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Radical-promoting "free" iron level in the serum of rats treated with ferric nitrilotriacetate: comparison with other iron chelate complexes.

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Abstract

Iron plays a critical role in the production of activated oxygen species and the activity of chelated iron in the biological system depends on the chemical forms of the chelators. In the present study, we used ferric nitrolotriacetate (Fe-NTA, molar ratio of iron to chelators = 1:3), ferric ethylenediaminetetraacetate (Fe-EDTA, 1:3 complex) and ferric Desferal (Fe-Des, 1:1.1 complex) to see their “free” iron content in aqueous solutions in vitro and in the serum obtained after a single intraperitoneal injection of the chelates to rats (7.5 mg of iron/kg). ”Free” iron was measured by the bleomycin-assay system. When Fe-NTA was dissolved in water, ”free” iron increased linearly with total iron concentration up to 10 microM, whereas Fe-EDTA and Fe-Des showed no ”free” iron with corresponding iron concentrations. When these three ferric chelates were dissolved in normal rat serum, ”free” iron in Fe-NTA increased abruptly between 40 microM and 60 microM iron concentrations, then increased slowly up to 100 microM. Fe-Des did not show any ”free” iron at comparable iron concentrations. Fe-EDTA had an intermediate ”free” iron level in the serum. Among the ferric chelate complexes, Fe-NTA showed a much faster increase of and a higher content of ”free” iron in the serum than the other two complexes after a single injection of the chelates into rats.(ABSTRACT TRUNCATED AT 250 WORDS)

KEYWORDS: ”free” iron, ferric nitrilotriacetate (Fe-NTA), ferric ethylenediaminetetraacetate (Fe-EDTA), ferric desferrioxamine (Fe-Desferal, Fe-Des), free radecal

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Radical-Promoting "Free" Iron Level in the Serum of Rats Treated with Ferric Nitrilotriacetate: Comparison with Other Iron Chelate Complexes

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Iron plays a critical role in the production of activated oxygen species and the activity of chelated iron in the biological system depends on the chemical forms of the chelators. In the present study, we used ferric nitrilotriacetate (Fe-NTA, molar ratio of iron to chelators = 1:3), ferric ethylenediaminetetraacetate (Fe-EDTA, 1:3 complex) and ferric Desferal (Fe-Des, 1:1.1 complex) to see their "free" iron content in aqueous solutions *in vitro* and in the serum obtained after a single intraperitoneal injection of the chelates to rats (7.5 mg of iron/kg). "Free" iron was measured by the bleomycin-assay system. When Fe-NTA was dissolved in water, "free" iron increased linearly with total iron concentration up to 10 μM, whereas Fe-EDTA and Fe-Des showed no "free" iron with corresponding iron concentrations. When these three ferric chelates were dissolved in normal rat serum, "free" iron in Fe-NTA increased abruptly between 40 μM and 60 μM iron concentrations, then increased slowly up to 100 μM. Fe-Des did not show any "free" iron at comparable iron concentrations. Fe-EDTA had an intermediate "free" iron level in the serum. Among the ferric chelate complexes, Fe-NTA showed a much faster increase of and a higher content of "free" iron in the serum than the other two complexes after a single injection of the chelates into rats. Furthermore, the results of the hepatic function tests and histopathological observations of the kidney and the liver showed that Fe-NTA was the most toxic among the three iron complexes and that the kidney was more vulnerable than the liver. In summary, Fe-NTA keeps iron both soluble and highly reactive in blood by providing "free" iron.

Key words : **"free" iron, ferric nitrilotriacetate (Fe-NTA), ferric ethylenediaminetetraacetate (Fe-EDTA), ferric desferrioxamine (Fe-Desferal, Fe-Des), free radical**

Iron is a key element in catalytic production of hydroxyl radicals and other active oxygen species, which initiate lipid peroxidation and destroy almost all the known biomolecules (1). However, iron-protein complexes such as ferritin and hemoglobin are unable to produce active oxygen species unless iron is released (2). Significance of the catalytic role of iron in the production of active molecular species of oxygen *in vivo* for the pathogenesis of tissue damage and carcinogenesis has been suggested since we reported that iron complex of nitrilotriacetate (Fe-NTA) caused

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severe nephrotoxicity and renal cell carcinoma at high rates in rats and mice (3).

Whether a given iron chelate complex catalyzes the formation of active oxygen species and induces lipid peroxidation *in vivo* depends on its iron-binding and iron-releasing capacity, and its distribution and metabolism in the body. The bleomycin-assay method developed by Gutteridge *et al.* seems to be one of the methods suitable for the detection of radical-promoting "free" iron *in vivo* (4). The iron-specific release of thiobarbituric acid-reactive substance (TBARS) from DNA by bleomycin provides a unique way to detect and to measure a trace amount of loosely-bound iron by catalytic redox cycling of the iron. The present study was undertaken, using this bleomycin-method, to examine dynamics of the "free" iron content in three iron complexes, namely ferric nitrilotriacetate (Fe-NTA), ferric ethylenediaminetetraacetate (Fe-EDTA) and ferric Desferal (Fe-Des) both *in vitro* and *in vivo*. Desferal (Des) has been used in the treatment of iron overload because it binds ferric iron to form iron complex which is rapidly excreted (5). Among other chelators the reactions of ethylenediaminetetraacetic acid (EDTA) with these metals are quite complicated. EDTA is usually used to bind iron in laboratory and in biological systems (6). Biochemical and histopathological studies of the kidney and the liver of the rats treated with different iron chelate complexes were also made to clarify whether the *in vivo* toxicity of an iron chelate can be explained by the presence of "free" iron in the serum.

Materials and Methods

Animals. Male Wistar rats (6–7 weeks old) weighing 150–180g were obtained from Charles River Japan, Inc. (Shizuoka, Japan). They were divided randomly into groups and were provided with commercial rat food and tap water *ad libitum.*

Chemicals. Calf-thymus DNA (typeI) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Chelex-100 was from Bio-Rad Laboratories (Richmond, CA, USA). Bleomycin sulphate was a gift from Nippon Kayaku Co. (Tokyo, Japan). $Fe(NO_3)_3 \cdot 9H_2O$ was purchased from Wako Pure Chemical Co. (Osaka, Japan), nitrilotriacetic acid disodium salt, Desferal were from Nippon Ciba-Geigy Co. (Takarazuka, Japan), and EDTA was from Nakalai Chemical Co. (Kyoto, Japan), respectively. All reagents were of the highest quality available from suppliers.

Preparation of iron chelate complexes and test samples. Metal chelate complexes were prepared freshly before each experiment by mixing solutions of $Fe(NO_3)_3 \cdot 9H_2O$ with each of nitrilotriacetic acid disodium salt, EDTA at an iron-to-chelator molar ratio of 1:3, and with Desferal at 1:1.1, and pH of the mixtures was adjusted to 7.4 with sodium bicarbonate (7). For an *in vitro* study, ferric chelates were dissolved either in water or in normal rat serum. To obtain serum from animals treated with ferric chelates, animals were divided into Fe-NTA, Fe-EDTA and Fe-Des groups, each consisted of 35 rats. The ferric chelates were injected intraperitoneally (i. p., 7.5 mg iron/kg body weight). After rats were anesthetized with pentobarbital sodium (Nembutal, Dainihonseiyaku, Osaka, Japan), the blood samples were removed from retroorital sinus 0, 10, 20, 30, 40, 50, 60 min after the injection. Five rats were used at each point. "Free" iron (see below), serum non-heme iron concentrations (see below) as well as hepatic and renal functions were examined by analyzing serum samples with autoanalyser (Toshiba, TPA-50s). The livers and kidneys were removed for histopathological observation 48 h after iron chelate injection.

"Free" iron measurement. "Free"iron content was measured by the bleomycin assay (8). Briefly, the reaction mixture consisted of 0.5 ml calf-thymus DNA (1 mg/ml), 0.05 ml bleomycin sulphate (1 mg/ml), 0.1 ml $MgCl_2$ (50 mM), 0.1 ml sample, 0.05 ml HCl (10 mM), 0.1 ml Milli-Q water and 0.1 ml ascorbic acid solution (1.4 μg/ml) were mixed in plastic tubes and incubated for 2 h at 37°C with shaking. Then 1 ml of 0.02 M Desferal was added to stop the reaction, and the contents were transferred to glass tubes and mixed with 1 ml of 1 % (W/V) thiobarbituric acid (in 50 mM NaOH) and 1 ml of 25 % (V/V) HCl. The mixtures were heated at 100°C for 15 min, cooled, and the absorbance of chromogen was measured at 532 nm.

Serum non-heme iron concentration. The serum non-heme iron concentration was measured by internationally standarized method (9).

Histopathological observations of the livers and the

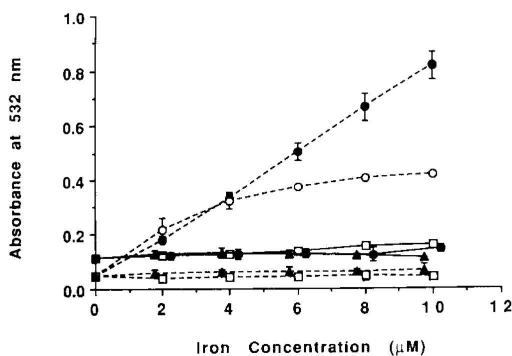
kidneys. The portions of the liver and kidney of rats were fixed in 10 % formalin for 3 days. The specimens were dehydrated, embedded in paraffin, sectioned at 4 μm, and mounted on glass slides for routine histologic staining.

Statistical analysis. Data were analyzed by student's t test. All the results shown were the means ± SD of five or more assays, which differed by less than ± 5 %.

Results

The results of bleomycin assay for "free" iron in ferric chelate complexes dissolved in water or in normal rat serum are shown in Fig. 1. Both Fe-EDTA and Fe-Des in water did not show "free" iron in the iron concentration range tested. On the contrary, Fe-NTA and freshly prepared $FeCl_3$ solution in water showed high "free" iron content. "Free" iron content in Fe-NTA increased linearly with total iron concentration up to 10 μM, and that in $FeCl_3$ showed linearity up to 4 μM concentration of iron. Ferric chelate complexes dissolved in serum, on the other hand, did not show any "free" iron in the range of iron concentrations tested. Fig. 2 shows "free" iron content in the iron chelate complexes dissolved in serum at higher iron concentrations. Fe-NTA showed abrupt increase in "free" iron content at the iron concentration of 60 μM, while Fe-EDTA showed a gradual increase. "Free" iron was significantly higher ($P < 0.001$) in Fe-NTA and Fe-EDTA than in Fe-Des and normal serum for iron concentration above 40 μM in normal rat serum. Fe-Des did not show any "free" iron.

Time courses of "free" iron productions in sera of the three groups of animals treated with Fe-NTA, Fe-EDTA or Fe-Des are shown in Fig. 3. The "free" iron content in the Fe-NTA group showed a rapid increase, reaching a plateau in 10 min after an i.p. injection of Fe-NTA, and then decreased after 30 min. In 60 min the "free" iron content decreased to the control level. "Free" iron content started to increase 20 min after Fe-EDTA injection at a slower rate than that for Fe-NTA, reaching the highest level in 40 min and decreasing to the control level in 60 min. Fe-Des injection did not show any significant increase in "free" iron content during the period of experiment. The serum "free" iron in rats



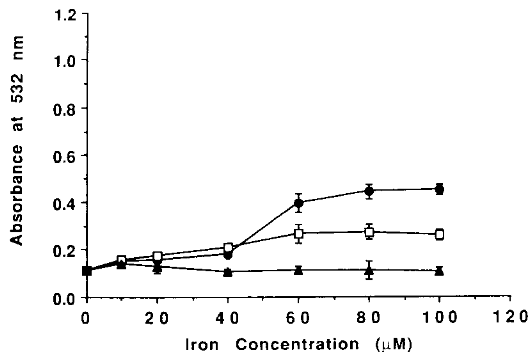
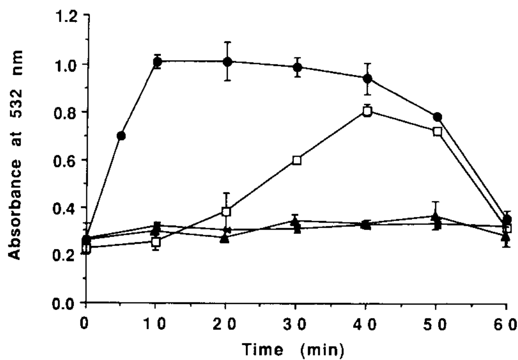


Fig. 1 (left) "Free" iron contents of the three iron chelate complexes dissolved in serum and in water analyzed by bleomycin method. The absorbance at 532 nm represents the thiobarbituric acid-reactive products. Ferric nitrilotriacetate (Fe-NTA, ●); ferric ethylenediaminetetraacetate (Fe-EDTA, □); ferric desferrioxamine (Fe-Des, ▲); ferric chloride ($FeCl_3$, ○). Dotted line: the iron chelate complexes dissolved in water; solid line: the iron chelate complexes dissolved in serum.

Fig. 2 (right) "Free" iron contents of ferric chelate complexes dissolved in serum as determined by the bleomycin method. The absorbance at 532 nm represnts the thiobarbituric acid-reactive products. Ferric nitrilotrdiacetate (Fe-NTA, ●); ferric ethylenediaminetetraacetate (Fe-EDTA, □); ferric desferrioxamine (Fe-Des, ▲).



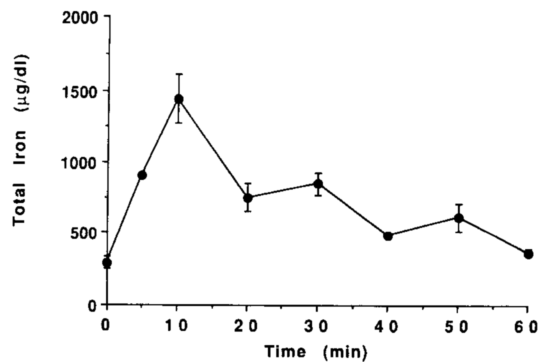


Fig. 3 (left) Time-dependent formation of "free" iron in the serum of rats treated with iron chelate complexes. Ferric nitrilotriacetate (Fe-NTA, ●); ferric ethylenediaminetetraacetate (Fe-EDTA, □); ferric desferrioxamine (Fe-Des, ▲); saline-treated control (×).
Fig. 4 (right) Serum non-heme iron concentration in the ferric nitrilotriacetate-treated animals after injection of iron chelate.

injected with Fe-NTA and Fe-EDTA was significantly higher ($P < 0.001$) than that in rats injected with saline, from 5 to 50 min in Fe-NTA injected and from 20 to 50 min in Fe-EDTA injected.

The serum non-heme iron levels in the Fe-NTA-treated animals increased, reaching the maximum in 10 min after the i.p. injection. It decreased rapidly after 20 min, but was significantly high ($P < 0.001$) from 5 min after Fe-NTA injection until 60 min, markedly different in the time course from the "free" iron content (Fig. 4).

After injection of the iron-chelate-complexes, serum GOT level increased steadily in the Fe-NTA-treated group (Table. 1) and transiently at one hour in the Fe-EDTA group. The serum GPT level also increased in the Fe-NTA-treated group in 48 h.

The routine hematoxylin and eosin staining of kidneys of the experimental animals showed that the morphologic abnormalities were different among the groups of animals treated with different iron-chelate-complexes. In the Fe-NTA-treated rats (Fig. 5.B), generalized proximal tubular necrosis often accompanied by regeneration, and occlusion of tubular lumina by casts were clearly noted. The nuclei of regenerative cells were large and irregularly shaped with prominent nucleoli. In both Fe-EDTA-(Fig. 5.C) and Fe-Des-(figures not shown) treated animals, there were no necrosis, nuclei and tubular sizes were orderly, and there were no signs of regeneration.

Table 1 Serum levels of GOT, GPT after injection of iron chlate complexes to rat

Time (hr)	Iron chelate complexes					
	Fe-NTA		Fe-EDTA		Fe-Des	
	GOT (IU/l)	GPT (IU/l)	GOT (IU/l)	GPT (IU/l)	GOT (IU/l)	GPT (IU/l)
0	86	35	84	37	87	36
1	150	47	130	77	89	61
48	265	142	81	43	70	42

Fig. 5 Histopathological findings of the rat kidney 48 h after iron chelate complexes (7.5 mg/kg) treatment. (A, B): Saline-treated control; (C, D): Fe-NTA-treated group: proximal tubular necrosis is evident (arrow); (E, F): Fe-EDTA-treated group; (A, C, E: X20; B, D, F: X40).

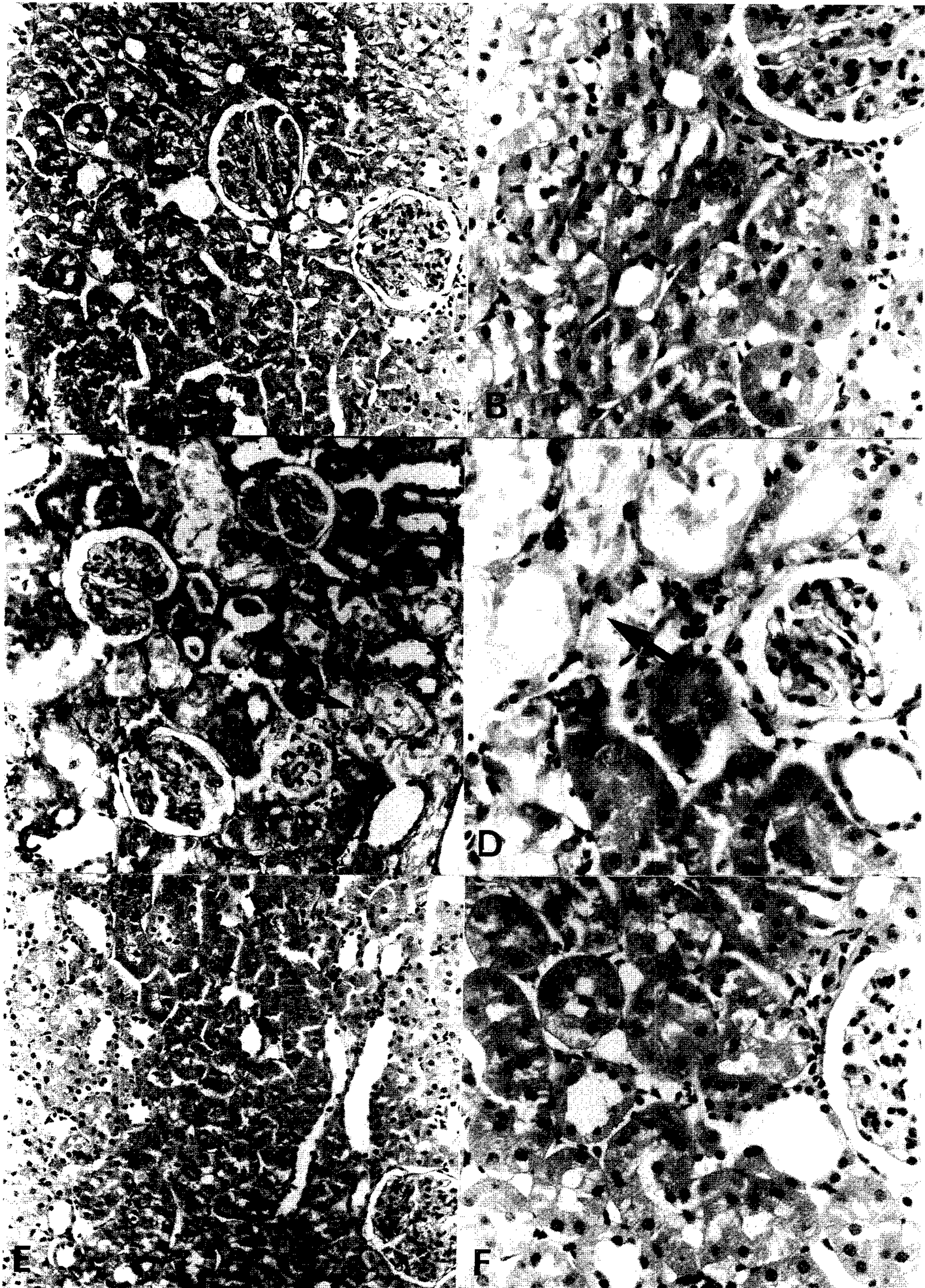


Fig. 5

The microscopic observation of the liver showed no abnormalities in all the groups (figures not shown).

Discussion

It is well-known that oxygen radicals are closely involved in the cytotoxicity of many organs and cause various diseases. In the absence of suitable transition metal catalysts, the reactions involving oxygen radicals would be impossible to take place (10). Among the transition metals, iron is most frequently involved in several aspects of oxidative stress. The following forms of iron in the biological system accelerate free radical reactions: the hemoglobin (4), the storage protein ferritin (11), and the intracellular mobile iron pool which consists of iron bound to low molecular weight chelators, such as citrate, ATP or ADP (12–14). However, it is the "free" iron, which is mobilized from iron deposit or is given from outside, that seems responsible for free radical-mediated lipid peroxidation and for the various damages to biological systems (11).

Among three iron chelate complexes we examined, Fe-NTA induces nephrotoxic and hepatotoxic effects, such as proximal tubular necrosis and some hepatic dysfunctions in rats and mice (15, 16). The morphological alteration induced by Fe-NTA was considered an acute cellular damage induced by iron-mediated lipid peroxidation. The injection of Fe-NTA induced also renal cell carcinoma in rats and mice in the prolonged administration (17, 18). EDTA is one of the most common iron chelator used in laboratories, and is administered to animals often as a calcium chelator rather than an iron chelator. The chelation of essential calcium *in vivo* limits its clinical use. EDTA penetrates cell membranes rather poorly, thus serving primarily as an extracellular rather than as an intracellular chelator. EDTA is also toxic to the kidney; the renal tubules appear relatively sensitive to EDTA, resulting in hydropic degeneration of the proximal tubules, progressing to total destruction. Mild effects of EDTA disappear after cessation of the therapy (19). EDTA stimulates the catalytic activity of iron, such as to promote the iron-catalyzed Haber-Weiss reaction, because Fe(III)-EDTA chelates are reduced by O_2^- quite rapidly (20). Fe(III)-EDTA significantly increased the ascorbyl radical in synovial fluid (6). However, negative observation of EDTA effect on OH・ formation are also reported (21). The mild increases of serum "free" iron level of Fe-EDTA *in vivo* (Fig. 3) and the reported mild toxic effect of EDTA itself are consistent with our results *in vivo* (Table. 1)

Iron overload is often treated with Desferal, as Desferal has a binding constant for Fe(III) several orders of magnitudes greater than for any other metals and is virtually specific for the chelation of Fe(III)(22). Desferal and Fe(III) complex (Fe-Des) are rapidly and almost completely excreted, mainly in urine but also in bile, so removing iron from the body (5). Desferal drastically reduce the catalytic activity of iron in the airoxidation of ascorbic acid. These results may be all explained by the fact that Desferal binds Fe(III) so tightly that iron can not be reduced by O_2^- and hence Desferal inhibits OH・ formation (23). The results of our "free" iron measurement were consistent with these observations.

In the present study, we used the sensitive and specific bleomycin assay method that can be used for the determination of "free" iron in biological systems. The method employs the anti-tumor antibiotic bleomycin, which binds to and degrades DNA. Degradation is accompanied by the formation of a product similar to malondialdehyde that react with thiobarbituric acid to form a chromogen. Degradation by bleomycin is absolutely dependent on the presence of Fe(II), and the rate of degradation can be used to assess the "free" iron content of a system. Where it has been possible to test, bleomycin-detectable iron is able to accelerate such radical reactions as lipid peroxidation, thus allowing to measure iron con-

tent available *in vivo* to accelerate radical reactions. Our results showed clearly that not only in water, but also in the serum of iron-chelate-treated animals, Fe-NTA was the most abundant "free" iron source among the iron-chelates used. These interesting observation provides sufficient evidence to support the iron-promoted lipid peroxidation in the nephrotoxic and carcinogenic effects of Fe-NTA, but not of other ferric chelates. Hamazaki *et al.* (24) compared the ability of Fe-NTA to initiate lipid peroxidation *in vitro* with that of other ferric chelates, including Fe-EDTA and Fe-Des. The results demonstrated that Fe-NTA could initiate the peroxidation of lipids in liposomal configuration on addition of a reducing agent although the initiation by Fe-EDTA was very weak, and Fe-Des could not initiate peroxidation of lipids. Their results also showed that Fe-NTA could start lipid peroxidation only when it was reduced by some reductants such as ascorbic acid, NADPH or cysteine. The present result that Fe-NTA is less toxic in the liver than in the kidney may mean that Fe-NTA is reduced less efficiently in the liver than in the kidney. Electron spin resonance techniques demonstrated that in the presence of H_2O_2, Fe-NTA can give a strong signal of 5,5-dimethyl-1-pyrroline-1-oxide-trapped hydroxyl radical adduct, but Fe-Des gives no signal at all (25). Therefore, it is quite clear that some chelates, such as Fe-NTA, keep iron both soluble and highly reactive, but some others, such as Desferal, bind Fe(III) tightly and render it unreactive in addition to be rapidly excreted. Also others, such as EDTA which not only binds Fe(III) but also other divalent and trivalent metal demonstrated mild toxic effects under some experimental conditions.

Differences of "free" iron content in water and in serum were quite striking. Serum seems to confer some protection against free radical reaction by preventing "free" iron to form from iron chelate complexes. Mode of protection may differ from one iron chelate to another. "Free" iron in Fe-NTA increased abruptly when iron concentration reached 40 μM *in vitro* (Fig. 2). This corresponds to the saturation concentration of rat serum iron binding capacity and the end point of iron transfer from Fe-NTA to rat serum transferrin. It is well known that iron bound to transferrin does not have any "free" iron (unless iron is released at low pH). Bleomycin-detectable iron is often present in the serum of patients suffering from the iron-overload disease, idiopathic hemochromatosis, in which the transferrin is at or near iron saturation (6, 26). However, there is no obvious stoichiometric relationship between the total non-heme serum iron and the presence of bleomycin-detectable "free" iron in idiopathic hemochromatosis (27) and in knee-joint synovial fluid from patients with rheumatoid arthritis. Bleomycin-detectable iron was only present if total plasma iron was greater than approximately 40 μmol/l (28). Our *in vivo* data showed some discrepancy between serum non-heme iron (Fig. 4) and "free" iron contents (Fig. 3). After initial increase of serum iron and "free" iron, serum iron decreased rapidly, while "free" iron was kept at plateau for about 40 min. Our observation, together with a clinical one, suggest that the iron that binds loosely or non-specifically to transferrin or probably to other serum proteins also becomes a source of "free" iron.

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