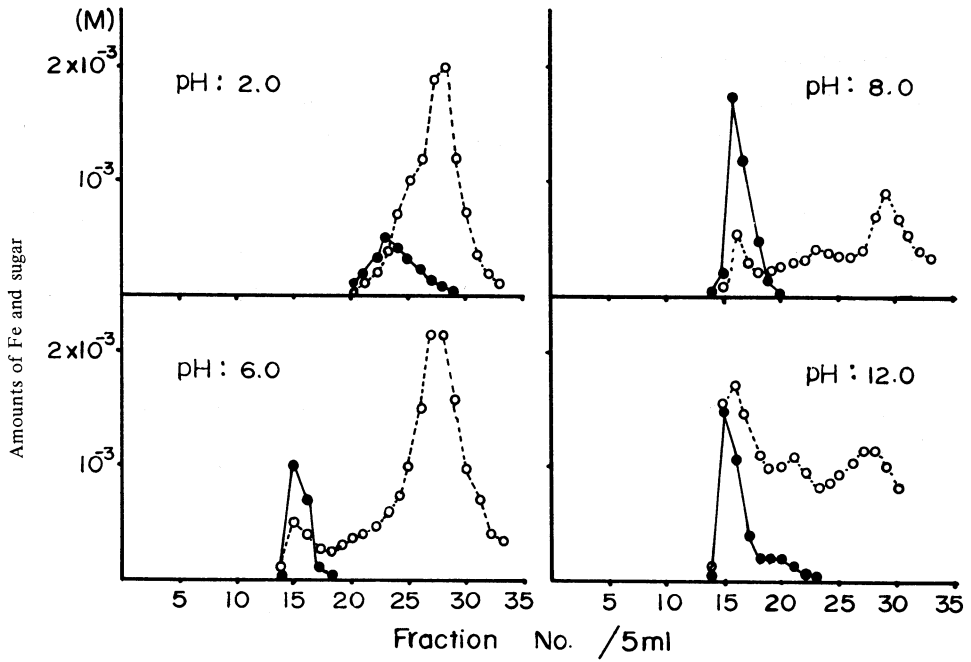
Fig. 2.　Gel-chromatography of Fe(III)-lactose complex with BRITTON-ROBINSON buffer solutions
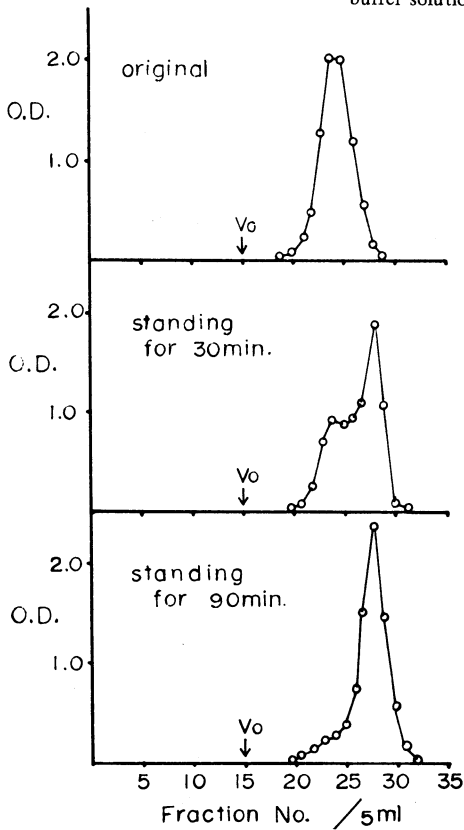

Fig. 3.　Effects of alkaline conditions on the gel-chromatograms of lactose

carried out. A gel-filtration of 0.5 M lactose solution of pH 9.5 was performed under the same conditions as in Fig. 2. The results are shown in Fig. 3. Only one peak of lactose was observed with the fresh solution, but the second peak corresponding to a hexose appeared with the solution after standing for 30 min at 50°C. When the solution was kept for 90 min at 50°C, only the second peak could be seen. Thus, we have evidence that lactose was decomposed and that it produced hexoses during the gel-filtration.

2.   Effects of the components of eluents

As the BRITTON-ROBINSON buffer contains phosphates, the use of this buffer is liable to cause a ligand-replacement between sugars and phosphates. For this reason the bicarbonate buffer which does not have these inconveniences was chosen for the eluent. The result obtained with a bicarbonate buffer solution (μ = 0.1, pH 9.5) is shown in Fig. 4. The conditions of the gel-filtration were the same as those in Fig. 2 and 3. The molar ratio of Fe/sugar of the peak at V_0 was found to be almost 1.
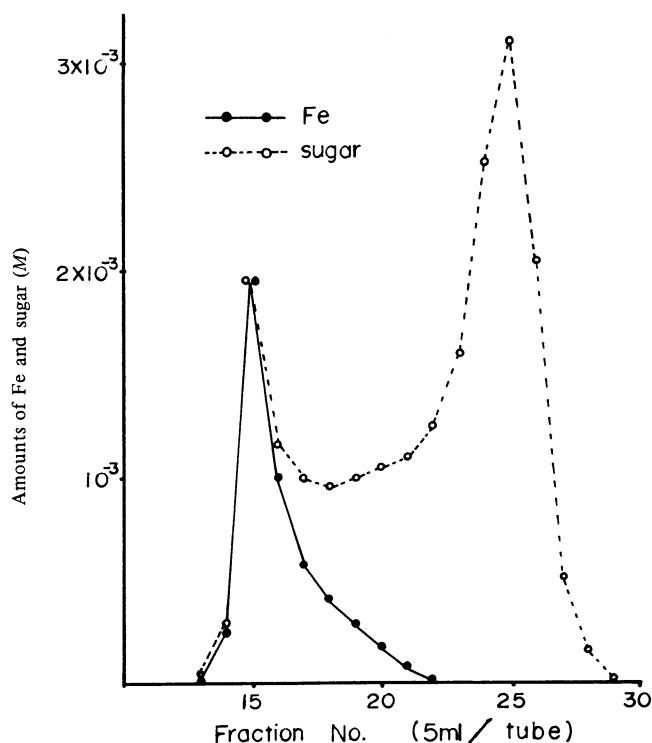

Fig. 4.   Gel-chromatography of Fe(III)-lactose complex with
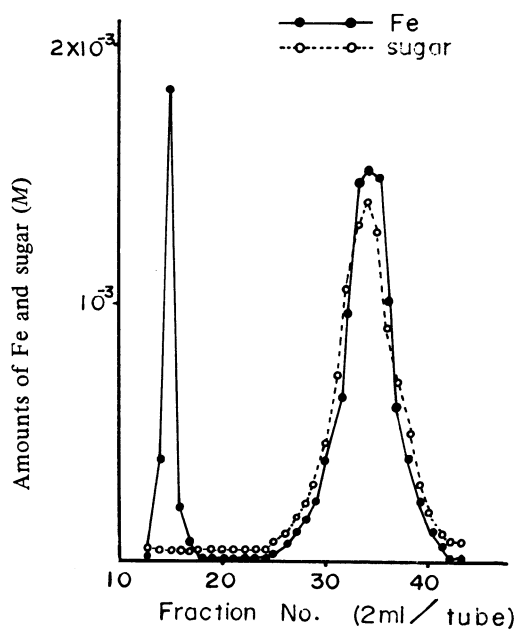bicarbonate buffer solution   (pH 9.5, μ = 0.1)

Fig. 5. Gel-chromatography of Fe(III)-lactose
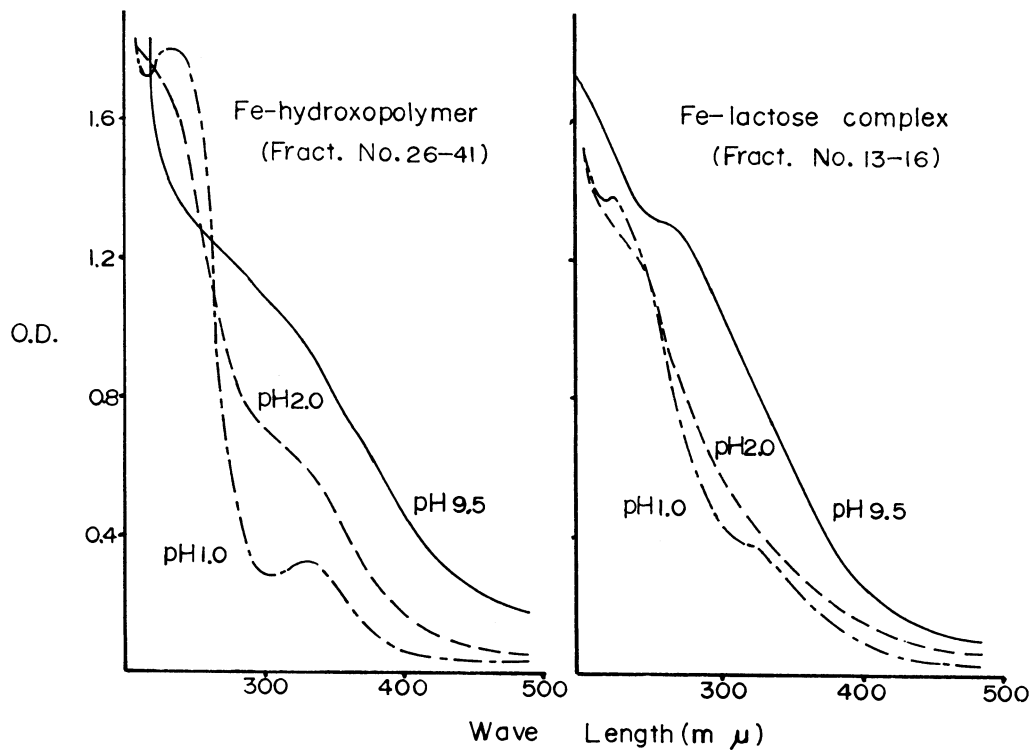complex with a Sephadx G-50 column

Fig. 6. Absorption curves of the two fractions in Fig. 5.

3.   Fractionation of the Fe-lactose complex with a Sephadex G-50 column

The fractions containing the Fe-lactose complex were pooled, lyophilyzed, and rerun through a column of Sephadex G-50. Except for the volume (2 m*l*) of the fractions, the eluent and other conditions of the gel-filtration were the same as those in Fig. 4. As shown in Fig. 5, the fraction of Fe was divided into two peaks of free Fe and the Fe-lactose complex at about fraction No. 15 and No. 34 (Kd = 0.5), respectively. Considering their elution patterns, the Fe-lactose complex seems to be a polymer of molecular weight 5,000 ~ 10,000. Fig. 6 shows the absorption spectra of free Fe and the Fe-lactose complex. The solid curves indicate that both free Fe and the Fe-lactose complex are polymerized in the solutions at pH 9.5. However, when the sample solutions of pH 9.5 were acidified to pH 1 (chain lines) or pH 2 (dotted lines), the absorption maximum at about 280 mμ was remarkably reduced and the patterns of the spectra changed : The positions of λ_{max} were at 240 mμ and 335 mμ.

DISCUSSION

1.   Structure of the Fe(III)-lactose complex

According to KIYAMA *et al.*,[11)] who studied on the hydrolytic polymerization of Fe-ions, the λ_{max} of Fe-aquo-ions is at 240 mμ and that of the dimer of the aquo-ion at 335 mμ. They also concluded that the Fe-hydroxopolymer, indicating a "general absorption" without any λ_{max} in the absorption curve, was composed of the above mentioned dimers as the unit. In Fig. 6, the solid curve of free Fe can be considered as one of the general absorption. Accordingly, the results suggest that the Fe(III)-lactose complex prepared in the manner described in Fig. 1 is really a polymer. The molecular weight of the polymer seems to be 5,000 ~ 10,000 on the basis of the molecular shieve effects of Sephadex G-50. The Fe(III)-lactose polymer complex is depolymerized on the acid side of neutrality and decomposed into free Fe-ions and free sugars in strongly acidic solutions below pH 2. Such a result was obtained by the
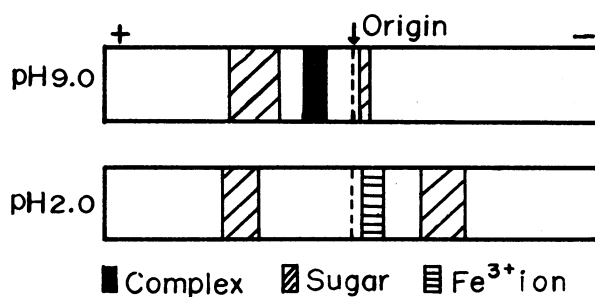

Fig. 7.   Paper electrophoresis of Fe(III)-lactose complex

paper electrophoresis reported in the previous paper (Fig. 7)[10] and further evidence on this point was obtained in this experiment as shown in Fig. 3.

SPIRO *et al.*[12] proposed models for the structures of monomer, dimer, and polymer of the Fe-sugar complex (Fig. 8). As shown in Fig. 8, every model is composed of Fe and sugar in the molar ratio of 1 : 1 and the sugar coordinates to the Fe-ion
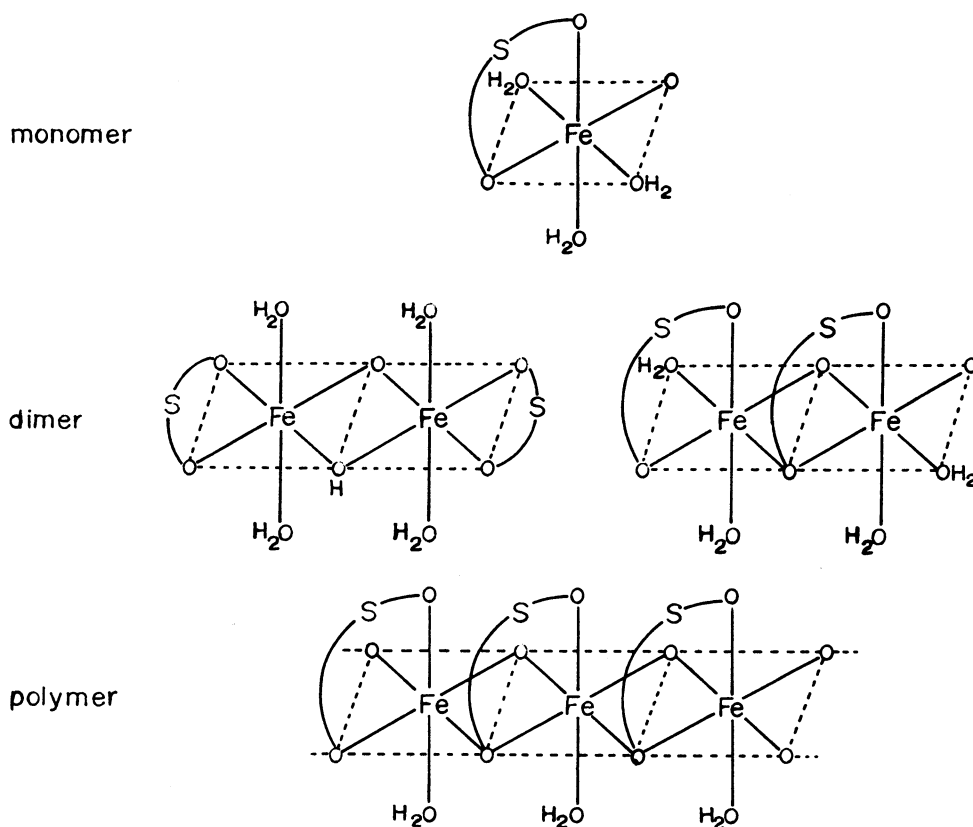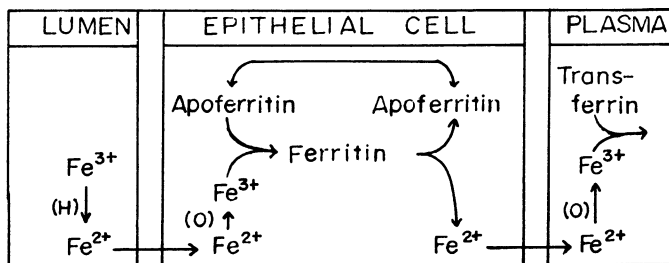
monomer

dimer

polymer

Fig. 8. SPIRO's models of Fe-sugar complexes

just like a cover that coats the Fe-hydroxopolymer. Since the Fe-lactose complex prepared according to the method outlined in Fig. 1 gave the molar ratio of Fe/sugar of 1 and was charged negatively,[10] the above models probably apply in the case of the Fe(III)-lactose complex in this experiment.
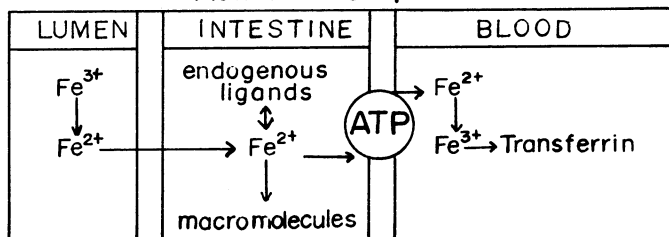
2. Nutritional significance of Fe-complexes

SALTMAN *et al*[6] classified the mechanism of Fe-absorption through the intestinal wall into three types as shown in Fig. 9. The absorption of Fe-complexes corresponds to the type c, namely, the passive transport mechanism. This is based on the studies by CHARLEY *et al.*[5] and STITT *et al.*[13]

a. Mucosal block mechanism

LUMEN	EPITHELIAL CELL	PLASMA
Fe^{3+} (H)↓ Fe^{2+}	Apoferritin ⇄ Apoferritin → Ferritin ← Fe^{3+} (O)↑ → Fe^{2+} Fe^{2+}	Trans-ferrin Fe^{3+} (O)↑ → Fe^{2+}

b. Active transport mechanism

LUMEN	INTESTINE	BLOOD
Fe^{3+} ↓ Fe^{2+}	endogenous ligands ⇕ → Fe^{2+} → ⓐⓉⓅ ↓ macromolecules	→ Fe^{2+} ↓ Fe^{3+}→Transferrin

c. Chelation mechanism

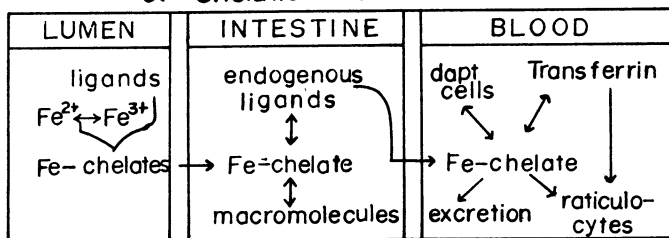| LUMEN	INTESTINE	BLOOD
ligands Fe^{2+}⇄Fe^{3+} Fe-chelates	endogenous ligands ⇕ → Fe-chelate ⇕ macromolecules	dapt cells Transferrin ↘ ↗ ↓ → Fe-chelate ↓ ↙ ↘ excretion raticulo-cytes

Fig. 9. The mechanisms of iron-metabolism proposed by SALTMAN

It is a well-known fact that, in a simple salt form, Fe(II) ions are more easily absorbed through the intestinal wall than Fe(III) ions. The authors consider that the reason for this is as follows. As the pK value of Fe(II) ions is 9.5, the ions exist as monomers in neutral or alkaline solutions. On the other hand, the values of pK_1 and pK_2 of Fe(III) ions are 2.2 and 3.3, respectively. Therefore, under the same conditions as above, Fe(III) ions are polymerized hydrolytically to a polymer complex with a high molecular weight. Such behavior of Fe(III) ions was confirmed also by the present research as shown in Fig. 4, 5, and 6. On the molecular weights of Fe-polymers, some reports were made by SPIRO et al.[14] and ALLERTON et al.[15] They succeeded in obtaining a globular Fe-hydroxopolymer complex with a diameter of 7 mμ and a molecular weight of 150,000. According to TERATO et al.,[9] such a high molecular

weight compound could not be transported through the intestinal wall by the passive mechanism : They reported that Fe(III)-hydroxopolymer complexes of molecular weights less than 10,000 were absorbed through the intestinal wall but not those of molecular weights more than 10,000. On the other hand, SALTMAN [16] was of the opinion that Fe(III) ions as well as Fe(II) ions were apt to be easily absorbed in the presence of such a chelating agent as EDTA. Similar chelating effects were observed with citric acid[7][17] and fructose.[2][13] The effect of EDTA is ascribed to the small molecular weight of the Fe(III)-EDTA complex which exists as a monomer in solution. However, in the case of the Fe(III)-citric acid complex the case is different, namely, the complex exists as a high polymer of molecular weight 210,000.[12][18] In addition, the Fe-complexes with fructose, ribose, arabinose, glucose, gluconic acid, and galacturonic acid are known to form high spin polymer complexes. They do not exist as monomer forms in solution. The Fe(III)-lactose complex in this study seems to have a molecular weight of 5,000 ~ 100,000. The molecular weight of the Fe(III)-fructose complex prepared by BATE et al.[19] is 15,000. From these facts, the effects of sugars and their derivatives are considered to be different from that of EDTA, that is, they depress incompletely the polymerization of Fe-aquo-ions. Although their chelating effects are not so strong as EDTA, which completely depresses the polymerization, they probably make the passive transport of Fe(III) ions through the intestinal wall possible.

SUMMARY

Purification by the gel-filtrations with Sephadex G-15 and G-50 was performed on a Fe(III)-lactose complex preparation containing free sugars with their derivatives. The most desirable result was obtained with a bicarbonate buffer solution (pH 9.5, $\mu = 0.1$) as the eluent. The purified Fe(III)-lactose complex was composed of Fe and sugar in the molar ratio of 1, and was a polymer of molecular weight 5,000 ~ 10,000. The structure of the polymer was probably similar to that proposed by SPIRO et al. The molecular weight of the polymer is very small compared to the molecular weight (250,000) of the Fe(III)-hydroxopolymer formed in alkaline solutions. Accordingly, the addition of sugars in Fe(III) solutions brings about the considerable inhibition of the polymerization of Fe-hydroxo-ions and makes the passive transport of Fe through the intestinal wall possible.

REFERENCES

1) IMAMURA, T., NAITO, H. and AOKI, T.: *Eiyo to Shokuryo*, **22**, 361–366 (1969).

2) BERSIN, T. and SCHWARZ, H.: *Ann.*, **623**, 138–141 (1959).

3) SALTMAN, P. and CHARLEY, P. J.: *Fr. M.*, **1495**, Oct. 22 (1962). (CA 58, 7794).

4) CHARLEY, P. J., SARKAR, B., STITT, C. F. and SALTMAN, P.: *Biochim. Biophys. Acta,* **69**, 313–321 (1963).

5) CHARLEY, P. J., STITT, C. F., SHORE, E. and SALTMAN, P.: *J. Lab. Clin. Med.,* **61**, 397–410 (1963).

6) SALTMAN, P.: *J. Chem. Educ.,* **42**, 682–687 (1965).

7) HOPPING, J. M. and RULIFFSON, W. S.: *Am. J. Physiol.,* **210**, 1316–1320 (1966).

8) MORGAN, O. S., WEIR, D. G., GATENBY, P. B. B. and SCOTT, I. M.: *Lancet,* **2**, 861–863 (1969).

9) TERATO, K., FUJITA, T. and YOSHINO, Y.: *Digestive Diseases,* **18**, 121–128 (1973).

10) IMAMURA, T. and KAWAMOTO, H.: *J. Fac. Fish. Anim. Husb., Hiroshima Univ.,* **13**, 181–188 (1974).

11) KIYAMA, M and TAKADA, T.: *Bull. Chem. Soc. Jap.,* **46**, 1680–1680 (1973).

12) THOMAS, G., SPIRO, T. G., PAPE, L. and SALTMAN, P.: *J. Am. Chem. Soc.,* **89**, 5555–5559 (1967).

13) STITT, C., CHARLEY, P. J., BUTT, E. M. and SALTMAN, P.: *Proc. Soc. Exptl. Biol. Med.,* **110**, 70–71 (1962).

14) SPIRO, T. G., ALLERTON, S. E., RENNER, J., TERZIS, A., BILS, R and SALTMAN, P.: *J. Am. Chem. Soc.,* **88**, 2721–2726 (1966).

15) ALLERTON, S. E., RENNER, J., COLT, S. and SALTMAN, P.: *J. Am. Chem. Soc.,* **88**, 3147–3148 (1966).

16) SPIRO, T. G. and SALTMAN, P.: in "Structure and Bonding", Vol. 6, pp. 116–156, Springer-Verlag, Berlin, Heidelberg, New York (1969).

17) HOPPING, J. M. and RULIFFSON, W. S.: *Am. J. Physiol.,* **205**, 885–889 (1963).

18) SPIRO, T. G., BATES, G. and SALTMAN, P.: *J. Am. Chem. Soc.,* **89**, 5559–5562 (1967).

19) BATES, G., HEGENAUER, J., RENNER, J. and SALTMAN, P.: *Bioinorganic Chemistry,* **2**, 311–327 (1973).

ゲルクロマトグラフィーによる鉄（Ⅲ）－乳糖錯体の精製

今村経明・畑中千歳・河本秀一

　前報[10]に述べた鉄－糖錯体の調製法では，溶液中に生成した錯体をEt-OHで沈でんさせ，遠心分離する方法をとった。この方法では未反応の遊離糖もEt-OHで沈でんするため，調製した標品にかなりの量の遊離糖が混入していることが予想できる。そこで，鉄（Ⅲ）－糖錯体と遊離糖を分別するために Sephadex によるゲル濾過を試みた。

　溶離液の pH や種類によっては，ゲル濾過中に鉄（Ⅲ）－糖錯体が分解することが分った。この点を中心に種々検討した結果，bicarbonate buffer (pH 9.5, $\mu = 0.1$) を用い，はじめに Sephadex G-15，その V_0 フラクションについて G-50 でゲル濾過することにより，Fe／糖＝1 の組成比をもった鉄（Ⅲ）－乳糖錯体を純粋に得ることができた。しかしこの錯体はモノマーではなく，分子ふるい効果から推定してM.W.＝5,000 ～10,000 のポリマーであった。この分子量は，Fe（Ⅲ）－アコイオンがアルカリ性溶液中で形成するポリマーの分子量に比べて著しく小さい。

　以上の実験結果に基づいて化学構造を考察した結果，SPIRO らの coating 説をとった。そして糖が Fe（Ⅲ）に配位することによって Fe（Ⅲ）－アコイオンの重合を抑制すると考え，Fe（Ⅲ）の腸管吸収，とくに受動輸送に対する寄与を考察した。