JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2002,** *50,* 871–877 **871**

Iron Derivatives from Casein Hydrolysates as a Potential Source in the Treatment of Iron Deficiency

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The properties of an Fe^{3+}−peptide complex containing 5.6% Fe, obtained by the reaction of ferric chloride with an enzymatic hydrolysate of casein, are described. The major site of iron binding corresponds primarily to the carboxylate groups and to a lesser extent to the peptide bonds. The Fe^{3+}−peptide complex is insoluble at acid pH and completely soluble at neutral to alkaline pH. When soluble, the Fe^{3+} is tightly bound to the complex peptide mixture but can be displaced and complexed by a low molecular weight ligand such as cysteine. Its efficacy in relation to iron sulfate was compared in rats. Both iron sources were administrated in Milli-Q water by gastric gavage to male Wistar rats (180−200 g) after an 18 h fast with water ad libitum. Fe^{3+} from the Fe^{3+}−peptide complex was transferred to the blood in a dose-dependent manner (1−8 mg of Fe/kg), and the serum iron levels were significantly higher ($p < 0.001$) than in a similar group of rats treated with iron sulfate. In the comparative kinetics experiments, the rats received 4 mg of Fe/kg. Both iron sources presented maximum absorption, as indicated by the elevation of serum iron levels, 30 min after administration, and the $AUC_{0\rightarrow12h}$ of the Fe^{3+}−peptide complex was significantly higher ($p < 0.05$) than that observed with iron sulfate. The simultaneous administration of free peptides (0−192 mg) with the Fe^{3+}−peptide complex or iron sulfate did not modify the extent of absorption of iron from both sources, suggesting that the absorption is due to the complex formed and probably not to exchange reactions in the gastrointestinal tract. In the hemoglobin repletion experiments carried out on newly weaned rats with anemia induced by a low-iron diet, supplementation of the diet with the the Fe^{3+}−peptide complex was as efficient as supplementation with iron sulfate in the conversion from diet to hemoglobin iron. These results, taken together, suggest that the Fe^{3+}−peptide complex is a potential compound for use as an iron source in biological situations.

KEYWORDS: Protein hydrolysate; casein; iron complexes; iron deficiency; iron supplementation

INTRODUCTION

The prophylactic or therapeutic administration of oral formulations containing non-heme iron compounds, such as iron sulfate, presents a wide range of inconveniences due to the low bioavailability and side effects of these preparations (*1*, *2*), thus making iron supplementation programs difficult (*2*). These side effects have been attributed to the acidity of iron sulfate and free ionic iron (*3*, *4*), which can damage the gastric and intestinal wall. Furthermore, some experimental evidence suggests that iron sulfate can lead to the formation of $OH^{\bullet}$ radicals, which can initiate the peroxidation of lipids from biological membranes, inactivation of enzymes, and DNA damage (*5*, *6*), explaining, at least in part, the toxicity of oral pharmaceutical preparations containing Fe(II) (*3*, *4*, *7*, *8*).

New strategies, delivery systems, or iron compounds can play an important role in the treatment of iron deficiency. A program of oral iron supplementation with iron sulfate administered every 3 days or once a week produced a positive effect on iron status and reduced side effects (*9*, *10*). Pharmaceutical preparations designed to minimize the peak concentration of iron in the gastrointestinal lumen have been used, but they suffered from slow liberation and reduced absorption (*1*). A gastric delivery system of iron sulfate based on floating beads (*1*) administered in a single dose to pregnant women has shown the same efficacy as three doses per day of iron sulfate tablets, without the side effects of the latter (*11*). A new compound, NaFe(III)EDTA,

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10.1021/jf0111312 CCC: $22.00
Published on Web 01/09/2002

has been utilized in food fortification and has shown a positive effect on iron status (*12*). Another type of compound, in which iron is complexed with chemically modified proteins, for example, succinylated casein, ITF282, with 5% Fe (*5*) or glutarylated lyzozyme, ABC1020, with 3.3% Fe (*13*), has provided good results in experiments involving hemoglobin repletion in rats and has shown efficacy comparable to that of iron sulfate in rats and human beings, but with higher tolerance (*14*).

We have extended this strategy for iron delivery by using an enzymatic hydrolysate of casein as the ligand. We describe the preparation and physicochemical properties of an Fe^{3+}−peptide complex prepared by reacting ferric chloride with a peptide hydrolysate at pH 7.8 and the separation of an insoluble Fe^{3+}−peptide complex by precipitation at pH 3.5. The high bioavailability in rats of Fe^{3+} in the complex was demonstrated by comparing its transfer to blood, the kinetics of iron absorption, and hemoglobin repletion in rats with that of iron sulfate.

MATERIALS AND METHODS

Casein Hydrolysate and Chemicals. The preparation and characterization of the casein hydrolysate obtained by hydrolysis with pancreatin and Proteomix are described in Freitas et al. (*15*). Briefly, this hydrolysate was prepared by dispersion of casein in water (16% w/v), at 37−40 °C at pH 7.6−7.8 for 24 h, using ammonium hydroxide to maintain the pH. The quantity of enzyme was 1.8 and 0.15 of pancreatin and Proteomix, respectively, reported as percent (w/w) relative to casein. One-third of the total amount of enzymes was added to the hydrolysate at 0, 9, and 18 h. The hydrolysate contained 18% free amino acids, 62% peptides with 2−9 residues/peptide, and 20% peptides with 10−20 residues/peptide (reported as number-average percent).

The casein hydrolysate was obtained from Biobrás S.A., Montes Claros, MG, Brazil. All other chemicals were of reagent grade.

In biological experiments, animals received ad libitum tap water and a standard chow (Nuvilab CR1, Nuvital Nutriments Ltda., Colombo, PR, Brazil) containing, according to the manufacturer, no less than 50 mg of Fe/kg of chow.

In the hemoglobin repletion experiment the components used for preparation of basal and low-iron diets were casein (Barbosa and Marques, Governador Valadares, MG, Brazil), starch, and corn oil (Refinações de Milho Brasil, Sumaré, SP, Brazil), and a premix of vitamins and minerals (*16*) was purchased from Roche (Rio de Janeiro, RJ, Brazil). Mille-Q (Millipore, Bedford, MA) water was used to prepare solutions and for the drinking water provided to the rats.

The biochemical analyses were performed using commercial kits for serum iron (Companhia Equipadora de Laboratórios Modernos, São Paulo, SP, Brazil) and for hemoglobin (Labtest, São Paulo, SP, Brazil), respectively.

Preparation of the Fe^{3+}—Peptide Complex. The Fe^{3+}−peptide complex was prepared by adding 2.5 g of $FeCl_3 \cdot 6H_2O$ slowly with constant mixing to a solution containing the casein hydrolysate (10%, w/v) at 27 °C. The pH of the solution was maintained at 7.8 by the dropwise addition of 0.1 N NaOH. The addition of $FeCl_3 \cdot 6H_2O$ was stopped when free Fe^{3+} was detected in the mixture after reaction with potassium thiocyanate (KSCN). When solutions of Fe^{3+} salts and thiocyanate ions are mixed, an intense red color develops, due largely to $[Fe(SCN)(H_2O)_5]^{2+}$ (*17*). We used this reaction in the qualitative determination of Fe^{3+}. The insoluble form of the Fe^{3+}−peptide complex was obtained by adjusting the pH to 3.5 with 0.1 N HCl. The precipitate was resuspended in 1 mM HCl and filtered. This procedure was repeated three times to remove loosely bound Fe^{3+}. Then the product was lyophilized and stored at 25−27 °C.

Analytical Methods. *Characterization of the Fe^{3+}−Peptide Complex.* The iron content of the complex was determined by atomic absorption spectroscopy (Atomspech, Hilger and Watts) after digestion with concentrated boiling sulfuric acid containing H_2O_2. Before analysis by infrared (IR) spectroscopy, an appropriate amount of sample was compressed with KBr, sealed between IR transparent windows, and analyzed using a Nicolet-FT infrared instrument.

Solubility. This test was carried out by dispersion of 20 mg of Fe as the Fe^{3+}−peptide complex or iron sulfate ($FeSO_4 \cdot 6H_2O$) in 20 mL of Milli-Q water. The pH was adjusted to 2.0 and 3.0 with 0.1 N HCl and to pH 5.0, 6.0, 7.0, and 7.5 with 0.5 M $NaHCO_3$. The dispersions were kept at 37 °C for 90 min with constant shaking and then centrifuged at 760*g* for 10 min. The total iron content of the supernatant was determined by atomic absorption spectroscopy.

Release of Iron from the Fe^{3+}−Peptide Complex. An aqueous solution, prepared immediately before use, containing one of the following low molecular weight ligands, was added to a dispersion of the Fe^{3+}−peptide complex, pH 5.0, containing 20 mg of Fe/20 mL: ascorbic acid, citric acid, cysteine, and sodium chloride (Fe/ligand molar ratio, 1:3) and EDTA (Fe/EDTA molar ratio, 1:1). The mixture was incubated at 37 °C with constant shaking, and aliquots were removed after 30, 60, 90, and 120 min and centrifuged; total iron was determined by atomic absorption spectroscopy in the supernatant to measure the iron released from the complex. pH 5.0 was chosen because at this pH value the Fe^{3+}−peptide complex has low solubility (see **Figure 2**). If iron is released/displaced by any ligand utilized, they would form a soluble compound easily detectable in the supernatant.

Biological Experiments. *Animals and Diets.* Animals were from the central animal house of the Ribeirão Preto Campus, University of São Paulo, and were maintained in accordance with the *Guide for the Care and Use of Laboratory Animals* (*18*), at 25 ± 1 °C, in individual stainless steel metabolic cages, on a 12 h dark−light cycle, with food and water ad libitum. The animals were acclimatized to the local animal house during 3 days before experiments and received tap water and standard chow ad libitum. The hemoglobin levels of all the animals were ≥12 g/dL.

Blood samples were taken by cardiac puncture, under ether anesthesia. The serum iron was determined according to a colorimetric method using ferrozine, 3-2(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,3-triazine (*19*).

Increase of Serum Iron Levels After Oral Administration. Groups of male Wistar rats weighing 180−200 g and fed standard chow were used. All animals were fasted for 18 h before the administration of the Fe^{3+}−peptide complex or iron sulfate. The compounds were dispersed in Milli-Q water, and 0.0 (controls), 0.5, 1.0, 2.0, 4.0, or 8.0 mg of Fe/kg were administered by gastric gavage. The rats were sacrificed 2 h after administration.

Comparative Kinetics of Iron Absorption After Oral Administration. Groups of male Wistar rats weighing 180−200 g were fed a standard chow. All animals were fasted for 18 h before the administration of the iron compounds. In the comparative oral kinetics experiment the Fe^{3+}−peptide complex and iron sulfate, dispersed in Milli-Q water, were administered (20 mL/kg) by gastric gavage at a dose corresponding to 4 mg of Fe/kg. Groups of rats were sacrificed 0.5, 1.0, 2.0, 4.0, 8.0, and 12 h after drug administration.

The maximum serum concentration of iron and time to reach it after the oral administration were determined from concentration−time curves. The area under the serum concentration−time curve (AUC) was computed using the linear trapezoidal rule (*20*) up to 12 h ($AUC_{0\rightarrow 12h}$). All results are expressed as mean ± SEM.

Effect of Free Peptide (Casein Hydrolysate) on Iron Absorption by Rats. Male Wistar rats (180−200 g) were fed a standard chow and then fasted for 18 h but with free access to Milli-Q water. The Fe^{3+}−peptide complex or iron sulfate was administered orally (20 mL/kg) at 4 mg of Fe/kg by gastric gavage. Free peptide (casein hydrolysate) was added to each iron source immediately before administration in the following amounts: 0, 12, 24, 48, 96, and 192 mg. The groups of rats were sacrificed 2 h after administration of each compound. The pH values of the solutions were 4.1 for iron sulfate, 3.5 for the Fe^{3+}−peptide complex, and 6.0 for the iron sulfate and Fe^{3+}−peptide complex solutions containing free peptide.

Antianemic Activity in Newly Weaned Rats with Anemia Induced by Low Diet Iron. The hemoglobin repletion model and the basal diet used in this experiment were described by the AOAC (*21*). Anemia was induced by reducing the iron in the basal diet to 8 μg of Fe/g of diet (low-iron diet). In the depletion period, newly weaned rats (50−55 g)

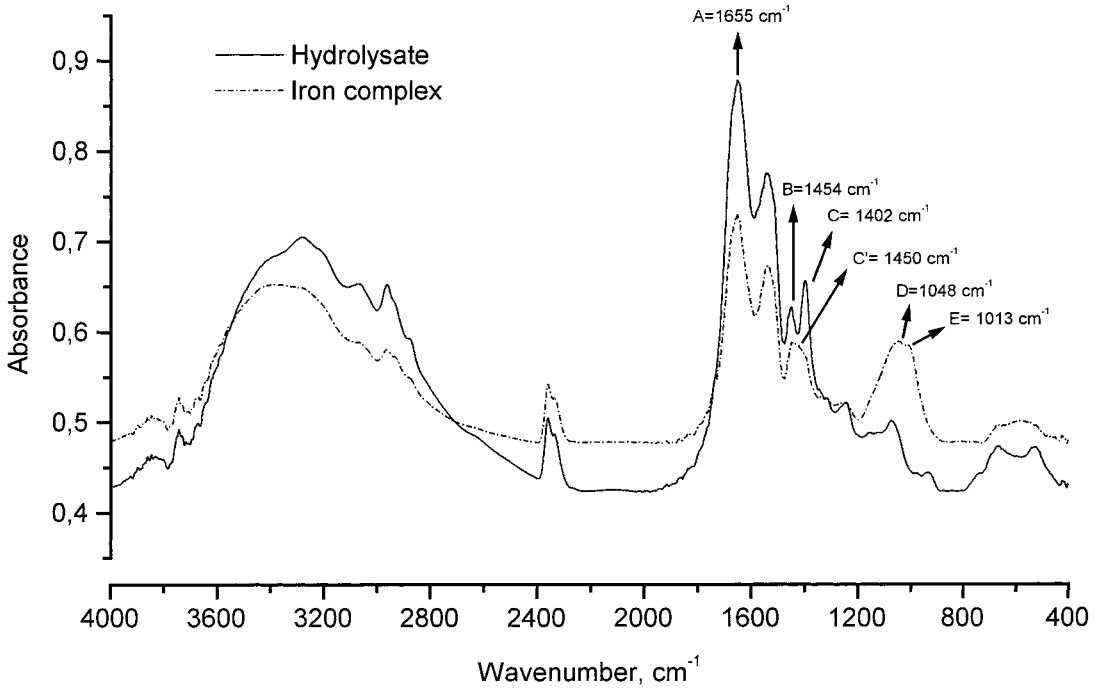

Figure 1. Infrared spectra of casein hydrolysate and the Fe^{3+}–peptide complex. The major iron binding sites were the carboxyl groups (movement of the C band and occurrence of the D–E band) and the peptide bond (narrowing of the high-frequency region) of the casein hydrolysate.

were maintained in individual stainless steel metabolic cages, at 25 °C, with a 12 h dark−light cycle, with low-iron diet for 35 days. Blood was obtained from the tail vein, collected in heparinized tubes, and hemoglobin was determined according to a cyanometahemoglobin method (*22*). The animals were separated into three groups to provide similar hemoglobin levels. In the repletion period (14 days), one group was maintained with the same diet (low-iron diet) as used during the depletion period (control group). One group received diet supplemented with iron sulfate (25 μg of Fe/g of diet), and another received diet supplemented with the Fe^{3+}−peptide complex (25 μg/g of diet). Diets and water were provided ad libitum. At the end of the repletion period, animals were weighed, and blood was collected to determine hemoglobin levels.

The hemoglobin iron was calculated as described by Mahoney et al. (*23*), assuming that the total blood volume of the rat was 6.7% of the body weight and the hemoglobin iron was 3.33 mg of Fe/g of hemoglobin. Bioavailability was calculated by the transfer from diet iron to hemoglobin iron, also according to Mahoney et al. (*23*), by the ratio of gain in hemoglobin iron (milligrams) and total iron ingested during the repletion period; bioavailability is reported as percent. The relative bioavailability of the Fe^{3+}−peptide complex was calculated as the ratio between percent bioavailability of the Fe^{3+}−peptide complex and percent bioavailability of iron sulfate.

Statistical Analysis. The statistical analysis was carried out by using the statistical package STATISTICA for Windows, version 4.5. ANOVA was performed on log transformed data when variances were unequal, followed by Duncan's test for multiple comparison of means.

RESULTS

Preparation of the Fe^{3+}—Peptide Complex. The composition of the complex obtained by the addition of different amounts of Fe^{3+} to the peptide solution is documented in **Table 1**, which reports the measured and expected iron content of the insoluble complex obtained by reducing the pH from 7.8 to 3.5. The KSCN reaction for free Fe^{3+} in the supernatant was negative from 1.0 to 2.5 g of $FeCl_3 \cdot 6H_2O$/10 g and positive above 2.6 g of $FeCl_3 \cdot 6H_2O$/10 g. The fact that the iron content measured by atomic absorption spectroscopy was 0.29−0.47% higher than expected was due to the presence of 0.3% Fe^{3+} in the peptide hydrolysate. Although products with >5.61% iron could be obtained with >2.8 g of $FeCl_3 \cdot 6H_2O$/10 g, the preparation of the complex was standardized on the basis of 2.5 g of $FeCl_3 \cdot 6H_2O$/10 g and the positive KSCN reaction in order to avoid washing the precipitate to remove loosely bound iron after pH adjustment to 3.5. When soluble ferric ions react with potassium thiocyanate, an intensely red complex develops; therefore, we utilized this reaction to verify the presence of ferric ions.

Table 1. Iron Content in the Fe^{3+}–Peptide Complexes Prepared with Different Relative Amounts of $FeCl_3 \cdot 6H_2O$[a]

mass (g) of $FeCl_3 \cdot 6H_2O$/10 g of peptide	KSCN reaction in the filtrate	measured iron concn (%) in the precipitate	expected iron concn (%) in the precipitate
1.0	negative	2.36	2.06
2.0	negative	4.45	4.13
2.2	negative	4.83	4.54
2.4	negative	5.42	4.95
2.5	**negative**	**5.61**	**5.13**
2.6	positive	5.61	5.39
2.8	positive	5.69	5.78
3.0	positive	5.84	6.19
4.0	positive	7.76	8.26

[a] $FeCl_3 \cdot 6H_2O$ was added dropwise to a stirred solution of peptide mixture (10%, w/v), maintained at pH 7.8 by the addition of NaOH. After an additional 30 min, the pH of the solution was reduced to 3.5 with 0.1 N HCl, the precipitate was filtered, and its iron content was determined by atomic absorption spectroscopy. The expected amount of iron in the precipitate was calculated on the basis of the total Fe^{3+} and peptide, assuming all had been precipitated. Data are reported as the mean of three independent experiments.

Characterization of the Fe^{3+}—Peptide Complex by Infrared Spectroscopy. Figure 1 shows the infrared spectra of the peptide hydrolysate and the Fe^{3+}−peptide complex. The absorption bands in **Figure 1** at 1656 cm^{-1} (A), 1454 cm^{-1} (B), and 1402 cm^{-1} (C) can be assigned to vibrations of the COO^- groups with (B) also reflecting the contribution of the N−H of the amide bond. Upon coordination with Fe^{3+} the C band corresponding to the carboxyl group is shifted from 1402 to 1450 cm^{-1} and denoted C′. Furthermore, an additional band

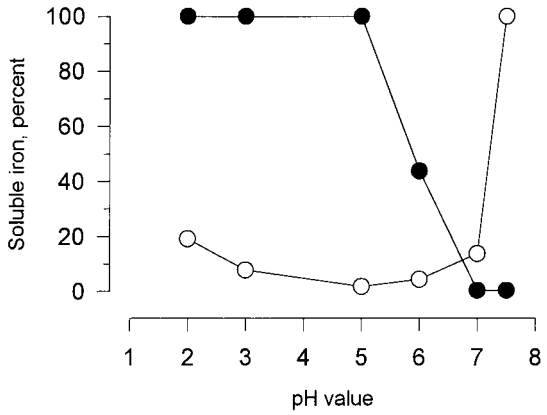

Figure 2. Solubility of the Fe^{3+}–peptide complex. The aqueous dispersions (1 mg/mL) of Fe^{3+}–peptide complex (○) or iron sulfate (●) had their pH adjusted to 2.0 and 3.0 with 0.1 N HCl and to 5.0, 6.0, 7.0, and 7.5 with 0.5 M $NaHCO_3$. The dispersions were kept at 37 °C under constant shaking for 90 min.

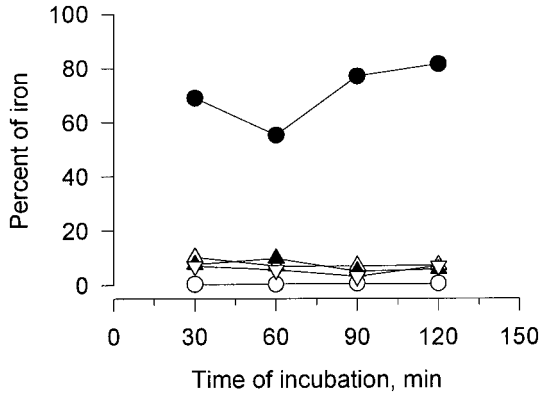

Figure 3. Iron release from the Fe^{3+}–peptide complex. NaCl (○), cysteine (●), ascorbic acid (△), and citric acid (▲) at a molar ratio of 1:3 and EDTA (▽) at a molar ratio of 1:1 were added to an aqueous dispersion of the Fe^{3+}–peptide complex (10 mg/mL, pH 5.0). Iron release from an aqueous dispersion of the Fe^{3+}–peptide complex was the same as that observed after the addition of NaCl.

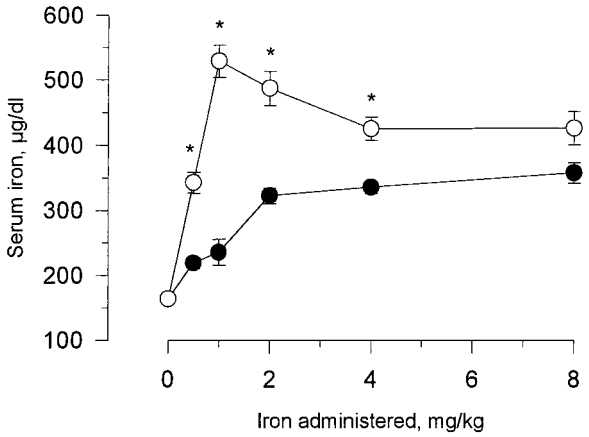

Figure 4. Serum iron after oral administration of the Fe^{3+}–peptide complex or iron sulfate to rats. An aqueous dispersion of the Fe^{3+}–peptide complex (○) or iron sulfate (●) was administered by gastric gavage to rats ($n = 21$ for controls and $n = 9$ for each dose of iron) as 0.0, 1, 2, 4, and 8 mg of Fe equiv/kg. After 2 h, the rats were sacrificed under ether anesthesia, and the blood was obtained by cardiac puncture for iron determination by a colorimetric method using ferrozine. Data are reported as means ± SEM.

D−E (range of 1048−1013 cm^{-1}) appears in the chelate spectrum, which can be attributed to C−O−Fe. Absorption at high frequency (3300 cm^{-1}) at the N−H and O−H bonds refers to water of hydration. With the coordination of Fe^{3+} the Fe−N bond replaces N−OH (hydrogen bonds) and is responsible for the sharpening of the 3300 cm^{-1} band, more visible in the transmittance plot. These data suggest that the principal site of Fe^{3+} bonding corresponds primarily to the carboxylate groups and to a lesser extent to the peptide bonds.

Solubility of the Fe^{3+}—Peptide Complex. The solubility of the iron present in the complex as a function of pH is shown in **Figure 2** together with similar data for iron sulfate. Below pH 6.0 most of the iron of the Fe^{3+}−peptide complex was insoluble, whereas the iron of iron sulfate was soluble. Above pH 7.0 the inverse was true. Total soluble iron was determined by atomic absorption spectroscopy. Furthermore, the iron of the Fe^{3+}−peptide complex was qualitatively KSCN negative at all pH values, indicating that the iron is not free. The figure demonstrates that the iron of the Fe^{3+}−peptide complex differs from that of iron sulfate in valence and solubility and that it is chelated at pH values from 2.0 to 7.5.

Release of Iron from the Fe^{3+}—Peptide Complex by NaCl and Ligand Agents. When a dispersion of the Fe^{3+}−peptide complex was incubated in water or NaCl at pH 5.0 for up to 2.5 h, none of the iron (<0.6%) was released into the solution (**Figure 3**), indicating that the iron was strongly bound. Similarly, neither ascorbic acid, citric acid, nor EDTA, all potential ligands of Fe^{3+}, released >15% of the bound iron. In contrast, cysteine competed effectively with the peptide(s) and mobilized 60−80% of the iron. The lower value at 60 min was observed in three experiments and may be related to the fact that the Fe^{3+}−peptide complex was insoluble at this pH (see **Figure 2**), and thus the reaction was heterogeneous; that is, it has soluble and insoluble components.

Iron Absorption from the Fe^{3+}—Peptide Complex in Vivo. The data in **Figure 4** demonstrate that iron from the Fe^{3+}−peptide complex, administered by gastric gavage, is transferred to the blood of rats in 2 h in a dose-dependent manner, increasing the serum iron levels when compared to iron sulfate. The Fe^{3+}−peptide complex transferred significantly more iron to the serum at doses of 0.5, 1, 2, and 4 mg/kg.

Comparison of the Kinetics of Iron Absorption from the Fe^{3+}—Peptide Complex and Iron Sulfate. The data in **Figure 5** show the kinetics of iron absorption in rats, evaluated by the increase of serum iron, from Fe^{3+}−peptide complex or iron sulfate, both administered at a dose of 4 mg of Fe/kg. The same maximal absorption levels were reached within 30 min after administration for both iron sources (495.31 ± 11.45 and 476.64 ± 7.92 for iron sulfate and the Fe^{3+}−peptide complex, respectively). However, from 1 to 4 h, administration of the Fe^{3+}−peptide complex promoted a higher elevation of serum iron level compared to iron sulfate ($p < 0.05$). The plateau observed between 1 and 4 h after the oral administration of the Fe^{3+}−peptide complex lasted longer than that observed after iron sulfate administration. The basal levels of serum iron were reached within 8−12 h for both iron sources. The $AUC_{0\rightarrow 12h}$ of the Fe^{3+}−peptide complex was significantly higher ($p < 0.05$) than that observed with iron sulfate (2678.37 ± 103.17 vs 2312.57 ± 80.86, respectively).

Effect of Free Peptides on Iron Absorption from the Fe^{3+}—Peptide Complex and Iron Sulfate. The presence of increasing concentrations of peptides in the Fe^{3+}−peptide complex or iron sulfate dispersions administered to rats by gavage did not modify the serum iron levels obtained with both iron sources (**Figure 6**). The animals that received the Fe^{3+}−

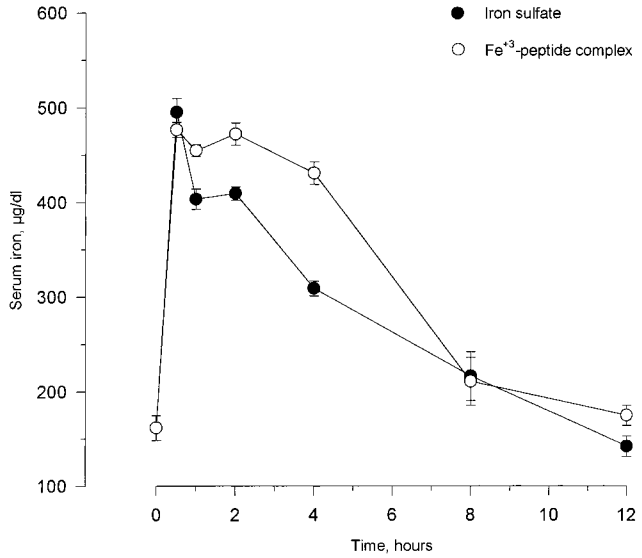

Figure 5. Comparative kinetics of iron absorption after oral administration of the Fe^{3+}–peptide complex or iron sulfate to rats. A solution containing 4 mg of Fe/kg from iron sulfate or the Fe^{3+}–peptide complex was administered to rats ($n = 9$) in a final volume of 5.0 mL by gastric gavage. Blood was collected by cardiac puncture after 0.5, 1, 2, 4, 8, and 12 h, and serum iron was determined by colorimetric reaction with ferrozine. The vertical lines in the points indicate the SEM.

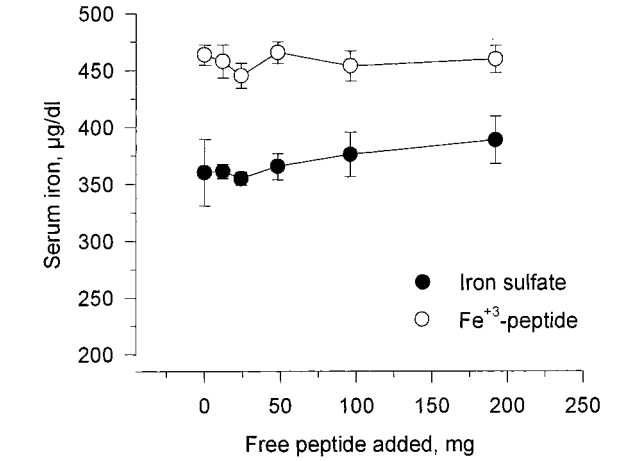

Figure 6. Effect of free peptides on the iron absorption from the Fe^{3+}–peptide complex or iron sulfate in rats. A solution containing increasing amounts of 4 mg of Fe/kg and free peptide was administered to rats ($n = 6$) by intragastric gavage, in a final volume of 0.5.0 mL (pH 4.12 for iron sulfate and pH 3.5 for the Fe^{3+}–peptide complex and for the casein hydrolysate dispersions). Blood was collected after 2 h by cardiac puncture, and serum iron was determined by colorimetric reaction with ferrozine. The vertical lines in the points indicate the SEM.

peptide complex presented higher serum iron levels than the rats that received iron sulfate ($p < 0.010$, Duncan's test following ANOVA).

Antianemic Activity in Newly Weaned Rats with Anemia Induced by a Low-Iron Diet. The antianemic activity was evaluated as percent of bioavailability of the Fe^{3+}−peptide complex compared to iron sulfate. **Table 2** shows that all groups presented weight gain in the repletion period, with the control group presenting the smallest weight gain, as expected. Hemoglobin levels were higher in groups supplemented with the Fe^{3+}−peptide complex or iron sulfate (10.88 ± 1.51 and 12.36 ± 0.41, respectively) compared to the control group, although the difference was not significant. The efficacy of conversion from dietary iron to hemoglobin iron was statistically similar for both iron sources but slightly lower for the Fe^{3+}−peptide complex (62.9) compared to iron sulfate (69.4).

Table 2. Bioavailability of Iron from Fe^{3+}–Peptide Complex Compared to Iron Sulfate, Evaluated by Hemoglobin Recovery in Anemic Rats

	groups		
parameter	control	Fe^{3+}–peptide	$FeSO_4$
Fe, mg/kg	8.0	25.0	25.0
initial wt, g	204.1 ± 7.1	189.7 ± 11.0	210.7 ± 20.4
final wt, g	230.4 ± 5.8	263.7 ± 21.1	274.5 ± 9.7
Δ (final – initial)	25.5	73.9	63.8
initial hemoglobin, g/dL	6.4 ± 0.4	5.8 ± 0.3	5.9 ± 0.2
final hemoglobin, g/dL	5.7 ± 0.1	10.9 ± 0.6	12.4 ± 0.2
Δ (final – initial)	–0.7	5.1	6.5
diet consumption, g	206.4	260.4	278.0
ingested iron, mg	1.65	6.52	6.95
hemoglobin iron, mg		4.10	4.82
bioavailability, %		62.9	69.4
% of bioavailability compared to iron sulfate		91	

[a] Data are presented as mean ± SEM ($n = 6$). The rats were 56 days old when the 14 day repletion period began.

DISCUSSION

We describe the preparation of a new class of iron chelates based on the reaction of $FeCl_3 \cdot 6H_2O$ with a mixture of peptides obtained by the enzymatic hydrolysis of casein.

The rationale for this approach is based on the known ability of carboxylate groups from amino acids and proteins to complex with iron (*24*) and the studies in which carboxyl groups are introduced into casein by reaction with succinic anhydride (*5*) and into lysozyme by reaction with glutaric anhydride (*13*). This approach was facilitated by the fact that we already have a methodology for preparing partial enzymatic hydrolysates and because this hydrolysate was biochemically (*15*) and nutritionally (*25*) characterized. Therefore, the use of this hydrolysate and its iron derivative in human beings should not raise toxicological concerns.

Although not a homogeneous compound, we characterized the Fe^{3+}−peptide complex in terms of infrared spectroscopy, solubility as a function of pH, transfer of iron to other chelating compounds, and absorption from the gastrointestinal tract to the blood of rats and compared some of the properties to those of $FeSO_4$.

The infrared spectra (**Figure 1**), solubility experiments (**Figure 2**), and competition experiments (**Figure 3**) indicated that the iron of the Fe^{3+}−peptide complex is Fe^{3+} and is in the form of a tightly bound chelate even when soluble (**Figure 2**). When insoluble at pH 5.0, the Fe^{3+} cannot be released from the Fe^{3+}−peptide complex by NaCl or even by weak chelating/reducing agents such as ascorbic and citric acid or EDTA. Indeed, of the compounds tested only cysteine competed effectively and solubilized the iron from the insoluble complex, suggesting that the complex is not so strong as to prevent transfer of iron to metal-binding proteins in the intestinal epithelium or in the cytoplasm (*26*).

The differences in the solubility of the Fe^{3+}−peptide complex and $FeSO_4$ deserve special comment. As shown in **Figure 2**, $FeSO_4$ is soluble at the acid pH observed in the stomach. In contrast, the Fe^{3+}−peptide complex would be expected to be insoluble in the stomach, and even when solubilized in the duodenum by its approximately neutral pH, it is important to note that the Fe^{3+} is still chelated. This difference between free

and chelated iron may be important for some of the side effects of $FeSO_4$ in vivo because it is believed that free-form Fe^{2+} at acid pH may be responsible for injury to the gastric mucosa (*7*).

We compared the biological effects in rats of the Fe^{3+}−peptide complex to iron sulfate, because the latter is widely used in the prevention and/or treatment of iron deficiency anemia. No technique or animal model is perfect for bioavailability studies of compounds for therapeutic or nutritional use. The rat model is limited as a model for human beings by differences in eating behavior, energy expenditure per body area, and heme iron absorption (*27*). However, data obtained in parallel mineral studies on humans and rats were generally consistent (*28*). The determination of serum iron after oral iron administration to intact animals under normal conditions may be considered to be an index of iron transfer from the intestinal lumen to the blood compartment. This transfer ultimately includes iron uptake by enterocytes, cytoplasmic transit, and true transfer to the blood compartment.

In the present study, we demonstrated that the transfer of iron from the iron−peptide complex to blood was somewhat more effective (10−30%) than $FeSO_4$ at low doses of 0.5−4 mg of Fe/kg in increasing serum iron levels during the 2 h period following administration by gastric gavage. Pizarro et al. (*29*), using milk fortified with ferric glycinate, demonstrated that iron absorption was 13.1 and 9.6% at doses of 6 and 15 mg/L, respectively, in healthy humans with hemoglobin levels >12 g/L. These investigators suggested that iron absorption as ferric glycinate may be controlled by iron stores.

Because in our study iron was present in the complexed form, the greater transfer of iron to the blood compartment from the Fe^{3+}−peptide complex may be attributed to other uptake processes of higher affinity, such as receptor-mediated endocytosis, as is the case for the process of heme iron uptake, considered to represent the most bioavailable form of iron (*26*) or to a carrier-mediated process in the membrane (*30*). Because soluble free iron can produce free radicals and induce membrane lipid peroxidation (*31*) or peroxidation of proteins or DNA (*5*, *6*), the increased amount of intracellular iron after doses >4 mg of Fe/kg may simultaneously inactivate iron-responsive proteins (*32*) or inhibit the intracellular transport system (*33*), thus reducing net iron transfer to blood.

It should be remembered that non-heme iron from the human diet is mostly presented as Fe^{3+}, which precipitates at pH >3.0 if it is not reduced and complexed or chelated (*26*). A generally accepted theory is that iron needs to be in the ferrous state to be readily absorbed, simply because the pH of the intestinal lumen, particularly at the site of the brush border surface, is near neutral or even slightly alkaline due to the mucus layer protecting the enterocytes. Ferrous iron is the only form that is soluble at that pH. Thus, the bioavailability of iron(III) is conditioned to its solubility at duodenal pH. These considerations, taken together with the results illustrated in **Figure 2**, may favor the idea of a potential therapeutic use of the iron−peptide complex. In vitro experiments have suggested some processes of inorganic iron uptake, among them electrogenic Fe(III) transport (*34*), the formation of Fe(II) complexes with free fatty acids facilitating their transfer through the membranes (*35*), Fe(III) binding to membrane proteins such as glycoproteins (*36*), integrins (*37*), melanotransferrin (*38*), or major histocompatibity class I molecules (*39*), in addition to paracellular absorption (*26*). There still is no conclusive evidence that might permit us to state that iron is taken up in the II or III form (*26*, *40*), although the activity of ferrireductases involved in the process of iron absorption (*26*, *40*) or in cytoplasmic transit (*30*) has been reported.

The comparative kinetic data (**Figure 5**) obtained after oral administration of the Fe^{3+}−peptide complex or iron sulfate to rats demonstrated a maximal absorption 30 min after administration of both iron sources, not observed for ABC1020 or ITF282, which presented a T_{max} of ∼2 h (*5*). However, Franzone et al. (*13*) and Cremonesi and Caramazza (*5*) have used a low dose of iron compounds (1 and 2 mg of Fe/kg, respectively) compared to our study (4 mg of Fe/kg). We believe that the kinetic pattern can be relatively compared on the basis of the results obtained for iron sulfate, which were similar in these studies and in ours. The Fe^{3+}−peptide complex also presented higher serum iron levels compared to iron sulfate, 1 h after administration, which persisted for as long as 4 h. This pattern was not observed for ABC1020, which produced lower serum iron levels compared to iron sulfate. The kinetic pattern of ITF282 was similar to ours, with a long duration of high serum iron levels, but this pattern were equal to or lower than that of iron sulfate, except at 4 h, when the levels were higher. The pharmacokinetic parameter $AUC_{0\rightarrow12}$ was significantly higher for the Fe^{3+}−peptide complex compared to iron sulfate. For ITF282 and ABC1020, the AUC values were smaller than that for iron sulfate (*5*, *13*).

It has been proposed that amino acid chelates could be absorbed like peptides in the jejunum rather than as non-heme iron in the duodenum (*41*). Our results do not support this hypothesis because the increase of peptide concentration did not increase iron absorption from ferrous sulfate or reduce the iron absorption from the Fe^{3+}−peptide complex. On the other hand, if this absorption mechanism were to be confirmed, there would be a risk of iron overload for the population in iron supplementation programs. In our experiment, increasing the free peptide concentration did not alter the profile of serum iron from the Fe^{3+}−peptide or iron sulfate (**Figure 6**), indicating that the complexation did not occur during the administration or during the gastrointestinal transit time. The Fe^{3+}−peptide complex itself is absorbed more than iron sulfate, and other mechanisms of iron absorption should be proposed for this case.

The efficacy of the Fe^{3+}−peptide complex in hemoglobin regeneration was demonstrated in hemoglobin repletion experiments in anemic rats. Its efficacy in converting dietary iron to hemoglobin iron was similar to its efficacy in converting iron sulfate to hemoglobin iron, like the other compounds described in the literature, that is, ITF282 (*5*), ABC1020 (*13*), and iron glycine (*42*), which presented the same efficacy as iron sulfate.

The Fe^{3+}−peptide complex utilized in our study is composed of nutrients (casein hydrolysate) that are utilized by the body. Moreover, the iron remains chelated in conditions similar to those occurring in the gastrointestinal tract, thus suggesting that the complexed iron is less aggressive to the gastrointestinal mucosa and less reactive with other food components. These data, taken together, suggest that the Fe^{3+}−peptide complex could be a potential iron delivery source in programs of treatment or supplementation of patients with iron deficiency anemia.

ACKNOWLEDGMENT

We gratefully acknowledge Dr. Lewis Joel Greene for useful discussions of the research.

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Received for review August 21, 2001. Revised manuscript received October 30, 2001. Accepted October 30, 2001. We gratefully acknowledge Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) for research support and Fundação Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for granting a Master's fellowship to M.V.C.

JF0111312