Iron-Supplemented Cow Milk. Identification and Spectral Properties of Iron Bound to Casein Micelles

Jack Hegenauer,* Paul Saltman, Diane Ludwig, Larry Ripley, and Arthur Ley

Because transition metals may cause "oxidized" flavors and odors in dairy products, the physical chemistry of iron bound to casein phosphoproteins may greatly influence the nutritional and organoleptic properties of iron-fortified milk. Centrifugal, spectrophotometric, and chromatographic evidence is presented to determine the distribution of iron in milk supplemented with ionic, chelated, or polynuclear iron complexes. With most iron donors, iron added at low concentration sedimented with the casein micelle and could be recovered with isoelectric casein. With the nitrilotriacetate (NTA) or lactobionate chelates of iron(III), the casein fraction of skim milk became saturated after addition of 10–20 mmol of Fe/L of milk. α-Casein was the principal iron-binding protein in milk. Iron donated by ferrous salt or ferric NTA was bound as the iron(III)-oxyphosphate complex on the phosphorylserine residues of casein. Ferrous salts may cause organoleptic deterioration of supplemented milk because the iron not bound to casein is capable of interacting with oxidizable milkfat. This oxidative instability may be reduced by use of chelated iron(III) supplements such as ferric nitrilotriacetate and ferric lactobionate that donate iron rapidly and specifically to the casein phosphoproteins, which effectively remove iron from the lipid phase.

Inadequate intake of dietary iron causes the high incidence of anemia which recent nutritional surveys have identified among young children, adolescents, and women of menstrual age (HANES, 1974; Nutrition Canada, 1973). Because of its wide consumption in the United States, milk is a logical vehicle for delivering supplemental iron to these iron-deficient groups. In a recent study of lipid peroxidation in iron- and copper-supplemented milk, we showed that reactions implicated in the development of "oxidized" flavor can be reduced if milk is supplemented rationally with newer chelated forms of these trace elements (Hegenauer et al., 1979a,b). It is of interest that the ferric chelates of the organic acids, nitrilotriacetate (NTA) and lactobionate, show not only the least tendency to oxidize milkfat but also the highest nutritional availability relative to a ferrous sulfate standard in conventional bioavailability tests using liquid milk diets (Hegenauer and Saltman, 1978).

The strong affinity of caseins for iron has been recognized for some time (see Carmichael et al., 1975) and is attributable to clustered phosphorylserine residues in these phosphoproteins. In animal species that secrete milk of high iron content, considerable iron is associated with the casein fraction (Loh and Kaldor, 1974). Iron binding by phosphoproteins may have important nutritional consequences, too, since we have shown previously that the gastrointestinal absorption of iron bound to purified casein or to casein micelles is equal or superior to ferrous salts for many animals (Carmichael et al., 1975). It is thus important to clarify the physical chemistry of iron bound in these phosphoproteins in order to understand—and possibly manipulate—the nutritional and organoleptic properties of iron-fortified milk.

In this report, we present centrifugal, spectrophotometric, and chromatographic evidence for the distribution of iron in milk supplemented with well-defined complexes that are representative of the principal classes of iron compounds: ionic, chelated, and polynuclear. The polynuclear iron(III)-polyphosphates and "ferripolyphosphate-whey protein" complexes described by Jones et al. (1972) were not included in these studies because their molecular composition is largely undefined, their chemical reactivity is limited in some cases by their insolubility, and their ligand-exchange behavior changes with age. Although different commercial products were supplemented for this study, we did not investigate the effects of processing variables, such as homogenization or pasteurization, on the stability of the iron–phosphoprotein complexes. We draw particular attention in this study to the facile exchange of iron between caseins and the ferric chelates of NTA and lactobionate, which appear to be promising milk and food supplements on the basis of relative inertness in promoting lipid oxidation (Hegenauer et al., 1979a,b) and on nutritional and sensory evaluations which will be presented elsewhere.

Department of Biology, University of California—San Diego, La Jolla, California 92093.

MATERIALS AND METHODS

Source of Milk. Raw, homogenized (pasteurized), and skimmed (pasteurized) milk were obtained from a Southern California dairy prior to commercial distribution and were used within 1 day of receipt.

Iron and Radioiron Complexes. General methods for preparing iron(II) salts and iron(III) chelates and polynuclear complexes have been described (Hegenauer et al., 1979a). Radioiron was incorporated by equilibrating acidic solutions of iron(II) or iron(III) with $^{59}FeCl_3$ ("carrier-free" in 0.5 N HCl, New England Nuclear Corporation, Boston, MA) prior to addition of ligand and neutralization (Carmichael et al., 1975). Measurements of ^{59}Fe were made under identical geometries with a Nuclear-Chicago gamma well scintillation detector [2-in. NaI(Tl) crystal] and spectrometer calibrated for the 1.10 + 1.29 MeV emissions of ^{59}Fe. A portion of each radioiron solution was set aside to allow iron concentration to be determined by reference to the counting rate of ^{59}Fe and the specific activity of the $^{55.847}Fe$ used in each experiment.

Microcentrifugation. For analytic centrifugation, 1 volume of milk was mixed with 0.01 volume of radioiron complex to give a final iron concentration of 1.0 mM with a specific activity of about 0.5 Ci ^{59}Fe/mol of Fe. After 3 h at room temperature, the incubation mixture was pipetted to a height of 7.5 cm into several borosilicate melting point capillaries (previously sealed at one end) and centrifuged in a water cushion at 25 000g for 3 h at 4 °C in a Servall HB-4 swinging-bucket rotor (Loh and Kaldor, 1974). Following centrifugation, the capillaries were wrapped with pressure-sensitive tape, scored at 0.5-cm intervals with a diamond pen, and allowed to freeze slowly

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at −18 °C. The capillaries were then cracked apart, and the segments were placed in tubes for direct gamma well counting or were extracted with water prior to measurement of protein concentration with a Folin-Ciocalteu reagent (Hartree, 1972).

Titration of Milk with Iron. To compare the ability of different iron complexes to donate iron to milk proteins, we assessed the iron-binding capacity of milk in four ways: (1) by equilibrium dialysis as a measure of undialyzable metal, (2) by isoelectric precipitation of casein to measure casein-bound iron, (3) by high-speed centrifugation to estimate iron bound to sedimentable casein micelles, and (4) by precipitation of proteins with trichloroacetic acid (Cl_3CCOOH) to measure acid-stable iron complexation.

Milk (10 mL), radioiron solution (sp. act. ~0.1 Ci ^{59}Fe/mol of Fe), and 0.15 M NaCl were mixed thoroughly in a final volume of 20 mL and incubated in the cold for 24 h. In dialysis experiments, 2 mL of incubated milk and 2 mL of an identical mixture prepared without ^{59}Fe were placed on opposite sides of a dialysis membrane (No. 20 Visking tubing, Union Carbide Corp.) using the technique described by Malmstrom (1953). Cells were shaken gently in the cold, and 1-mL samples of the diffusate and retentate compartments were taken for ^{59}Fe measurements after 48 h. Nondialyzable iron (%) = ^{59}Fe in retentate/(^{59}Fe in retentate + ^{59}Fe in diffusate) × 100. Casein was precipitated isoelectrically from incubated milks by adding an equal volume of 1.0 M sodium acetate buffer, pH 4.60 (El-Negoumy, 1966); after incubation for 30 min at room temperature, isoelectric casein was removed by centrifugation at 25000*g* for 15 min. Casein was sedimented from skim milk incubation mixtures by centrifugation at 42000*g* for 2 h (King et al., 1959). Casein-bound iron was established as the difference in ^{59}Fe content between 1-mL samples from the uncentrifuged incubated milk or isoelectric suspension and a 1-mL sample removed from the top 2 cm of the supernatant following centrifugation., Casein-bound phosphorus was determined in a similar way after wet-ashing by the method of Allen (1940). Proteins were precipitated from incubated milk by adding an equal volume of 10% (w/v) Cl_3CCOOH and centrifuging immediately at 8000*g* for 5 min. Precipitable radioiron was estimated as described for casein.

Ion-Exchange Chromatography of Caseins. Casein samples were prepared for chromatography by isoelectric precipitation from 10 mL of milk after incubation with iron as described above. Precipitated caseins were washed once by suspension/centrifugation with 0.5 M sodium acetate buffer (pH 4.60) and redissolved in 10 mL of urea–bisulfite buffer [5 M urea (deionized with mixed-bed resin)/5 mM $NaHSO_3$/5 mM Na_2SO_3, pH 7.12], and dialyzed under nitrogen for 24 h against 1 L of urea–bisulfite buffer in the cold. The dialyzed sample was applied to a column (1.5 × 25 cm) of DEAE-cellulose (Whatman "DE-52") previously equilibrated with urea–bisulfite buffer (Hegenauer et al., 1979c). A small amount of unadsorbed protein was removed by washing the column with about 100 mL of buffer, and caseins were eluted with a linear gradient (Hegenauer et al., 1965) formed between 250 mL of urea–bisulfite buffer (starting) and 250 mL of 0.3 M NaCl in urea–bisulfite buffer (limiting). Flow rate was about 0.5 mL/min; to prevent oxidation of bisulfite, eluting buffers were bubbled continuously with nitrogen to exclude air. Major casein fractions in the eluate were identified by gel electrophoresis (El-Negoumy, 1966).

Spectrophotometry of Iron-Supplemented Milk. Milk was supplemented by the addition of 1 mL of iron solution/L of milk and stored in the cold for up to 7 days.

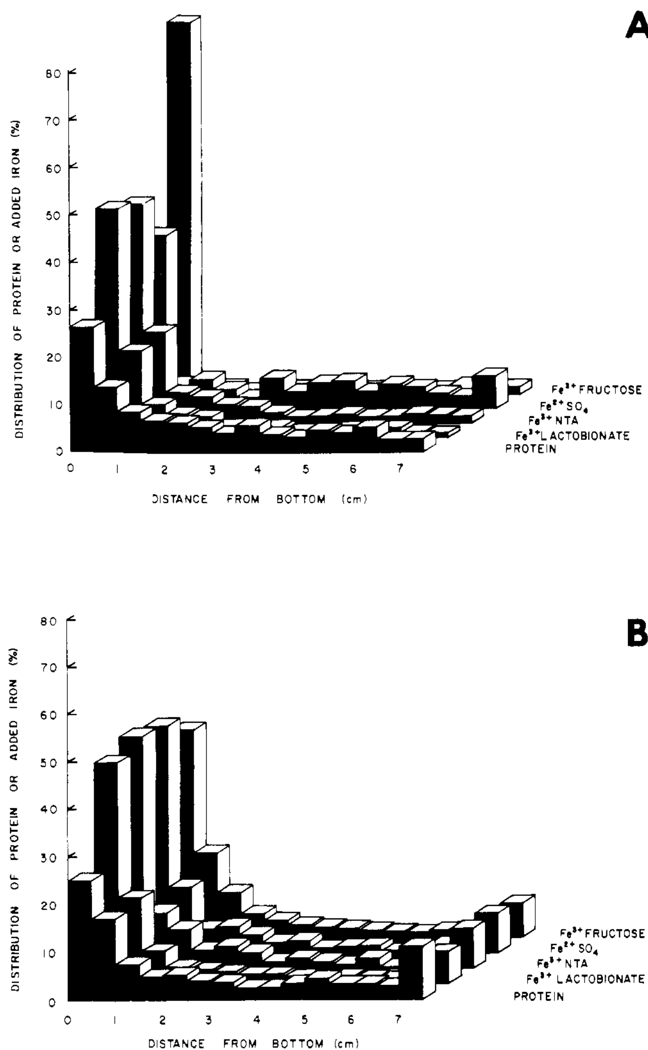

Figure 1. Density distribution of iron in supplemented raw (A) or homogenized (B) milk. Radioiron complexes were added at a final concentration of 1 mM and allowed to stand 3 h before centrifugation in a microcapillary system. Experimental points are the average of four experiments. Protein distribution was measured after centrifugation of unsupplemented milk.

The single-beam spectrophotometric apparatus used in this study was capable of measuring light transmission and reflectance in solutions with high light scatter. The instrument consisted of a Cary monochromator, a tungsten lamp whose intensity was regulated with neutral density filters, and a GaAs photomultiplier; fiber optics were used as light guides. Absorbance scales were calibrated with neutral density filters. Spectral data were stored in a multi-channel analyzer and plotted by computer after subtraction of a "background" or control sample. For transmission measurements, the photomultiplier light guide was positioned at 180° from the incident light passing through a 2-mm sample path length; reflected light was detected at an angle of 20° from incident.

RESULTS

Iron-Binding Components in Milk. The casein micelle is the principal sequestrant of iron added to milk. During centrifugation of iron-supplemented raw and homogenized milk, the distribution of iron from a variety of complexes roughly paralleled the distribution of protein (Figure 1). The iron concentration chosen for these experiments (1 mM) was higher than the recommended pediatric level (0.25 mM) but well below the concentration required to "saturate" casein (see below). Conditions of

centrifugation were sufficient to sediment completely only the "heaviest" casein micelles and to float the milkfat; we did not observe significant fractionation of other milk solutes. Longer centrifugation did not alter the distribution of either protein or iron. Inspection of Figure 1 shows that the iron/protein ratio of the centrifugal region was considerably greater than the ratio in the middle portion of the capillary tube which contained proteins of "neutral" density, including the iron-specific binding proteins, lactoferrin and transferrin. The high relative iron content of the sedimentable proteins demonstrates that the intact casein micelle can bind significant quantities of added iron even when it is "saturated" with calcium ion.

Raw Milk. We observed distinct differences in the way different complexes delivered iron to the proteins of raw milk. Ferric ethylenedinitrilotetraacetate (EDTA) donated very little iron to the casein micelle, as expected from the stability of this iron chelate; thus distribution of iron was about the same in all regions of the centrifuged capillary (results not shown). Ferrous sulfate, a common iron supplement, donated less iron to the micelle (and left more iron in the supernatant) than any iron complex except ferric EDTA. The high iron content of the milkfat fraction of milk supplemented with ferrous salts is a salient feature of Figure 1A; the significance of this result for the organoleptic qualities of iron-supplemented milk will be discussed later. Ferrous sulfate, like polymeric ferric fructose, appeared to label only the heaviest (i.e., most centrifugal) casein micelles. We attribute this similarity to polymeric iron that was formed from iron(II) under the influence of oxygen and iron-binding ligands during the incubation period (Harris and Aisen, 1973). Physical adsorption of this polynuclear iron onto the casein micelle may have altered the density of the micelle sufficiently to increase its sedimentation velocity, so that only a single centrifugal zone of micelles was observed. The distribution of iron from the ferric NTA and ferric lactobionate chelates was indistinguishable: The major portion of added iron was bound to micelles; little iron was found in the supernatant, and virtually none in the milkfat fraction. In addition, the distribution of iron and micellar protein was similar enough to suggest that these chelates were capable of donating iron specifically to casein without perturbing the micellar size or density equilibrium in milk.

Homogenized Milk. The distribution of iron in homogenized milk supplemented with several iron complexes was virtually identical, with the exception of ferric EDTA. Ferric EDTA showed the "flat" distribution discussed for raw milk and donated little iron to the centrifugal caseins (results not shown). Physical adsorption of polynuclear iron, if it occurred at all, apparently did not alter the size and density distribution of the micelles to the degree observed in raw milk. The milkfat fraction of homogenized milk, however, bound a significant quantity of iron from all complexes (Figure 1B). Since homogenization also increased the protein content of the milkfat fraction, we may surmise that adsorption of casein onto the fat globule membrane (Fox et al., 1960) was responsible for the increased affinity of the milkfat for added iron. Addition of chelated iron (ferric NTA or ferric lactobionate) lead to a lower iron content of the milkfat than addition of iron as the ionic (ferrous salt) or polynuclear (ferric fructose) complexes.

Iron-Binding Capacity of Casein Micelles. Throughout the practical range of fortification (<1 mM Fe), the casein fraction bound about 90% of iron added to skim milk as the ferric NTA complex (Figure 2). Up to about 5 mM Fe, separation of the iron–casein by isoelectric precipitation or high-speed centrifugation gave equivalent results. At higher iron concentrations (5–10 mM), slightly more iron was associated with isoelectric casein (Figure 2). Only the casein fraction bound significant iron throughout a wide range of concentrations, since Cl_3CCOOH precipitation and equilibrium dialysis produced the same iron-binding curve and equivalence point as isoelectric precipitation of casein when iron was presented as the NTA chelate.

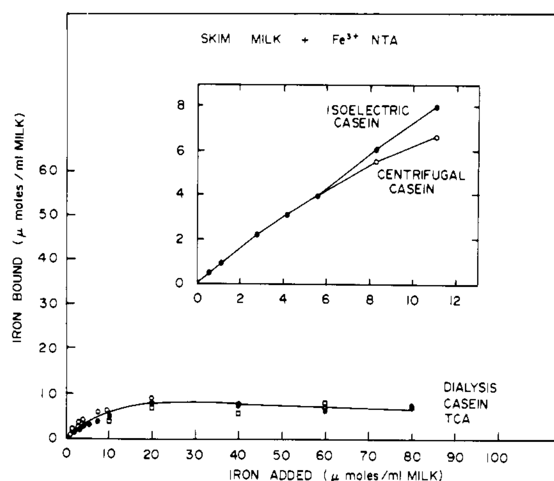

Figure 2. Iron binding by skim milk as a function of concentration of supplemental ferric NTA 1:1 chelate. Binding was estimated radioisotopically as undialyzable iron ("dialysis"), as iron precipitated with isoelectric casein ("casein"), or as iron associated with Cl_3CCOOH precipitates ("TCA"). Inset compares isoelectric precipitation and ultracentrifugation as means of estimating iron binding by the casein fraction. Datum points are the average of two measurements on each two replicate incubation mixtures.

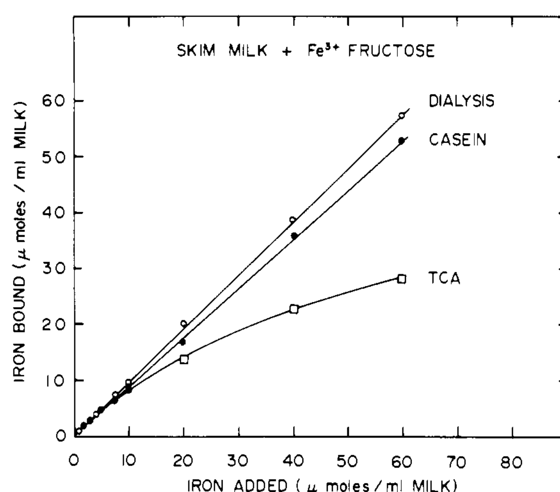

Figure 3. Iron binding by skim milk as a function of concentration of supplemental ferric fructose polymer complex. Bound iron was estimated as described in the caption of Figure 2.

The large molecular ferric fructose complex was essentially nondialyzable after addition of skim milk (Figure 3). A constant proportion (80–90%) of the added iron could be precipitated with isoelectric casein, so that binding saturation was not observed (Figure 3). Precipitation of iron–protein complexes with Cl_3CCOOH underestimated the iron-binding capacity of the casein fraction, especially at high iron concentrations. This result suggests that strong acid caused dissociation of the iron–casein complex. Very little iron from the ferric lactobionate chelate was dialyzable after addition of skim milk. At low iron con-

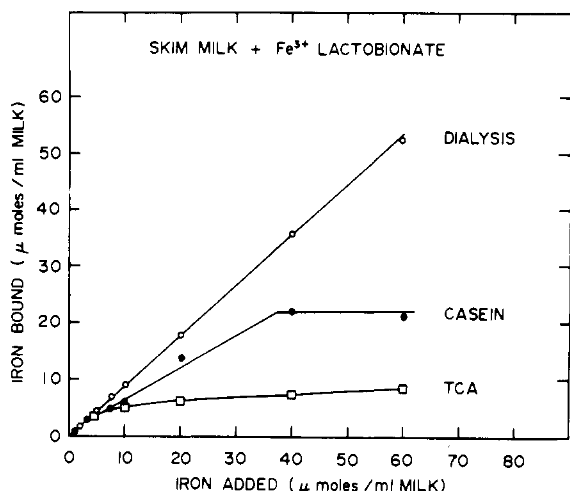

Figure 4. Iron binding by skim milk as a function of concentration of supplemental ferric lactobionate 1:1 chelate. Bound iron was estimated as described in the caption for Figure 2.

centrations (<5 mM Fe), nearly all this iron was associated with the casein fraction. With increasing iron concentration, casein bound an increasingly smaller fraction of the total iron and eventually became saturated (Figure 4), although this equivalence point was greater than that obtained with the ferric NTA chelate. Within the range of iron concentration relevant to human nutrition (<5 mM), however, data obtained from equilibrium dialysis, casein fractionation, or Cl_3CCOOH precipitation of milk protein gave an equivalent picture of the iron-binding capacity of milk for the lactobionate complex. Homogenized milk gave identical results for most of the experimental manipulations described above (data not shown).

Because iron experiences rapid ligand-exchange reactions with many macromolecules, nondialyzability is not a sufficient criterion of specific iron-binding; this situation is particularly evident in Figure 4, which shows that the casein iron-binding curve did not parallel the nondialyzability curve, particularly after the casein fraction had been saturated with added ferric lactobionate. The binding capacity may also be overestimated if macromolecular iron complexes like ferric fructose remain physically adsorbed onto the casein fraction during isoelectric or centrifugal isolation.

Distribution of Iron among Caseins. Iron that was bound to the casein micelles remained with individual caseins even after dissociation of the micelle and ion-exchange chromatography on DEAE-cellulose in urea buffers (Figure 5). Our chromatographic system, using urea for dissociation and bisulfite for anaerobiasis (Hegenauer et al., 1979c)) was patterned after the urea–mercaptoethanol system described by Thompson (1966). Thiol reductants like mercaptoethanol could not be used, however, because they reduced iron(III) and removed it from the caseins by chelation of iron(II). As judged by gel electrophoresis, chromatographic resolution was sufficient to fractionate α- and β-caseins with good purity and to separate κ-casein with some residual contamination by α-casein. When skim milk was supplemented with a low concentration of iron as the 1:1 ferric NTA chelate, chromatography of the isoelectric caseins showed that iron was distributed among the principal caseins in about the same proportion as the protein content (Table I). As the iron concentration of the supplemented milk was increased, a greater proportion of the additional iron appeared to associate with the α-casein fraction during chromatography (Table I). At concentrations of added iron >4 mM, the individual components of isoelectric casein were poorly resolved by DEAE-cellulose chromatography due to greater overlapping of elution peaks, so integration of peak areas was problematic. The presence of iron, however, did not alter the gradient elution position of any major casein fraction. This simple chromatographic analysis did not attempt to correct for differences in the molar absorptivities of the individual caseins (McKenzie and Murphy, 1970) or for possible redistribution of iron among the caseins during isolation, dissociation, and chromatography.

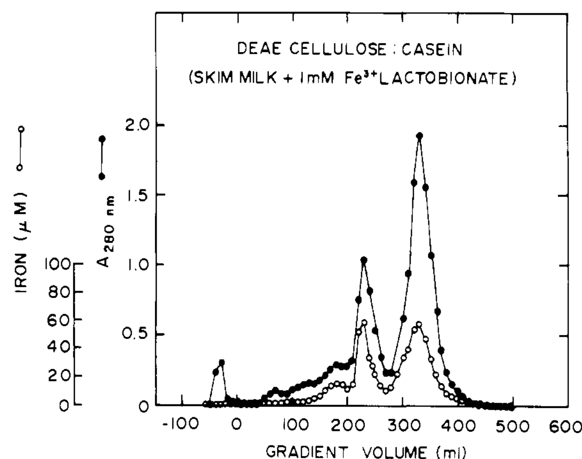

Figure 5. DEAE-cellulose chromatography of casein precipitated isoelectrically from skim milk supplemented with the $^{59}Fe(III)$ lactobionate 1:1 chelate (1 mM).

Table I. Distribution of Iron and Phosphorus in Whole and Fractionated Casein from Iron-Supplemented Milk

whole casein: iron, mmol/L of milk, added[b]	whole casein: iron, mmol/L of milk, bound	whole casein: phosphorus, mmol/L of milk, bound	bound Fe:P, M	casein fractions[a]: α	β	κ	γ
				Protein (%)[c]			
0		7.73		60.0	25.0	11.0	4.0
				Iron (%)[d]			
1	0.87	7.63	0.11	54.4	30.6	12.1	1.4
2	1.62	8.03	0.20	54.2	30.0	12.2	1.8
4	3.10	7.59	0.41	58.0	23.7	15.4	0.8
6	4.34	8.71	0.40	71.1	18.4	9.7	1.6
8	5.83	9.68	0.60	78.5	11.5	8.7	0.8

[a] Isoelectric casein precipitated from 10 mL of skim milk and chromatographed on DEAE-cellulose. [b] Iron added as $^{59}Fe(III)$ NTA 1:1 chelate. [c] Protein estimated by integrating areas of A_{280} elution profile. [d] Iron determined by integrating areas of ^{59}Fe elution profile.

Spectrophotometric Analysis of Iron Chelation in Supplemented Milk. Centrifugation and chromatographic experiments with iron-supplemented milk provide evidence that the calcium caseinate micelle and individual caseins can sequester iron but do not distinguish between adsorption or chelation as the mechanism of iron-binding in unfractionated milk. Spectrophotometric analysis, however, has been successful in differentiating iron(III) in polynuclear forms from iron(III) chelated to phosphorylserine residues, which have been implicated in iron-binding phenomena in caseins (Carmichael et al., 1975) and phosvitin (Webb et al., 1973). We could identify three classes of iron in supplemented skim milk by their electronic absorption spectra (Figure 6): "free" mononuclear chelates like ferric EDTA that have not undergone ligand

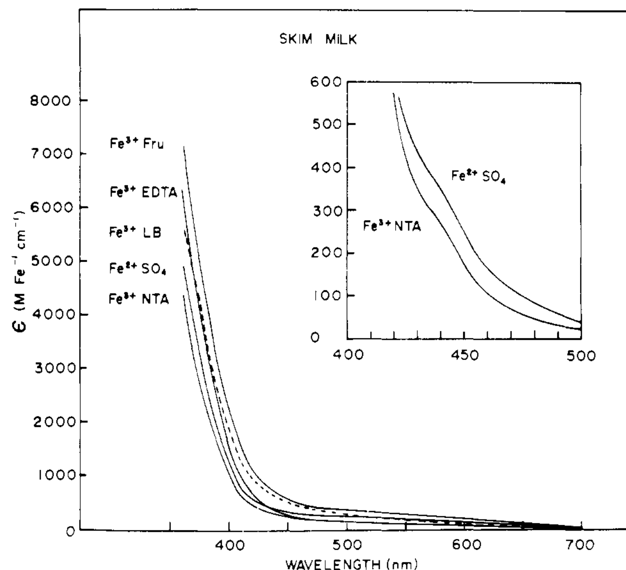

Figure 6. Electronic absorption difference spectra of iron-supplemented skim milk. Ferric fructose (Fe^{3+} Fru), ferric EDTA, ferric lactobionate (Fe^{3+} LB), ferrous sulfate, or ferric NTA was added at a final concentration of 1 mM and allowed to stand 2 days in the cold. Inset shows the 440-nm absorption band of the Fe(III)–diphosphorylserine chelate formed by casein in skim milk supplemented with 2 mM ferrous sulfate or ferric NTA. Spectra have been corrected arithmetically for light absorption and scattering by unsupplemented milk.

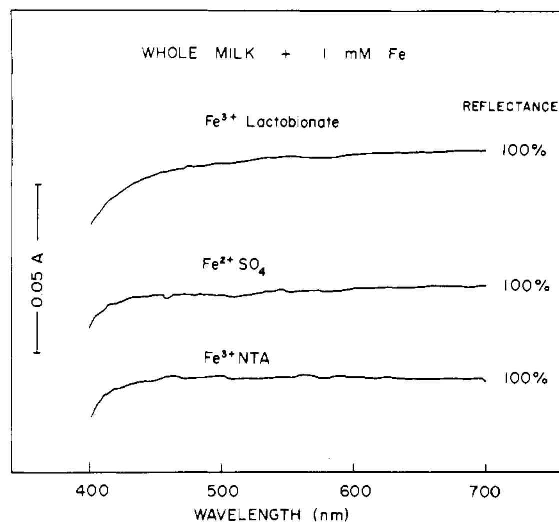

Figure 7. Reflectance difference spectra of iron-supplemented whole milk. Homogenized, pasteurized milk was supplemented with the iron complex at a final concentration of 1 mM and allowed to stand for 7 days in the cold. Reflected monochromatic light has been corrected arithmetically for reflectance of unsupplemented milk.

exchange with milk constituents show low-intensity, charge-transfer bands in the region of 400–450 nm; (2) polynuclear complexes like ferric fructose show intense ultraviolet absorption tailing into the visible region, with a shoulder at 470 nm (Bates et al., 1973); and (3) iron(III) oxyphosphate tetrahedral chelates, which arise by coordination of iron from ferric NTA by adjacent phosphorylserine residues of casein, show one or more weak absorption bands in the region of 420–450 nm (Webb et al., 1973). Figure 6 shows detail of the iron(III)–phosphorylserine 440-nm absorption band present in skim milk supplemented with iron(II) or iron(III). Because of its high absorbance and absence of the iron(III) oxyphosphate absorption bands, ferric lactobionate had many features of polynuclear iron after addition to milk. Except for ferric EDTA, >80% of the iron from these complexes was associated with the casein micelle during isoelectric precipitation, so that "free" iron complexes could have made only a minor contribution to the recorded spectra.

"Color" of Iron-Supplemented Milk. The strong absorption of ultraviolet and violet light by iron-supplemented milk has only slight influence on the appearance of milk. This absorption does, however, enrich reflected light in the longer visible wavelengths and imparts a faint yellow tint to homogenized milk supplemented with any of a variety of iron complexes. The reflectance spectra shown in Figure 7 are essentially the inverses of the absorption spectra of Figure 6 but do not provide the resolution of the latter. When ferric NTA was used as the supplement, most of the iron was donated to the casein phosphoproteins; since light absorption by the iron–phosphorylserine chelate is weak, reflectance was relatively high throughout the visible region. Ferric lactobionate, as discussed above, appeared to form polynuclear complexes during storage, so the electronic absorption spectra (Figure 6) exhibit the greater absorption of shorter wavelengths that is characteristic of these complexes; accordingly, the reflectance spectrum showed somewhat less contribution from these wavelengths (Figure 7). Milk supplemented with ferrous sulfate showed intermediate reflectance; this suggests that oxidation of ferrous sulfate had produced appreciable polynuclear ferric iron during prolonged storage.

DISCUSSION

Modes of Iron Binding in Milk. Iron added to whole or skim milk is bound almost exclusively by the phosphoproteins of the intact calcium caseinate micelle. For certain iron donors, iron added up to a concentration of about 5 mM is found associated with the casein micelle in centrifugation experiments (Figure 1). This iron remains firmly bound to the calcium-free caseins during isoelectric precipitation (Figures 2–4) and ion-exchange chromatography (Figure 5 and Table I). Iron may be complexed to caseins by nonspecific physical adsorption ("peptidization") of hydroxy-iron(III) polymers like ferric fructose or by formation of the specific iron(III)–diphosphorylserine chelate when iron is exchanged between mononuclear ferric NTA and contiguous phosphorylserine residues on the phosphoproteins (Webb et al., 1973). We have obtained presumptive spectrophotometric evidence that these chelates are formed in skim milk that has been supplemented with a "nonsaturating" concentration of ferric NTA (Figure 6). The favored stoichiometry of these iron–protein chelates is 1 Fe:2 P (Webb et al., 1973). In an earlier study (Carmichael et al., 1975), we observed the binding of 2.7 μmol of Fe/mL of skim milk that was "saturated" with the 1:2 ferric NTA chelate. If we assume that skim milk contains 7.7 μmol of casein P/mL (Table I), this Fe:P ratio (0.35) is lower than theoretical (0.5) and may be attributed to less successful competition of casein for iron in the presence of a higher NTA concentration. It is evident from inspection of Figure 2 and Table I that Fe:P ratios greater than 0.5 were obtained in several experiments using Fe(III) NTA. The anticipated range of iron fortification (<1 mM) would, however, be well below the theoretical and actual binding capacity of the casein fraction.

Since the total iron-binding capacities of transferrin in blood plasma and of lactoferrin in milk are both in the micromolar range, it is apparent that neither protein would play an important role in complexing supplemental iron in millimolar concentration. We found no evidence of substantial iron binding by the whey proteins in micro-

centrifugation experiments. The milkfat fraction of raw milk binds very little supplemental iron from any iron donor except ferrous sulfate (Figure 1A). Significant added iron may be complexed by milkfat after homogenization (Figure 1B), presumably because of the association of considerable casein with the emulsified fat globules (Fox et al., 1960).

Characteristics of Iron Complexes as Donors for Casein. *Ferric EDTA.* This strong chelate shows little tendency to exchange iron with casein, as determined by the small proportion of iron cosedimenting with casein micelles in centrifugation experiments (data not shown) and by the absence of iron(III) oxyphosphate absorption bands in spectrophotometric studies (Figure 6). Negligible iron exchange with phosphoproteins is consistent with the slow exchange rate of ferric EDTA with transferrin (Bates et al., 1967).

Ferric NTA. The facile exchange of iron we have observed between ferric NTA and casein is another example of the suitability of this chelate as an iron donor for many macromolecules of biological importance, such as transferrin (Bates and Wernicke, 1971), and the egg yolk phosphoglycoprotein, phosvitin (Webb et al., 1973). Ferric NTA is perhaps the only iron complex that can be used to "titrate" milk to determine its iron-binding capacity. Unlike other iron complexes used in this study, ferric NTA gave similar iron-binding kinetics with four different methods for determining iron complexation by milk proteins (Figure 2): saturating iron concentrations determined by equilibrium dialysis, casein precipitation, casein ultracentrifugation, and Cl_3CCOOH precipitation were virtually identical. As shown previously (Carmichael et al., 1975), donation of iron from ferric NTA to casein is complete within 2 h. Binding of iron by ligand exchange from ferric NTA to casein appears to be remarkably specific. Iron is bound to casein without disturbing the density and sedimentation properties of the micelle (Figure 1). We have estimated that the equilibrium constant for the formation of the iron(III)–phosphoprotein complex is about 10^{13} greater than for the formation of the calcium(II) complex (Hegenauer et al., 1979d). Iron would thus be capable of binding to phosphorylserine residues (Figure 6) even if the micelle were fully "saturated" with calcium(II). This iron is not lost during acid precipitation (Figure 2) or during ion-exchange chromatography (Table I).

Ferric Fructose. Iron may be complexed to caseins and other macromolecules by physical adsorption of oxo-bridged hydroxy-iron(III) polymers like ferric fructose. Since these complexes depend on electrostatic interactions between iron polymer and protein molecules, they are relatively weak and are easily dissociated by changes in pH or salt concentration. The sensitivity of such complexes to change in the ionic mileau may account for our observation (Figure 3) that, when ferric fructose was used to supplement milk, less iron was precipitated by strong acid (Cl_3CCOOH) than by weak acid (pH 4.6 buffer). Because of lack of stoichiometry in the formation of these nonspecific complexes, it is not appropriate to discuss iron-binding capacity of milk proteins saturated with polynuclear iron donors. The electronic absorption spectra of polynuclear iron (Figure 6) are relatively unaffected by physical adsorption. The intense light absorption of these complexes would mask the 440-nm absorbance of the iron(III) oxyphosphate chelate in supplemented milk, so it is not possible to determine the proportion, if any, of specifically chelated iron by spectrophotometric methods.

Ferric Lactobionate. In its ability to donate iron rapidly to the casein micelle (Figures 4 and 5), this low-molecular-weight chelate resembles ferric NTA. We found a close resemblance between the casein distribution of iron added as ferric NTA (Table I) and ferric lactobionate (Figure 5). It required more iron to saturate isoelectric casein with ferric lactobionate (Figure 4) than with ferric NTA (Figure 2); about the same amount of iron from either complex, however, was required to saturate Cl_3CCOOH-precipitable protein (Figures 2 and 4). We interpret these observations to mean that ferric lactobionate contains some polynuclear iron that is loosely adsorbed to the casein micelle in addition to low-molecular-weight chelates that exchange iron specifically with phosphorylserine residues. As discussed above, the weakly bound polynuclear iron is coprecipitated with isoelectric casein in weak acid, but only the firmly bound iron(III) oxyphosphate chelates survive precipitation by strong acid. The absorption spectrum of skim milk supplemented with ferric lactobionate (Figure 6) also showed more evidence of polynuclear iron than of the specific iron(III) oxyphosphate chelate. Ferric lactobionate thus displays the monomer–polymer equilibrium that characterized the "ferric citrate" system (Spiro and Saltman, 1969).

Ferrous Sulfate. Iron(II) may be bound to casein initially by ion exchange of calcium(II) from the micelle. The specific iron(III)–phosphoprotein chelate (Figure 6) would then be formed in situ by oxidation of casein-bound ferrous ion. Autoxidation of iron(II) may in some cases be accompanied by modification of phosphorylserine residues (Rosenstein and Taborsky, 1970). Some evidence for polynuclear iron in ferrous sulfate supplemented skim milk may be seen in the high base line absorbance that is superimposed upon the iron(III) oxyphosphate chelate absorbance band (Figure 6). Appreciable unbound ferrous ion is present soon after supplementation of raw milk; in addition, the milkfat fraction of raw milk binds a very large percentage of added ferrous ion but not of polynuclear or chelated ferric complexes (Figure 1A).

Ferric Polyphosphates. The distribution of iron added to milk as iron(III) polymetaphosphate has been studied by Basch et al. (1974). We have confirmed their major conclusion (data not shown) that iron added in the form of such complexes is associated with centrifugal and isoelectric casein. As discussed above for the case of the polynuclear ferric fructose complex, however, dialysis, sedimentation, and isoelectric precipitation cannot distinguish between adsorption and chelation as the mechanism of iron binding. Basch et al. (1974) also found that sodium [^{32}P]polymetaphosphate as well as [^{59}Fe]ferric polymetaphosphate were bound to casein micelles. It thus appears that much of the iron added as polynuclear phosphate complexes may be physically adsorbed to the calcium caseinate micelle as large molecular complexes. Aging of the "ferripolyphosphate" complex (Jones et al., 1972; Basch et al., 1974) is undoubtedly due to increase in polynuclearity of the iron(III) and to increase in size of the iron(III) polymetaphosphate polymer; such aging has been observed in the polynuclear hydroxy-iron(III) polymers (Sommer et al., 1973; Bates et al., 1973). Unpredictable changes in the reactivity of such polynuclear iron(III) systems may seriously limit their use in experimental or commercial situations where reproducibility and rapid ligand-exchange are required. It has been our experience that the insoluble "ferripolyphosphate–whey" complex (Jones et al., 1972) is of limited usefulness as a milk supplement because of rapid settling of the iron particles.

Implications for Nutritional and Organoleptic Qualities of Milk. Successful trace element fortification of dairy products requires chemical additives that will

provide good nutritional assimilation along with a degree of inertness that will minimize objectionable sensory or technological alteration. In earlier work (Hegenauer et al., 1979a,b), we showed that oxidative deterioration of iron- and copper-supplemented milks—as judged by the thiobarbituric acid reaction—could be reduced considerably when chelated forms of iron(III) and copper(II) were employed. The NTA and lactobionate chelates of these metals were particularly promising in these evaluations. With data for lipid peroxidation and for the distribution of iron in supplemented milk, we can perhaps now predict the organoleptic consequences of fortification with certain metal complexes. Previous work has identified the iron(III)–casein complex as a source of supplemental iron with exceptional bioavailability (Carmichael et al., 1975).

Ferrous ion has been used frequently—and somewhat thoughtlessly—as a source of supplemental iron, even though it has been shown repeatedly to promote oxidative changes in fortified milk (Wang and King, 1973). From earlier discussion, we can appreciate the diverse reactions of the ferrous ion and their deleterious consequences. For example, the casein fraction of raw milk binds iron(II) incompletely, leaving a disproportionately large amount of iron(II) to bind with the milkfat fraction (Figure 1). The affinity of iron(II) for the milkfat undoubtedly contributes to the rapid peroxidation of lipid observed in milks supplemented with ferrous ion. The tendency for iron(II) to undergo cyclic autoxidation and to form ferric hydroxide–protein complexes may contribute additional instability to supplemented milks during prolonged storage. High molecular weight hydroxy-iron(III) polymers like ferric fructose have been shown to rival ferrous sulfate in promoting lipid peroxidation of milkfat (Hegenauer et al., 1979a,b). In the present study, we could identify no unusual affinity of ferric fructose for milkfat that might account for its catalytic potential, but we did observe the nonspecific adsorption of polymeric iron onto the casein micelle and an alteration of the density equilibrium of the casein micelles in milk (Figure 1). Previous work (Carmichael et al., 1975) has shown that the nutritional availability of ferric fructose is not affected by complexation with casein. Polynuclear iron(III) polymetaphosphate complexes have also been shown to have good bioavailability (Jones et al., 1975), but from the physical–chemical standpoint they would seem to have little advantage over polynuclear ferric fructose as iron supplements for milk.

Chelated ferric iron provides a better means of controlling the distribution and reactivity of iron in supplemented milks. The strong ferric EDTA chelate catalyzes very little lipid peroxidation in raw and homogenized milk (Hegenauer et al., 1979a), but the present data show clearly that it donates little iron to casein. As a result, ferric EDTA added to milk would show the gastrointestinal absorption of the intact chelate rather than the enhanced bioavailability of the iron(III)–casein complex (Carmichael et al., 1975). There is evidence that ferric EDTA may be well absorbed but poorly utilized because of rapid urinary excretion of the undissociated chelate (Larsen et al., 1960).

The ferric NTA and lactobionate chelates, however, exchange iron readily with casein phosphorylserine residues, which then, by definition, bind iron more strongly than the original complex. This very specific chelation apparently serves to remove iron from the environment of oxidizable milkfat, since lipid peroxidation even in raw milk supplemented with these iron complexes is significantly lower than that observed with ferrous sulfate (Hegenauer et al., 1979a,b). Emulsification of milkfat with casein and micelles during homogenization may also provide a layer of iron-binding proteins (Fox et al., 1960) which prevents iron from contacting sensitive lipid. It is well known that homogenization significantly reduces oxidation in both unfortified and iron-fortified milks (Wang and King, 1973). We have not yet studied the effects of homogenization or pasteurization required for commercial processing on the stability of the iron-laden micelles following addition of iron complexes to raw milk. We feel that rational manipulation of ligand-exchange phenomena, using stable yet reactive transition metal chelators like NTA and lactobionate, holds the key to successful supplementation of dairy products with hematologically important metals. Experiments now in progress indicate that iron and copper bioavailability of fluid milk supplemented with these chelates is equal or superior to the inorganic ions by several criteria of biological assimilation.

CONCLUSIONS

Iron added to milk at the fortification level (<5 mM) is bound to micellar casein. Ferric iron presented as the small molecular chelates of NTA binds rapidly to the casein phosphoproteins to form the iron(III)-di-*O*-phosphorylserine chelate, which can be identified spectrophotometrically. Ferrous salts also form some iron(III) oxyphosphate chelates after autoxidation, but incomplete binding to the casein fraction permits some of the iron(II) to bind to milkfat. When used as a supplement for milk, the ferric lactobionate chelate shows characteristics of a monomer–polymer mixture, since both iron(III)-oxyphosphate chelates and adsorbed iron(III) oxyhydroxide polymers are present spectroscopically. When milk is supplemented with ferrous salts, the milkfat fraction may bind iron not immediately complexed by caseins; this iron may then catalyze lipid peroxidation and cause the extensive oxidative deterioration frequently observed in certain iron-fortified dairy products. Iron added as small molecular iron(III) chelates, however, binds rapidly to casein and insignificantly to milkfat. Such complexes of ferric iron may thus be more suitable iron donors for fortification programs in order to preserve the organoleptic stability of milk.

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Analysis of Vitamin K_1 in Some Green Leafy Vegetables by Gas Chromatography

Richard M. Seifert

A gas chromatographic method has been developed for the assay of naturally ocurring vitamin K_1. The vitamin was extracted with hexane from the freeze-dried plant, purified on an alumina column, and assayed directly by gas chromatography. Lettuce, cabbage, and spinach were found to contain 0.51, 0.76, and 3.30 mg/100 g (dry weight basis), respectfully. Convenient levels of sensitivity for the assay were 0.05–1.0 μg although lower levels are detected.

The K vitamins have an essential but not completely understood role in the hepatic synthesis of prothrombin and proconvertin which are required for the blood clotting process. There is also evidence to indicate that K vitamins are involved in electron transfer in the oxidative chain and associated phosphorylation (Cantarow and Schepartz, 1962). Vitamin K_2 (2-methyl-3-difarnesyl-1,4-naphthoquinone), from intestinal microflora, appears to provide adequate amounts for human needs. Nutritional requirements for vitamin K_1 (2-methyl-3-phytyl-1,4-naphthoquinone) have not been established, probably because all its functions are not completely understood. An assay method for vitamin K_1 would be an important tool to use in exploring the functional and related nutritive requirements of vitamin K_1 in man. Little attention has been given analytical assays for K_1 in biological systems. Schilling and Dam's (1958) colorimetric method was applied only to alfalfa, and interfering substances could affect the accuracy of the method. Aaron and Winefordner (1972), in their publication of a phosphorimetry method for pure K_1, reviewed the literature and found few quantitative determinations of naturally occurring K_1. The comprehensive review of gas chromatography (GLC) of the fat-soluble vitamins by Sheppard et al. (1972) discussed the results of various GLC assays for K_1 and analogues. Initially, Nair and Turner (1963), working with lipid extracts and synthetic mixtures, and later Carroll and Herting (1964) and Libby et al. (1967), using synthetic mixtures, showed the feasibility of separating K_1 by GLC. A number of other workers, reviewed by Sheppard et al. (1972) and cited by Aaron and Winefordner (1972), explored the possibilities of GLC assay. Sheppard and Hubbard (1971) described a GLC method for K_1 in liver tissue but did not report any results. Problems of thermal breakdown of pure K_1 were reported by Vetter et al (1967), although they obtained excellent resolutions of their Me_4Si derivatives of the reduced K_1. The elegant work of Dialameh and Olson (1969) in separating and applying direct GLC analysis of K_1 from liver tissue showed the feasibility of using GLC for assaying naturally occurring K_1. However, their method required a long separation procedure. A simple quantitative method with reported assay results for K_1 in human foods has not been demonstrated.

The purpose of the present work was to develop a simple, sensitive assay of naturally occurring vitamin K_1. An extraction and one-step separation procedure proceeded the direct GLC assay of extracts from three commonly eaten vegetables–spinach, lettuce, and cabbage.

Western Regional Research Center, Science and Education Administration, U.S. Department of Agriculture, Berkeley, California 94710.

EXPERIMENTAL SECTION

Materials. (1) Fresh spinach, iceberg lettuce, and head cabbage were obtained from local produce markets and immediately separated into individual leaves, frozen, and freeze-dried. (2) Vitamin K_1 (2-methyl-3-phytyl-1,4-naphthoquinone) used as a standard without further purification was obtained from Sigma Chemical Co, St. Louis, MO. Its purity was checked by GLC. (3) Dotriacontane from Eastman Kodak C., Rochester, NY, was purified by recrystallization from ether and its purity checked by GLC. (4) Ether was freshly distilled before use. (5) Hexane 95% from Matheson, Coleman & Bell Division of the Matheson Co., Norwood, OH, was distilled through a 20 plate still. (6) Heptane from Eastman Kodak (EK 2215) was used

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