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Iron speciation in intestinal contents of rats fed meals composed of meat and nonmeat sources of protein and fat*

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Our objective was to test the hypothesis that interactions of iron with protein and fat digestion products are responsible for the iron-absorption-enhancing properties of meat. Rats were fed meals labeled with $^{59}FeCl_3$ and ^{14}C-oleic acid, formulated from various combinations of protein and fat sources (lean beef, skim milk or egg white; tallow, butter or vegetable shortening). After 30 min, iron absorption was determined, and stomach and intestinal contents were collected and fractionated by gel filtration chromatography . Iron absorption was highest from the beef+tallow and beef+shortening meals. Different protein sources produced different chromatographic profiles of peptides in the intestinal lumen. Soluble iron in intestinal contents was associated with peptides, but not with amino acids, fatty acids or bile salt micelles. Results suggest that specific peptides produced during protein digestion may be one factor influencing iron bioavailability.

ABREVIATIONS

Abbreviations for the treatment groups: BB — lean beef + butter; BS — lean beef + vegetable shortening; BT — lean beef + tallow; ET — egg white + tallow; ES — egg white + vegetable shortening; MB — skim milk + butter; MT — skim milk + tallow; MS — skim milk + vegetable shortening.

INTRODUCTION

Meal composition is an important determinant of nonheme iron absorption. Ascorbic acid and meat enhance (Cook & Monsen 1976; Lynch & Cook, 1980), while dietary fiber, tea, phytate, casein and calcium salts inhibit iron absorption (Cook & Monsen, 1976; Hallberg & Rossander, 1982; Dawson-Hughes *et al.*, 1986, Siegenberg *et al.*, 1991; Torre *et al.*, 1991). Dietary factors which enhance iron absorption may form complexes with lumenal iron that remain soluble in the digesta. Dietary factors which inhibit iron absorption may promote iron polymerization and/or precipitation or may bind iron in complexes which do not release iron to mucosal receptors. Thus, knowledge of the speciation of iron in the gastrointestinal lumen is crucial to our understanding of the mechanisms involved in the enhancement or inhibition of iron absorption by various dietary factors. Few studies have investigated iron speciation in intestinal contents following a meal.

Our objective was to characterize iron species formed during *in-vivo* digestion. Meals composed of ferric citrate mixed with various combinations of protein and fat sources were labeled with $^{59}FeCl_3$ and ^{14}C-oleic acid and fed to rats. After 30 min, iron solubility, valence and association with selected digestion products were examined in lumenal digests.

MATERIALS AND METHODS

The study protocol was approved by the Institutional Animal Care and Use Committee at Cornell University.

Male Sprague Dawley rats (Camm Research Institute, Wayne, NJ) aged 52 days, left over from a previous study of the effects of protein and fat sources on iron absorption (Kapsokefalou & Miller, 1993), were used. The rats had been fed diets composed of various combinations of protein (lean beef, skim milk and egg white) and fat (tallow, butter and vegetable shortening) sources for 31 days at the time of the current study. All diets contained 200 g protein/kg and 200 g fat/kg and met or exceeded nutrient levels recommended for the rat (AIN, 1980). Iron was added as ferric citrate so that the final nonheme iron concentration in all diets was

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Table 1. Body weights and hemoglobin concentrations for the diet groups on the day of the experiment[a]

Group[b]	Weight (g)	Hb (g/liter)
BT	320·38	130·8
BB	304·35	131·0
BS	351·55	132·6
MT	321·01	130·5
MB	311·72	130·3
MS	302·07	132·5
ET	332·22	131·6
ES	330·32	131·8
Pooled SEM	21·82	10·5

[a]Mean (n = 4) for each diet group and pooled SEM (standard error of the mean).
[b]Abbreviations for the treatment groups — see text.

42 mg/kg. Details of diet composition and animal housing and care were reported previously (Kapsokefalou & Miller, 1993). Body weights and hemoglobin concentrations are shown in Table 1. Due to the time-consuming procedures involved, only four rats per treatment group could be managed. These rats were selected from groups of eight on the basis of iron absorption measured in the previous study so that iron absorption from each rat was similar to the mean of its diet group.

Each rat was studied according to the following protocol. The rat was deprived of food for 14 h. On the day of the experiment the rat was counted for ^{59}Fe activity remaining from the previous experiment. A 2 g meal from each rat's respective diet was diluted with 2 ml of water and mixed with 0·1 ml of ^{59}Fe-labeled $FeCl_3$ solution (314 MBq/liter in 0·1 mol/liter HCl) and 0·2 ml of $^{14}C_1$-labeled oleic acid solution (222 MBq/liter in toluene) (IFS1 and CFA243, respectively, Amersham, Arlington Heights, IL), held for 10 min to allow the toluene to evaporate, and offered to the rat. Meals were offered at timed intervals so that the digestive period was the same for all rats. All animals consumed their meals within 10 min. Thirty minutes after consumption of the meal the rat was anesthetized with methoxyflurane (Metofane^R, Pitman-Moore, Mundelein, IL). A longitudinal incision along the midline of the abdomen was made and the stomach and the small intestine were exposed. The esophagus and the pylorus were clamped with scissor clips and the stomach was removed from the carcass. The whole small intestine, from the pylorus to the ileocecolic orifice, was exposed, clamped with scissor clips, and removed from the carcass. Intestinal contents were removed by holding the intestine in a vertical position and gently squeezing between the thumb and forefinger to expel the contents into a centrifuge tube. The segment was flushed with 2 ml of 9 g/liter NaCl, 2 mmol/liter sodium taurodeoxycholate. (Sodium taurodeoxycholate at its critical micellar concentration (2 mmol/liter) was added to the flushing solution to protect bile salt micelles from disruption due to dilution.) Flushings were added to the intestinal contents. Stomach contents were collected in a separate centrifuge tube.

The above procedure was repeated for all four rats of each group. Intestinal contents of the four rats from each group were pooled. It was necessary to pool the intestinal contents in order to obtain samples large enough for further analysis. Stomach contents were pooled separately from the intestinal contents. The pooled stomach and intestinal contents of the four rats of each group were centrifuged at 10000 g for 20 min at 4°C. The supernatant was decanted and collected.

It should be mentioned that the 30 min digestive period was chosen after preliminary experiments showed that shorter digestion periods left most of the iron in the stomach and longer digestion periods allowed most of the iron to be absorbed, at least in the lean beef treatments.

Analysis of gastric and intestinal contents

Pellets and supernatants of the intestinal and stomach contents, emptied intestinal segments, emptied stomachs and carcasses (intact rat, including blood, minus stomach and small intestine) were counted in a small animal whole-body γ-counter at a window setting of 0·30 to 1·500 meV. Ferrous and total soluble iron were determined spectrophotometrically with ferrozine (Kapsokefalou & Miller, 1991) in all supernatants.

A 4 ml aliquot of the pooled intestinal supernatant was applied to a Sephacryl S-100 HR column (Pharmacia XK16, 70 cm length, Pharmacia LKB Biotechnology, Piscataway, NJ). The column was eluted with 0·15 mol/liter PIPES buffer (pH 7·0), 2 mmol/liter sodium taurodeoxycholate, 0·15 mol/liter NaCl, 0·2 g/ml NaN_3 at 1 ml/min using a pump (2132 MicroPerpex peristaltic pump, Pharmacia LKB Biotechnology). Fractions of 1·5 ml were collected. A mixture of ovalbumin (A5503, Sigma Chemical, St. Louis, Mo), two styrene polymers (Polysciences, Warrington, PA) and phenylalanine (P2126, Sigma Chemical), (Molecular weights (MW) 43000, 8000, 5400 and 165·2, respectively) were used to standardize the column. The void volume was determined with Blue Dextran 2000 (2 g/liter, Pharmacia LKB Biotechnology). Elution of the standards and of Blue Dextran was monitored at 260 and 640 nm, respectively.

Elution of iron was monitored by counting fractions for ^{59}Fe activity in a small sample γ-counter (Packard Minaxi 5530, Packard Instrument, Downers Grove, IL). Elution of lipids was monitored by counting for ^{14}C activity according to the following protocol: 0·4 ml of the collected fractions were mixed with 4 ml of Ecoscint, a liquid scintillation cocktail (LS271, National Diagnostics, Manville, NJ) and counted in a liquid scintillation counter (Packard Minaxi 4030, Packard Instrument, Downers Grove, IL). The instrument provided disintegrations/minute (dpm) after automatic correction for quenching. Disintegrations from ^{14}C were corrected for the contribution of ^{59}Fe. Elution of protein and protein digestion products was monitored at

280 nm. Elution of micelles was monitored using the Rhodamine 6G spectral shift method, as modified from Carey & Small (1969): a 0·5 ml aliquot of the eluted fraction was mixed with 0·5 ml of Rhodamine 6G (2·5 μmol/liter). The spectrum of the mixtures was scanned and the wavelength of maximum absorption was recorded (Spectronic 1001, Milton Roy for Baush and Lomb, Rochester, NY). In the presence of micelles, the maximum wavelength shifts towards higher values. The spectral shift results from the interaction of Rhodamine G molecules with the bile salt micelles.

Calculations

Iron absorption, extent of stomach emptying, and the distribution of ^{59}Fe in soluble and insoluble fractions of stomach and intestinal contents were calculated from ^{59}Fe activities found in the respective compartments. Results are expressed as a percentage of ingested ^{59}Fe. Formulas used for the calculations are shown below:

$$\% \, Fe_{Abs} = \frac{Fe_{Car}}{Fe_{Ingest}} \times 100$$

$$\% \, Fe_{StC,I} = \frac{Fe_{StC,P} + Fe_{St}}{Fe_{Ingest} \times 4} \times 100$$

$$\% \, Fe_{StC,S} = \frac{Fe_{StC,Sup}}{Fe_{Ingest} \times 4} \times 100$$

$$\% \, Fe_{IntC,I} = \frac{Fe_{IntC,P}}{Fe_{Ingest} \times 4} \times 100$$

$$\% \, Fe_{IntC,S} = \frac{Fe_{IntC,Sup}}{Fe_{Ingest} \times 4} \times 100$$

where Fe = ^{59}Fe; Abs = absorption; Car = carcass (whole body including blood and empty intestines minus stomach); St = empty stomach; Ingest = ingested (note: $Fe_{Ingest} \times 4$ is used where samples from four rats were pooled); IntC = intestinal contents; StC = stomach contents; S = soluble; I = insoluble; P = pellet; Sup = supernatant.

Since iron absorption occurs primarily in the intestine and not in the stomach (Conrad, 1987), activity in the empty stomachs was included in the insoluble iron fraction based on the assumption that residual ^{59}Fe activity in the flushed stomach represented unabsorbed iron trapped in the folds of the stomach. Activity in the flushed intestinal segments was included in the calculation of iron absorption based on the assumption that it represented iron that had been taken up by the mucosal cells, but had not yet been transferred to the circulation.

Statistical analysis

Because of pooling, solubility and valence data were not statistically analyzed. Animal carcasses and empty intestines were counted separately, therefore iron absorption data were statistically analyzed with ANOVA. ANOVA and regression analysis of the data (Snedecor & Cochran, 1980) were performed using Minitab software (Minitab, State College, PA). The confidence interval was 95% ($p < 0.05$).

RESULTS

The distribution of ingested ^{59}Fe activity between the carcass and the gastrointestinal lumen and the partitioning of ^{59}Fe among the soluble and the insoluble phases in stomach and intestinal digesta are presented in Fig. 1. Low recovery of ingested ^{59}Fe (around 85%) resulted from geometry differences between the meal and the samples. Iron absorption (percentage of ingested ^{59}Fe present in the carcass and empty small intestine) ranged from 4·6 to 19·7% and was more than two-fold higher from the BT and the BS meal compared with the other meals ($p < 0.05$). In both the stomach and the intestinal digesta, most of the iron was associated with the insoluble fraction, with the exception of the egg white meals (Fig. 1). An appreciable fraction of the soluble iron in the intestinal digesta of all treatments was in the ferrous form (Table 2), but digesta from animals fed the BT and the BS meals contained approximately twice as much as the others. There was no correlation between soluble iron in the intestinal lumen and iron absorption (r = 0·220, p = 0·601), but there was strong correlation between soluble ferrous iron and iron absorption (r = 0·980, $p > 0.001$, Fig. 2).

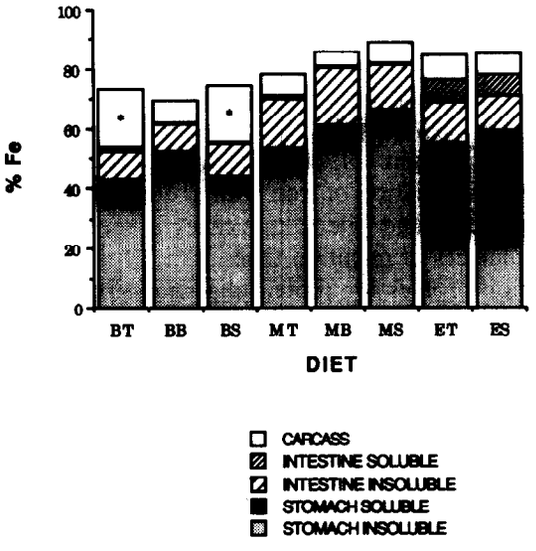


Fig. 1. Percentage of the administered iron found in the insoluble and the soluble fractions of the pooled stomach and intestinal digesta and in the carcasses of rats 30 min after they were fed ^{59}Fe-labeled meals. *Iron in the carcass was significantly higher in these groups ($p < 0.05$).

Table 2. Percentage of total iron present in the ferrous form in the soluble fraction of the stomach and intestinal contents of rats 30 min after they were fed ^{59}Fe-labeled meals of the experimental diets[a]

Group[b]	Fe(II) (%)	
	Stomach	Intestine
BT	57·30 ± 0·02	96·05 ± 0·70
BB	35·30 ± 0·03	41·87 ± 1·05
BS	43·50 ± 0·10	100·21 ± 1·10
MT	45·63 ± 0·83	32·71 ± 0·83
MB	38·54 ± 0·05	36·48 ± 0·78
MS	44·34 ± 1·23	47·82 ± 0·34
ET	50·98 ± 1·02	51·77 ± 0·36
EB	51·76 ± 0·18	45·65 ± 1·17
ES	52·67 ± 0·62	40·33 ± 0·54

[a]Mean and standard deviation of two determinations from the same pooled sample.
[b]For abbreviations for the dietary groups see text.

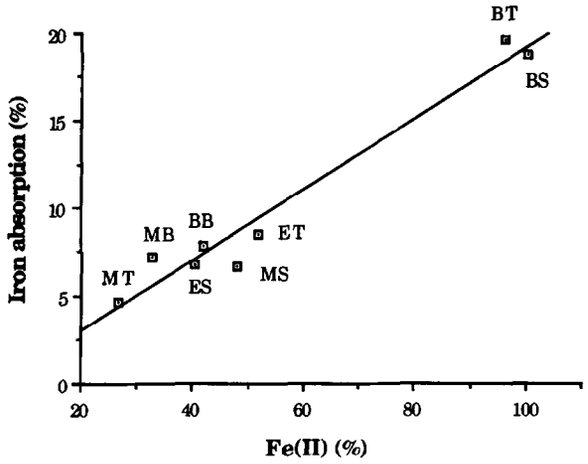


Fig. 2. Correlation of total soluble iron present in the ferrous form in the intestinal digesta with iron absorbed from the various meals.

Chromatograms of the soluble intestinal contents from rats fed the various meals are shown in Figs. 4–11. Comparisons of these chromatograms with standards (Fig. 3) yield information about the approximate molecular weight of iron complexes. In addition, apparent association of iron with protein and fat digestion products and bile salt micelles is revealed, based on similarities of retention times.

Elution profiles for protein digestion products (A_{280}) show a range of peptide molecular weights (Figs. 4–11). It is notable that profiles of the three protein sources are different and that the fat source had some effect on the size of the peptides within a given protein source. Iron-59 associated primarily with two of the beef protein peaks (MW > 10000 and 10000 < MW < 1000). In the milk digesta, ^{59}Fe was associated primarily with one peak (5000 < MW < 10000). In egg white digesta, ^{59}Fe was associated primarily with a higher molecular weight peak. No iron eluted in the low molecular weight region (MW < 1000) in any of the digesta. This suggests that soluble iron in the intestinal lumen is not

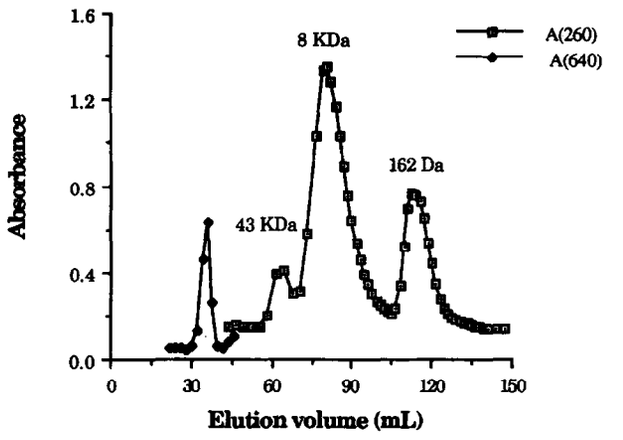


Fig. 3. Calibration of the Sephacryl S-100 HR column (useful MW range 1000–100000), eluted with 0·15 mol/liter PIPES buffer (pH 7·0), 2 mmol/liter sodium taurodeoxycholate, 0·15 mol/liter NaCl and 0.2 g/ml NaN_3 at 1 ml/min. Void volume was determined with elution of Blue Dextran at 640 nm. Elution of a mixture of ovalbumin (MW 43000), polystyrenes (MW 8000 and 5000) and phenylalanine (MW 165·2) was monitored at 260 nm. The two polystyrenes were not resolved.

associated with small peptides or amino acids, but with peptides of larger molecular weight.

In all meals, the ^{14}C-labeled fatty acids coeluted with the bile salt micelles, which confirms micellar solubilization of fatty acids in the lumen (Hernell *et al.*, 1990). In the beef and the milk meals, ^{59}Fe-labeled species did not coelute with ^{14}C-labeled fatty acids or bile salt micelles, suggesting that iron and fat digestion products in the intestinal digesta of the beef and of the milk meals were not associated. In the egg white meals, ^{59}Fe-labeled species and ^{14}C-labeled fatty acids and bile salt micelles did coelute. However, it is not clear whether this represented a coincidental coelution or a physical/chemical association between the two species. The fact that no interaction between egg white and source of fat was observed in the iron absorption from these diets in a previous study (Kapsokefalou & Miller, 1993) suggests that it may have been coincidental coelution.

DISCUSSION

In an attempt to understand the mechanism responsible for the iron-absorption-enhancing properties of meat, we formulated a two-part hypothesis:

1. Digestion products of meat protein promote the reduction of food iron from ferric to the more bioavailable ferrous form.
2. Soluble ferrous iron in the intestinal lumen forms lipophilic complexes with free fatty acids. These lipophilic iron complexes then partition into mixed micelles that form from biliary secretions and products of triglyceride digestion. As the micelles disaggregate near the absorptive surface of the brush border the lipophilic iron complexes are readily taken up by enterocytes.

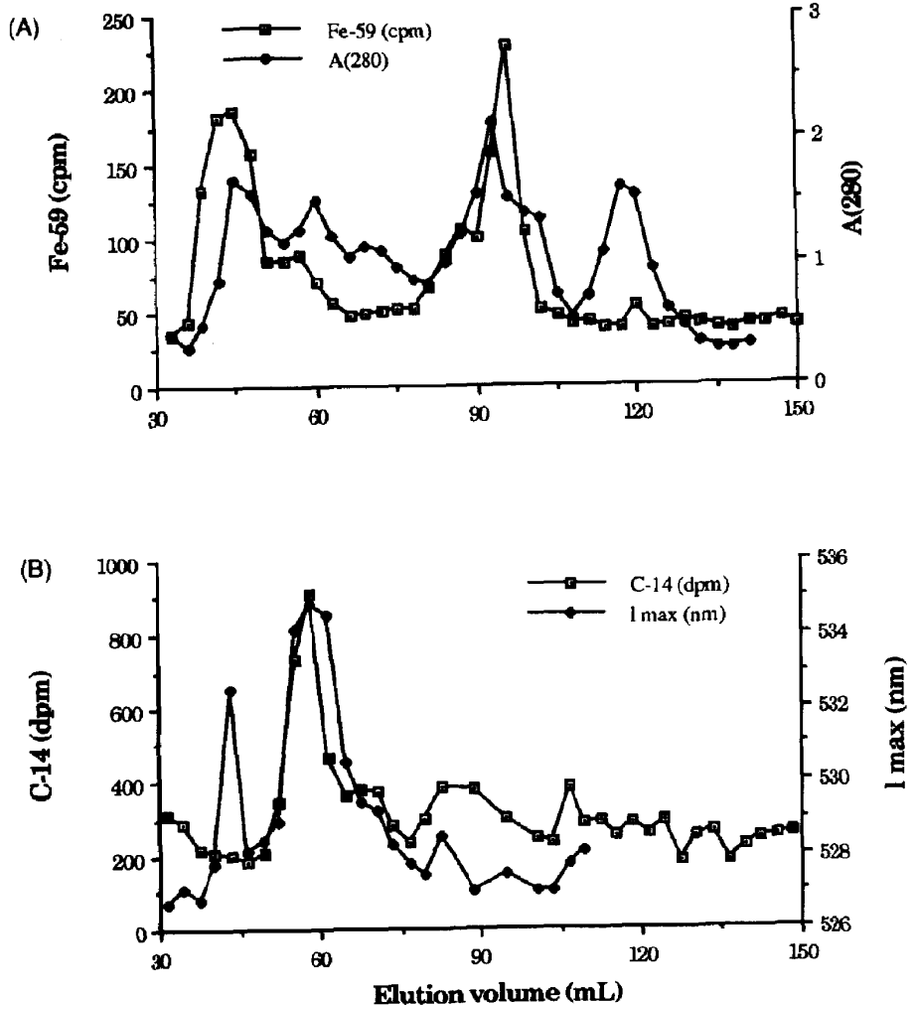


Fig. 4. Chromatograms showing the elution of ^{59}Fe-labeled iron chelates and protein digestion products (graph A), and ^{14}C-labeled fatty acids and bile salt micelles (graph B) from Sephacryl S-100 HR. An aliquot of the soluble phase of the intestinal digesta of a BT meal labeled with ^{59}Fe-labeled $FeCl_3$ and ^{14}C-labeled oleic acid was applied to the column and eluted with 0·15 mol/liter PIPES buffer pH (7·0), 2 mmol/liter sodium taurodeoxycholate, 0·15 mol/liter NaCl and 0·2 g/ml NaN_3 at 1 ml/min. Elution of ^{59}Fe and of ^{14}C is indicated as cpm and dpm, respectively. Elution of micelles is indicated by the shift towards higher values of the λ_{max} of the spectrum of solutions prepared by mixing aliquots of eluted fractions with Rhodamine 6G. Elution of protein digestion products is indicated by absorbance at 280 nm.

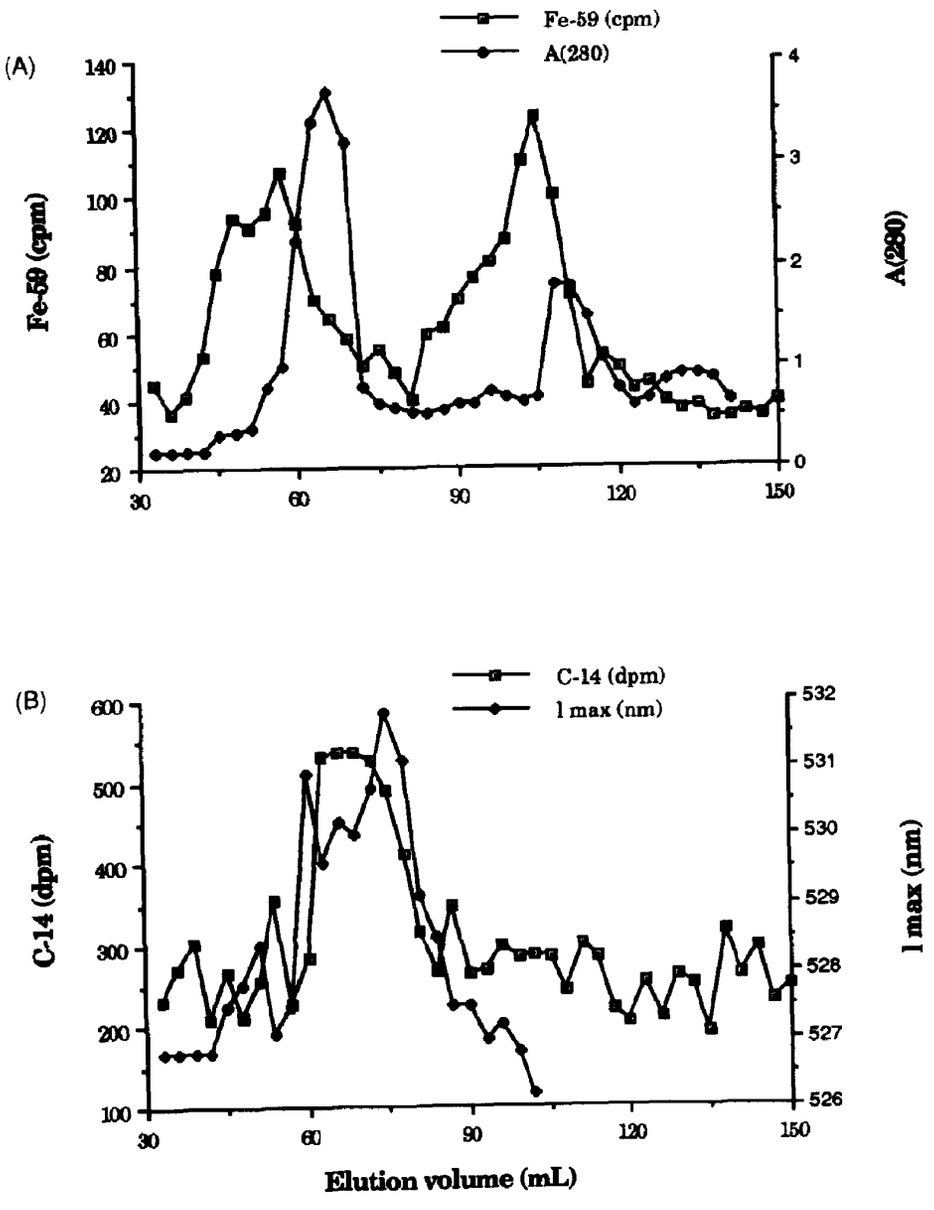


Fig. 5. Chromatograms showing the elution of ^{59}Fe-labeled iron chelates, protein digestion products (graph A), ^{14}C-labeled oleic acid and bile salt micelles (graph B) from the BB meal. Chromatography was performed as in Fig. 4.

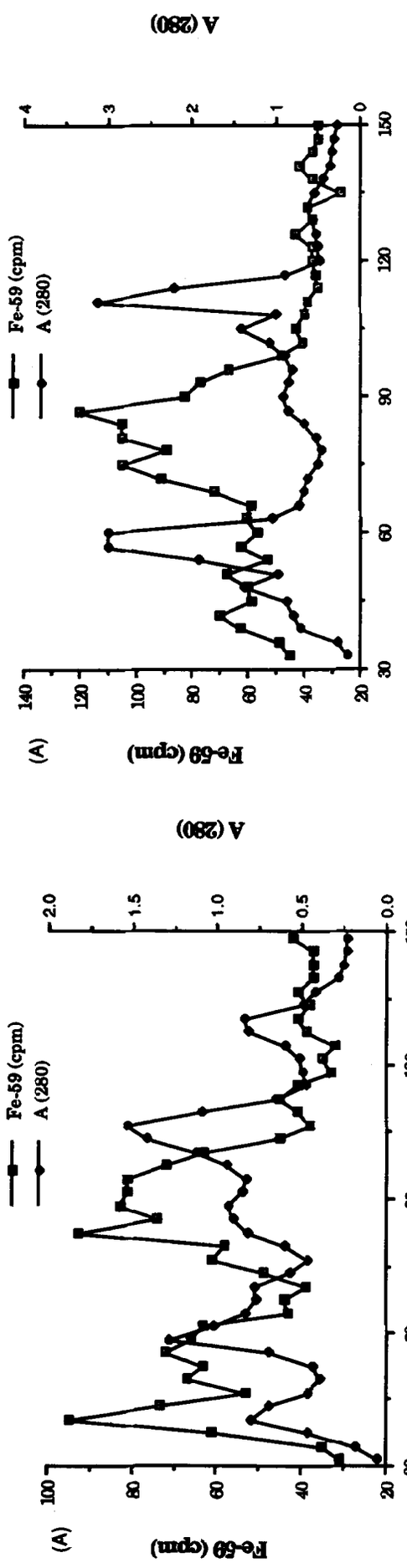


Fig. 6. Chromatograms showing the elution of ^{59}Fe-labeled iron chelates and protein digestion products (graph A), and ^{14}C-labeled oleic acid and bile salt micelles (graph B) from the BS meal. Chromatography was performed as in Fig. 4.

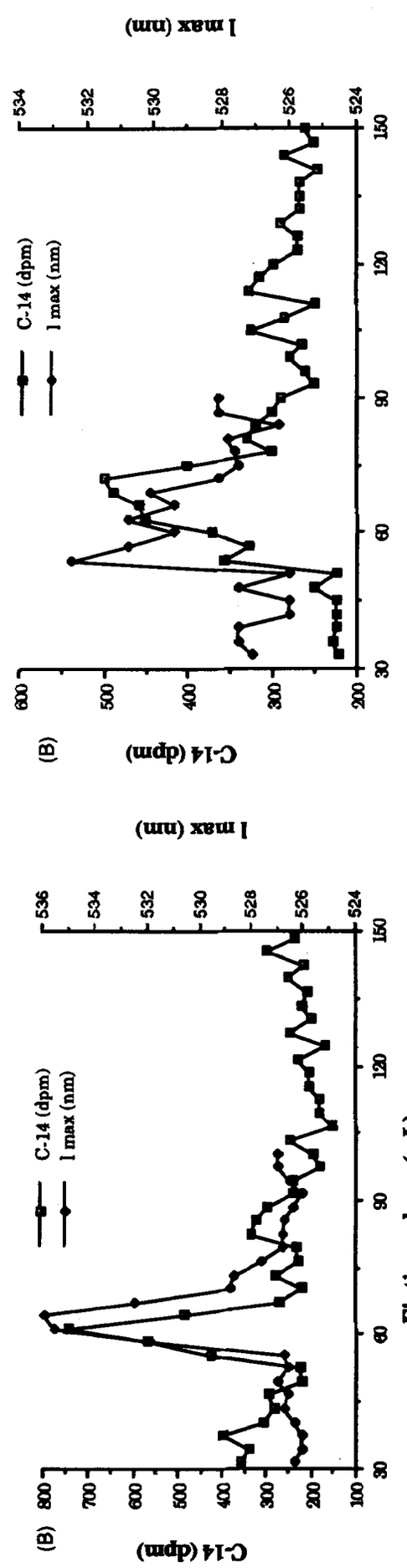
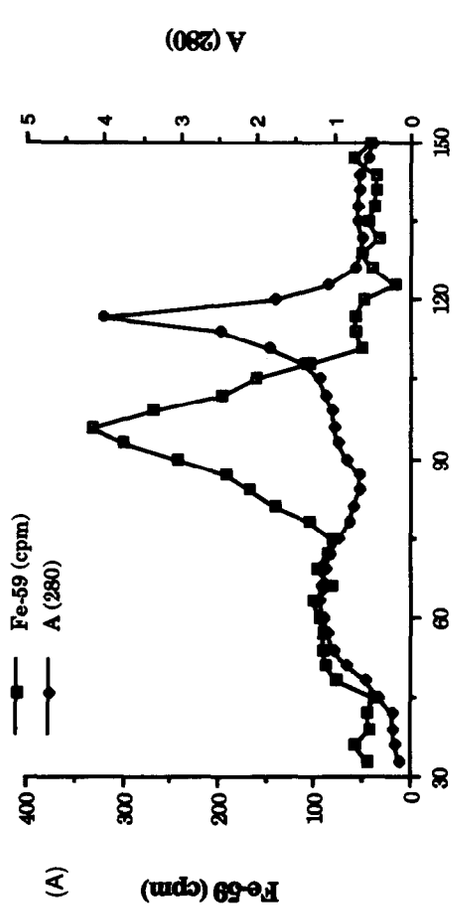


Fig. 7. Chromatograms showing the elution of ^{59}Fe-labeled iron chelates and protein digestion products (graph A), and ^{14}C-labeled oleic acid and bile salt micelles (graph B) from the MT meat. Chromatography was performed as in Fig. 4.



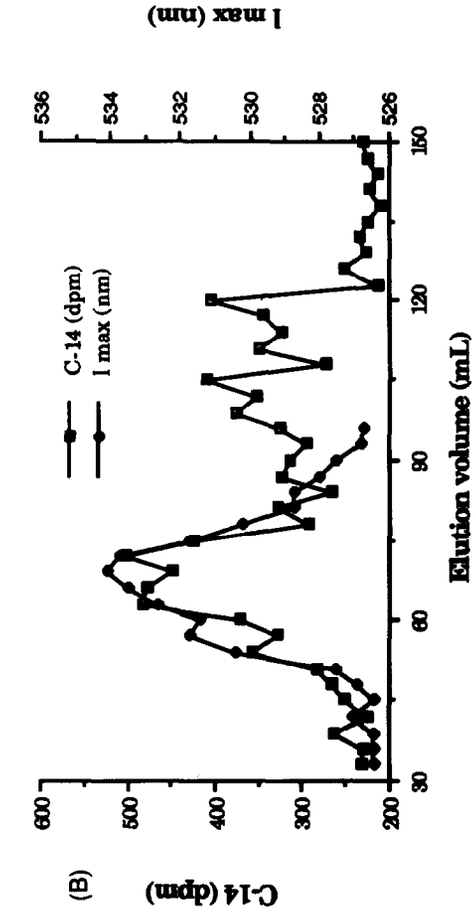
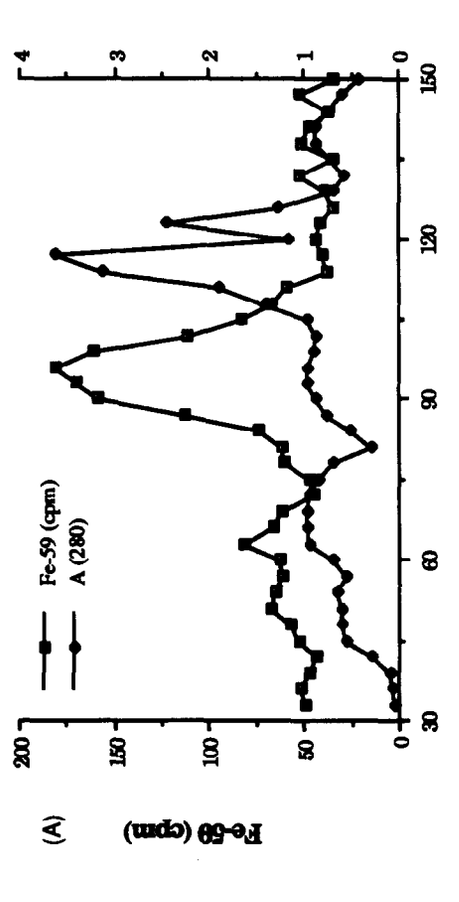


Fig. 9. Chromatograms showing the elution of ^{59}Fe-labeled iron chelates and protein digestion products (graph A), and ^{14}C-labeled oleic acid and bile salt micelles (graph B) from the MS meal. Chromatography was performed as in Fig. 4.



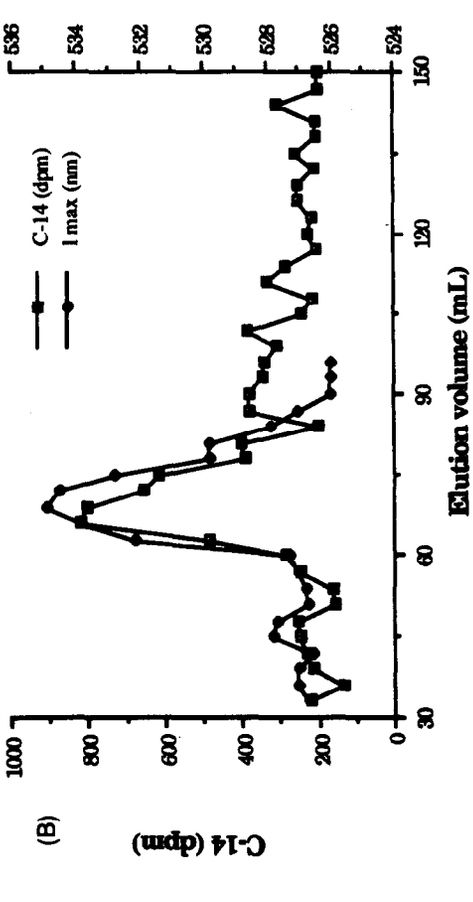


Fig. 8. Chromatograms showing the elution of ^{59}Fe-labeled iron chelates and protein digestion products (graph A), and ^{14}C-labeled oleic acid and bile salt micelles (graph B) from the MB meal. Chromatography was performed as in Fig. 4.

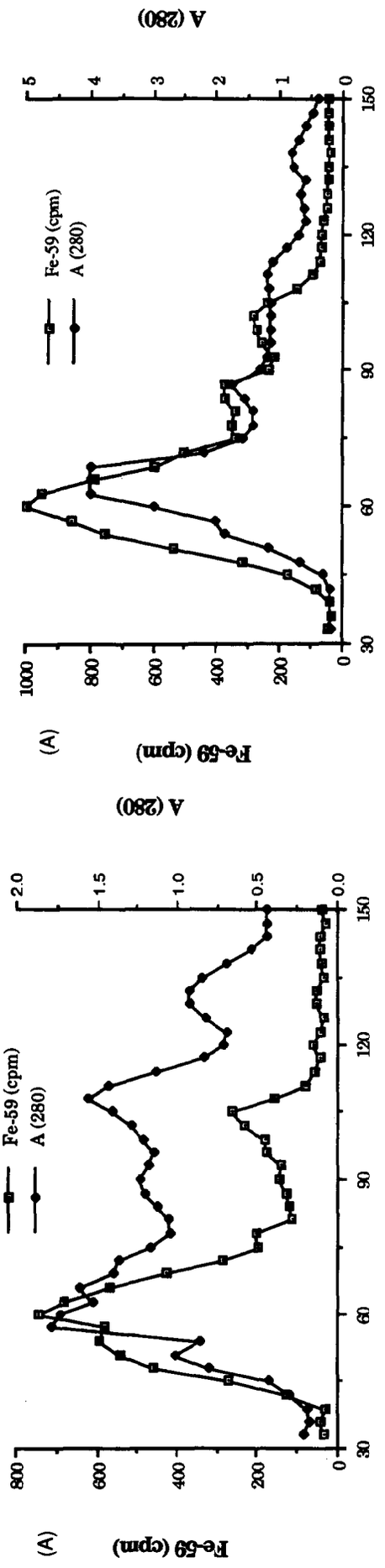


Fig. 10. Chromatograms showing the elution of ^{59}Fe-labeled iron chelates and protein digestion products (graph A), and ^{14}C-labeled oleic acid and bile salt micelles (graph B) from the ET meal. Chromatography was performed as in Fig. 4.

Fig. 11. Chromatograms showing the elution of ^{59}Fe-labeled iron chelates and protein digestion products (graph A), and ^{14}C-labeled oleic acid and bile salt micelles (graph B) from the ES meal. Chromatography was performed as in Fig. 4.

Part 1 of the hypothesis is supported by the data obtained. The proportion of ferrous iron in intestinal supernatant was elevated in two of the beef treatments (Table 2) and iron absorption was correlated with percentage ferrous iron (Fig. 2). Moreover, this part of the hypothesis is consistent with a considerable body of evidence from the literature. *In-vitro* enzymic digestion of beef and nonheme ferric iron mixtures produced increases in soluble ferrous iron (Kapsokefalou & Miller, 1991). Ferrous iron is more soluble and less likely to hydrolyze under conditions present in the gastrointestinal lumen (Forth & Schafer, 1987; May *et al.*, 1978). Also, ferrous iron complexes are less stable than ferric iron complexes and, therefore, reduction of the ferric iron associated with insoluble food components may promote its release into solution (Reddy *et al.*, 1986), leading to the formation of new iron complexes that may be more available for absorption. Reduction of iron appears to be a prerequisite for iron uptake into the mucosal cell (Wollenberg & Rummel 1987, 1990; Wien & Van Campen 1991), the step suggested as rate limiting in the iron absorption process (Nathanson *et al.*, 1985). It is noteworthy that iron solubility in the intestinal lumen was not correlated with iron absorption. This confirms earlier observations by Sato *et al.* (1987) and Miller & Berner (1989). Apparently, some soluble iron complexes do not readily release iron to brush border receptors.

Part 2 of the hypothesis was supported by a recent experiment (Kapsokefalou & Miller, 1993) where we studied iron absorption in rats from nine diets identical to the meals used in this study. Lean beef enhanced iron absorption compared to skim milk and egg white. Also, in the comparison of absorption from lean beef and other diets, there was a significant interaction between lean beef and fat source on iron absorption, suggesting that fat is involved in the enhancement of nonheme iron absorption by meat.

In the present study, however, iron was not associated with fatty acid or bile salt micelles in the digesta of the beef and milk meals. This leads us to reject the hypothesis that iron and fatty acids interact in the lumen and implies that the interaction that was observed in the earlier study (Kapsokefalou & Miller, 1993) between lean beef and fat was not lumenal. Simpson *et al.* (1988) have proposed that nonesterified fatty acids in the brush border membrane may influence iron uptake. Thus, it is possible that alterations in membrane fatty acids caused by dietary fat source could be responsible for the interaction. Unfortunately, we did not measure membrane fatty acid composition.

Chromatographic data from the present study do suggest that protein digestion products may influence iron absorption through complex formation with lumenal iron although the importance of this compared to ferrous iron formation cannot be determined from these data. The differences in the A_{280} elution patterns for the three protein sources and the apparent association of iron with peptides of different sizes clearly show that different protein sources yield different iron species during digestion. This observation may provide an answer to the often asked question: Why do protein sources with similar amino acid composition (e.g. beef, milk and egg) have markedly different effects on iron absorption? The absence of an iron peak in the molecular weight range of individual amino acids suggests that iron does not complex with amino acids in the intestinal lumen, but rather complexes with relatively large peptides. Thus, amino acid sequence may be more important than amino acid composition in determining the effect a particular protein may have on iron absorption. An alternative interpretation may be that concentrations of amino acid–Fe complexes in the lumen are low because they are absorbed very rapidly. However, this would not explain the observation that proteins from different sources with similar amino acid compositions (e.g. meat and milk) have markedly different effects on iron absorption.

To summarize, results reported here suggest that protein and fat source may affect the solubility, molecular weight and/or valence of iron species in the gastrointestinal lumen. Lean beef promoted the formation of soluble, ferrous iron in the lumen; however, reduction was not followed by iron–fatty acid complex formation. Our study clearly shows that different protein sources produce a different profile of peptides in the intestinal lumen and that enhancement of iron absorption by meat may be related to the formation of soluble ferrous iron, possibly chelated with peptides but not with amino acids or fat digestion products.

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