Effects of Supplemental Iron and Copper on Lipid Oxidation in Milk. 1. Comparison of Metal Complexes in Emulsified and Homogenized Milk

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Because of its wide consumption in the United States, cow milk is a good vehicle for delivering supplemental iron and copper to prevent anemia in infants, children, and adolescents, but transition metals may cause "oxidized" flavors and odors in dairy products. To help predict oxidative deterioration that may occur in commercially fortified milks and to complement organoleptic evaluations we describe the use of the thiobarbituric acid (TBA) test to quantitate lipid peroxidation due to iron and copper. Various chemical forms of iron and copper complexes—ionic, chelated, and polynuclear—are compared with respect to their ability to promote lipid peroxidation during short-term incubation and long-term cold storage in raw and pasteurized milk. Emulsification of milk fat prior to fortification greatly reduced lipid peroxidation by all metal complexes. Compared under any conditions to the simple ferrous and cupric salts, the iron(III) and copper(II) chelates of nitrilotriacetate and lactobionate produced significantly less lipid peroxidation at concentrations within the practical range of fortification.

Recent nutrition surveys in the United States and elsewhere continue to demonstrate the prevalence of anemia resulting from inadequate intake of iron, particularly among young children, adolescents, and women of menstrual age (HANES, 1974; Nutrition Canada, 1973). Because milk is widely consumed by infants, children, and adolescents, it is a logical vehicle for delivering supplemental iron during the period of rapid growth.

Recognizing that cow milk has an extremely low natural content of iron and copper, pediatricians now universally recommend the addition of these essential nutrients to milk-based formulas to improve the hematological status of bottle-fed infants. Levels of 6–12 mg of Fe/L and 0.4 mg of Cu/L of formula have been recommended for infants at risk of iron deficiency (Committee on Nutrition, 1976). Although there is still some conflicting evidence about the ability of milk to inhibit iron absorption (Carmichael et al., 1975), there is little argument that iron added to milk not only shows excellent biological availability, but can prevent iron-deficiency anemia (Demott, 1971; Wang and King, 1973b). Contamination of milk by metal ions, however, has long been known to promote the development of a characteristic "oxidized" odor and flavor, probably as a result of lipid peroxidation of milk fat (Shipe et al., 1978; Wang and King, 1973a, Forss et al., 1955a,b). The presence of transition metals—notably iron and copper—is thus assiduously avoided in every aspect of dairy processing. Can the nutritional advantages of adding iron and copper to milk be made compatible with the requirements of modern dairy technology to maintain stability and palatability?

A large proportion of the total iron in American diets is derived from fortified foods; in most cases, however, only chemically inert and nutritionally unassimilable forms of iron are used as additives to prevent oxidative deterioration of foods during storage. We are seeking a solution to this nutritional paradox by developing trace element complexes that display a useful balance of physical-chemical, nutritional, and organoleptic properties as new food additives. This and the following report (Hegenauer et al., 1979) will demonstrate that lipid peroxidation—and, by inference, some aspects of "oxidized" flavor—can be reduced when milk is supplemented rationally with newer chelated forms of iron and copper. The metal compounds selected for our studies are well characterized and reproducibly synthesized representatives of the principal classes of metal complexes: ionic, chelated, and polynuclear. To monitor oxidative deterioration of supplemented milks, we have used the well-known thiobarbituric acid (TBA) assay, which Dunkley and Jennings (1951) and King (1962) have adapted to correlate lipid peroxidation and organoleptic evaluation of iron-supplemented milks. To assist in establishing proper quality control procedures for dairy processing, we present information on interpreting the TBA reaction performed on iron- and copper-supplemented milk. In addition, we have simulated some significant variables that may be encountered in the commercial processing of supplemented milks and present data on the effects of homogenization and storage on the TBA reactivity of supplemented milk. The effects of heat treatment will be considered in the following paper.

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MATERIALS AND METHODS

Source of Milk. Raw milk and homogenized, pasteurized ("homogenized") milk was obtained in 1-qt cartons from a southern California dairy prior to commercial distribution.

Laboratory Milk Processing. *Ultrasonic Emulsification.* When we wished to control the degree of emulsification of milk fat or to differentiate the effects of emulsification and heating, we treated small samples of raw milk (25–50 mL) ultrasonically in a vessel thermostated at 30 °C, using a Model W140D Branson sonifier equipped with a Model L converter, 0.5-in. step horn, and macrotip and operated at maximum power (130 watts) for varying times.

Skim Milk. Raw milk was skimmed by centrifugation at 900*g* for 30 min at room temperature in a Servall GSA rotor. The subnatant was collected by aspiration without disturbing the fat layer.

Milk fat analyses were performed by extraction of total lipid (Folch et al., 1959).

Thiobarbituric Acid (TBA) Test. Many of our preliminary experiments quantitating iron-catalyzed lipid peroxidation in milk were monitored with the TBA reaction of King (1962). The data reported here, however, were obtained with a TBA test based on the method of Dunkley and Jennings (1951), whose paper should be consulted for rationale. We modified their method by (1) using less TBA to prevent crystallization, (2) combining citric (Porter et al., 1976) and phosphoric acids as both acidulants and metal chelators, (3) extracting the red pigment into cyclohexanone instead of isoamyl alcohol, and (4) using ammonium sulfate instead of pyridine to desorb

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the red pigment from the precipitated protein.

Reagents. 2-Thiobarbituric acid (Eastman) was crystallized from hot water. The TBA reagent was prepared immediately before use by mixing equal volumes of freshly prepared 0.025 M TBA (brought into solution by neutralizing with NaOH) and of 2 M H_3PO_4/2 M citric acid.

Assay Method. Reactions were terminated by pipetting 5.0 mL of milk sample into a 15-mL glass centrifuge tube containing 2.5 mL of TBA reagent. Tubes were mixed, placed without delay in a boiling water bath for exactly 10 min, and then cooled on ice. Five milliliters of cyclohexanone and 1 mL of 4 M ammonium sulfate were then added (in a well-ventilated atmosphere). Tubes were stoppered, shaken for 2 min, and centrifuged at 6000*g* for 5 min at room temperature. The orange-red cyclohexanone supernatant was decanted and its absorbance of 532 nm was measured spectrophotometrically in a 1-cm light path. To check the internal consistency of our assay procedure, as suggested by Gutteridge (1975), we added 1,1,3,3-tetramethoxypropane to milk samples as a malondialdehyde standard. Absorbance was linear with concentration at least to 10 μM malondialdehyde (molar absorptivity at 532 nm $\simeq 1.4 \times 10^5$ L mol^{-1} cm^{-1}). We have reported absorbance values as estimates of lipid peroxidation rather than malondialdehyde concentrations without positive evidence of identity (Gutteridge and Tickner, 1978). This modified TBA test gave a more reproducible assay for metal-catalyzed peroxidation than the methods described by King (1962) or Dunkley and Jennings (1951). All three methods gave a high background absorbance in the presence of iron. Absolute absorbance obtained with the three different TBA tests varied, of course, but all gave similar relative data and each was equally applicable to our comparative studies. Selection of appropriate "reagent blanks" and "controls" for the presence of metals alone in these experiments proved difficult because both iron and copper caused some lipid peroxidation at the temperature of the TBA assay, in addition to that catalyzed under the experimental conditions. Absorbances of TBA pigment are therefore not corrected for the absorbance of control samples to which no metal was added.

Iron and Copper Supplements. Iron and copper complexes were freshly prepared before each experiment. We chose for most experiments to work with solutions of 0.2 M iron and/or 0.02 M copper in order to avoid the addition of very concentrated solutions of ferrous and cupric ions, which are well-known precipitants of milk proteins. We did not observe protein precipitation, however, with the mononuclear or polynuclear metal chelates.

Ferrous Salts. Ferrous chloride or ferrous sulfate was prepared in oxygen-free water as a 0.2 M stock solution in 0.001 N HCl and filtered under nitrogen; these solutions could be stored frozen to retard oxidation.

Ferric Nitrilotriacetate (NTA). The ferric NTA 1:1 chelate was prepared (Carmichael et al., 1975, 1977) as a 0.2 M stock solution by dissolving $FeCl_3 \cdot 6H_2O$ (0.2 mol) in 600 mL of water and adding dropwise, with good mixing, a solution of 0.2 mol of disodium hydrogen nitrilotriacetate (Sigma Chemical Co.) in 200 mL of water; the chelate solution was then titrated to pH 5.5–5.8 with solid $NaHCO_3$ (added gradually to permit controlled evolution of CO_2) and diluted to 1 L. Since >98% of the chelate typically passed an Amicon PM-10 ultrafiltration membrane, iron was predominately in mononuclear, low-molecular-weight form.

Ferric Lactobionate. The ferric lactobionate chelate was prepared as a 0.2 M stock solution by dissolving $FeCl_3 \cdot 6H_2O$ (0.2 mol) and lactobionic acid (0.2 mol; hemicalcium salt, pentahydrate; Sigma Chemical Co.) in 700 mL of water, titrating to pH 6.8 by dropwise addition of 3 N NH_3, and diluting to 1 L. Potentiometric titration of the 1:1 chelate indicated that the calcium lactobionate complex was no longer present at pH 6.8. The chelate was of low molecular weight (>98% through the Amicon PM-10 ultrafilter).

Ferric Fructose. The 1:10 ferric fructose polynuclear complex (Bates et al., 1972, 1973) was prepared as a 0.2 M stock solution by dissolving $FeCl_3 \cdot 6H_2O$ (0.2 mol) and D-fructose (2.0 mol) in 800 mL of water and titrating to pH 8.0 with 5 N NaOH with rapid stirring; this pH was maintained with NaOH for about 60 min, and the solution was diluted to 1 L. The Amicon PM-10 filter passed 81% and the Amicon PM-30 filter passed 98% of this high-molecular-weight complex.

Other Iron(III) Complexes. The ferric ethylenediaminetetraacetate (EDTA) 1:1 chelate was prepared by combining equivalent disodium EDTA (adjusted to pH 8 with NaOH) and ferric chloride, followed by titration to pH 6.8 with NH_3. "Ferric citrate" solutions were prepared (Spiro et al., 1967; Avol et al., 1973) with 0.4, 1.0, or 5.0 equiv of citrate per equivalent of iron(III) by combining ferric chloride solution and trisodium citrate (solid or in solution), followed by titration to pH 6.8. The molecular size and reactivity of "ferric citrate", like that of ferric fructose, is determined by the ligand: iron ratio present during hydrolysis (Spiro et al., 1967; Bates et al., 1973).

Copper(II) Complexes. Aqueous solutions of cupric chloride or cupric sulfate were prepared at a variety of concentrations. Cupric chloride was used to prepare the lactobionate chelates to avoid precipitation of $CaSO_4$. The 1:1 copper(II) chelates were prepared as 0.2 M stock solutions in the manner described above, by mixing cupric salts and either disodium hydrogen NTA or hemicalcium lactobionate and titrating to pH 6.8 with 3 N NH_3 before dilution to final volume. The copper(II) fructose complex could not be prepared because the reducing sugar caused formation of insoluble Cu(I).

Iron/Copper Complexes. Stable combinations of iron and copper were prepared by mixing metal complexes having a corresponding anion or ligand before diluting to the desired concentration. Copper was not added directly to the iron(III) fructose solution.

Incubation and Sampling of Supplemented Milk. Except where noted, incubation mixtures contained 1 volume of milk, 0.02 volume of 1.0 M Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)/NaOH buffer (pH 6.8), and 0.005 volume of iron, copper, or water to achieve the desired metal concentration. Reactions were initiated by addition of iron. When milk was emulsified in the laboratory, Hepes buffer was added to the milk after ultrasonic treatment. Milk was placed in Erlenmeyer flasks holding about twice the volume of the initial reaction mixture and incubated in a 25 °C water bath with reciprocal agitation. In most cases, duplicate or triplicate samples were withdrawn from each of two identical incubation mixtures at predetermined intervals.

Sterile conditions were not maintained for short-term experiments designed to study the time course of oxidation at or near room temperature. For studies involving long-term cold storage, the original milk containers (cardboard cartons) were used as reaction vessels; additions were made and samples were withdrawn aseptically by piercing the cartons with sterile hypodermic needles after

Table I. TBA Reactivity of Raw Milk and Milk Fractions Supplemented with $FeSO_4$ (2 mM)

milk fraction	A_{532nm} 1cm: iron added before fractionation[a,e]	A_{532nm} 1cm: iron added after fractionation[b,e]
whole (raw) milk	1.955	(1.955)
raw skim milk	0.371	0.257
whey[c]	0.276	0.231
ultrafiltrate[d]	0.359	0.257

[a] Whole raw milk incubated with $FeSO_4$ (2 mM) and pH 6.8 Hepes buffer (20 mM) 3 h at 25 °C before fractionation in the cold. [b] Milk fractionated in cold, then fractions incubated as in *a* above. [c] Ultracentrifugal supernatant (Carmichael et al., 1975). [d] Ultrafiltrate (Amicon PM-30) of ultracentrifugal whey. [e] Results are average of determinations on duplicate samples from each of four incubation mixtures.

swabbing the surface with 70% ethanol. Iron and copper supplements were sterilized by passage through Millipore 0.45-μm membranes.

Statistical Analysis. Means of grouped data were compared by the two-sample *t* test. Probabilities (*P*) were computed by integrating the *t* distribution. Levels of significance ($2P \leq 0.05$) are based on two-tailed tests.

EXPERIMENTS AND RESULTS

Location of TBA-Reactive Material in Raw Milk. Raw milk is particularly susceptible to iron-catalyzed oxidation and provides a good test system for comparing experimental variables. The majority of the TBA-reactive material appeared to reside in the milk fat fraction (King, 1962), since iron-supplemented raw skim milk (1.0% fat) produced less than 20% of the TBA color produced by iron-supplemented raw whole milk (4.1% fat) (Table I). The TBA color produced by iron-supplemented whey or whey ultrafiltrate was only slightly less than that of raw skim milk and may be partly attributed to oxidation of unsaturated lipid, phospholipid (Tarassuk and Koops, 1960), or carbohydrate (Wilbur et al., 1949; King, 1962), which are known to react with TBA. Similar results (not shown) were obtained at a variety of iron concentrations. There was evidently little migration of the "oxidized" component, since TBA reactivity of milk fractions was similar whether they were supplemented before or after fractionation (Table I). Although chemical identification of the oxidizable component was beyond the scope of this study, we are probably justified hereafter in calling it a lipid (Forss et al., 1955a,b; Marcuse and Johansson, 1973).

Some Characteristics of the TBA Reaction Products in Iron-Supplemented Milk. The chemistry of the TBA assay has recently been reviewed by Gray (1978). Iron surely catalyzes the oxidation of more than one lipid or carbohydrate moiety having different rates of reaction with the TBA reagent. Since our conclusions about lipid oxidation in milk are drawn from data obtained with the TBA reaction, it is relevant to discuss the limitations of the assay and the character of the colorimetric product.

As described by Dunkley and Jennings (1951), a mixture of yellow (450 nm) and red (532 nm) pigments is formed during the TBA reaction with oxidized homogenized milk (Figure 1); very little color developed without heating. Malondialdehyde, in contrast, reacted rapidly with cold TBA to form a single condensation product absorbing at 532 nm. The degradation of TBA in hot acid, reported by Tarladgis et al. (1962), did not contribute significantly to the total absorbance. We chose to monitor only the more conventional "532-nm" pigment for these studies, but both pigments may be equally useful in drawing chemical correlates for grading sensory evaluation (Marcuse and Johansson, 1973).

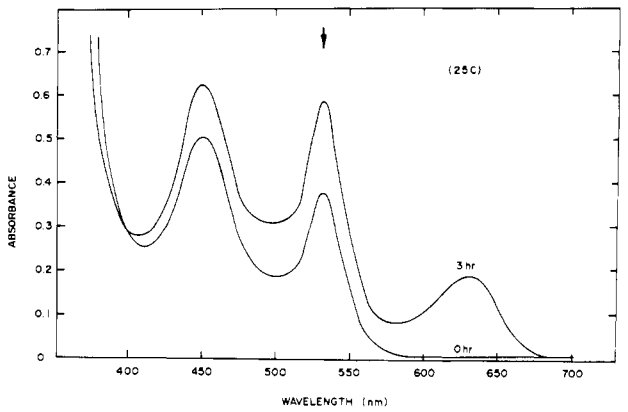

Figure 1. Absorbance spectra of TBA reaction products from iron-oxidized milk. Homogenized pasteurized milk was incubated with 20 mM Hepes buffer (pH 6.8) and 7.6 mM $FeSO_4$ for 0 or 3 h at room temperature before samples were removed for the TBA assay. A rapid oxidative reaction (discussed in text) involving iron at the high temperature of the TBA assay is evident in the unincubated ("0 h") milk sample. The arrow marks the position of the single 532-nm absorbance band obtained with authentic malondialdehyde. The 630-nm absorbance band is attributed to an iron-TBA chelate (see text).

As estimated by the TBA assay, significant oxidation could be observed immediately after the addition of iron to homogenized milk at room temperature (Figure 1). TBA reactivity of iron-supplemented milk did, however, increase significantly after a 3-h incubation. Note in Figure 1 that incubation at room temperature produced a proportionately greater increase in the 532-nm absorbance band than in the 450-nm band. TBA reactivity was also significant if unsupplemented milk was added to a TBA reagent containing iron; furthermore, delay of more than a few minutes between addition of incubated sample to the TBA reagent and heating at 100 °C frequently increased the TBA reactivity (Dunkley and Jennings, 1951) (data not shown). Taken together, these results suggest that a large share of the total TBA color is attributable to a reaction between metal complex and milk lipid accelerated by the temperature (100 °C) of the TBA reaction (Dunkley and Jennings, 1951). This reaction may be facilitated by dissociation of iron from the iron(III)–casein complex (Carmichael et al., 1975) by the acidulants used in the assay. It was thus not possible in all experiments to differentiate TBA reactivity due to "nonspecific" catalysis by iron at the high assay temperature from that due to "specific" catalysis at ordinary storage temperatures (i.e., the oxidation reactions leading to "off-flavor" in milk products). Because of these considerations, we did not attempt to devise appropriate "control" incubation conditions; rather, our data report the summation of specific and nonspecific reactions involving metal ions.

When homogenized milk was incubated for 3 h with high concentrations of supplemental iron, the TBA reaction product was frequently purple when extracted into the cyclohexanone phase. This color (Figure 1) may be attributable to a metal–TBA complex (Murphy and Svehla, 1978).

Effect of Emulsification and Skimming on Iron-Catalyzed Oxidation. Relatively brief ultrasonic treatment decreased the susceptibility of raw milk to oxidation by ferrous sulfate, a commonly used iron supplement (Figure 2). Under our assay conditions, oxidation

Table II. TBA Reactivity of Raw, Raw Emulsified, and Raw Skim Milk Supplemented with 1 mM Fe[a]

iron complex	A_{532nm}^{1cm} raw milk	emulsified milk[b,e]	Skim milk[c,e]
$Fe^{II}SO_4$	2.273	0.716	0.340
Fe^{III} fructose	1.492	0.601	0.386
Fe^{III} lactobionate	0.954	0.308	0.236
Fe^{III} NTA[d]	0.656	0.295	0.162

[a] Milk incubated with Fe (1 mM) and pH 6.8 Hepes buffer (20 mM) for 3 h at 25 °C. [b] Raw milk ultrasonically emulsified for 60 s at 30 °C as described in text. [c] Skimmed raw milk. [d] NTA = nitrilotriacetate. [e] Results are average of determinations on duplicate samples from each of two incubation mixtures.

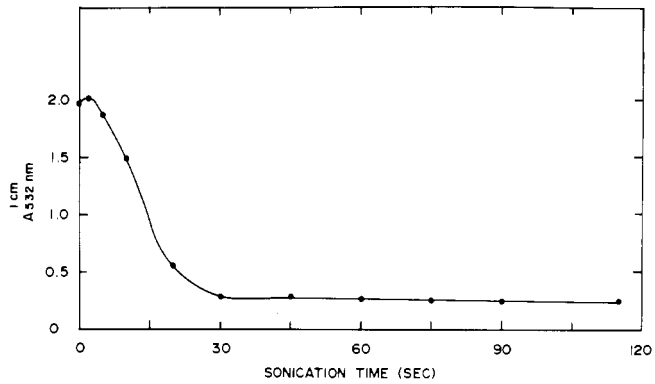

Figure 2. Effectiveness of ultrasonic emulsification in reducing susceptibility of raw milk to iron-catalyzed oxidation. Aliquots of raw milk were treated ultrasonically at room temperature before addition of Hepes buffer and $FeSO_4$. Final incubation conditions: 1.0 mM Fe; 20 mM Hepes: pH 6.8; 3 h at 25 °C. Datum points represent averages of two samples from each of two incubation mixtures. TBA reactivity of the oxidized product is indicated by A_{532nm}.

catalyzed by ferrous sulfate was suppressed to the same degree in raw milk emulsified ultrasonically for 60 s at 30 °C and in commercial homogenized, pasteurized milk (data not shown). We recognize the limitations of extrapolating laboratory-scale sonication to commercial high-pressure homogenization. Simulation of homogenization by sonication, however, allowed us to compare untreated, "homogenized", and skim milk prepared from the same batch of raw milk and thus to avoid variation in susceptibility to oxidation encountered among different batches of milk. Homogenization without pasteurization leads to lipolytic rancidity that is unacceptable in commercial practice. In this experiment, however, we attempted to differentiate the effect of emulsification from that of heating, which will be considered in the following paper.

Ultrasonic emulsification suppressed oxidation catalyzed by all of the principal chemical forms of soluble iron: ionic (ferrous sulfate), polynuclear (ferric fructose), and mononuclear chelated (ferric lactobionate, ferric NTA). As shown in Table II, TBA reactivities in emulsified raw milk were 30–45% of those observed in raw milk (4.0% fat); TBA reactivities in skim milk (1.1% fat) were 15–25% of those in raw milk. The relative catalytic activity of these iron complexes was the same in all three milk products, and ferrous sulfate produced two–three times as much TBA reactivity as ferric NTA (Table II).

TBA Reactivity of Iron-Supplemented Milk: Comparison of Iron Complexes. Table III compares several ionic, polynuclear, and chelated iron complexes, which are ranked in approximate decreasing order of activity in catalyzing oxidation in the TBA test of iron-supplemented milks. With few exceptions, iron compounds exhibited the same relative activity in raw and homogenized, pasteurized milks. As expected from the results in Table II, ferrous salts produced the greatest oxidation and ferric chelates the least; polynuclear complexes were intermediate. The difference between ferrous chloride and ferrous sulfate was statistically significant in this experiment; this result was not predicted from the predominantly ionic character of the ferrous ion. "Ferric citrate" complexes are, in general, an equilibrium mixture of polynuclear and mononuclear chelates; high ligand/Fe ratios favor the low-molecular-weight complexes (Spiro et al., 1967). The low-molecular-weight 1:5 ferric citrate catalyzed somewhat less TBA reactivity than the polymeric 1:0.4 complex, which was in turn less catalytically active than the polymeric ferric fructose complex. The 1:1 iron(III) chelates of lactobionate, NTA, and EDTA showed the least reactivity. The stability of ferric EDTA relative to ferric NTA is well recognized from its coordination chemistry and slower reactivity with transferrin (Spiro and Saltman, 1969). No information is presently available on the stability of the ferric lactobionate complex, but its greater catalytic activity relative to ferric NTA in these experiments may be related to polynuclear iron complexes generated by a monomer–polymer equilibrium.

Kinetics of Iron- and Copper-Catalyzed Oxidation: Effect of Metal Concentration. *Iron.* The degree of maximum ("plateau") oxidation observed in homogenized, pasteurized milk increased with increasing iron concen-

Table III. Comparison of Iron Supplements by TBA Reactivity Catalyzed by 0.5 mM Fe in Raw and Homogenized Pasteurized Milk[a]

iron compound	ligand/Fe ratio	A_{532nm}^{1cm} raw milk	homogenized milk
$Fe^{II}Cl_2$		0.916 ± 0.057[a]	0.311 ± 0.019[a]
$Fe^{II}SO_4$		0.849 ± 0.043[b]	0.270 ± 0.026[b]
Fe^{III} fructose	10.	0.566 ± 0.059[c]	0.223 ± 0.040[c]
Fe^{III} citrate	0.4	0.487 ± 0.012[d]	0.175 ± 0.015[d]
Fe^{III} citrate	1.	0.464 ± 0.031[de]	0.172 ± 0.010[de]
Fe^{III} citrate	5.	0.465 ± 0.027[def]	0.161 ± 0.009[def]
Fe^{III} lactobionate	1.	0.420 ± 0.027[g]	0.161 ± 0.006[dfg]
Fe^{III} NTA[b]	1.	0.385 ± 0.017[h]	0.159 ± 0.012[defgh]
Fe^{III} EDTA[c]	1.	0.250 ± 0.014[i]	0.106 ± 0.014[i]

[a] Results are expressed as mean ± standard deviation for six determinations (triplicate TBA assays from each of two incubation mixtures). Commercial raw or homogenized pasteurized milk incubated in original cartons with sterile Fe (0.5 mM) for 3 h at 25 °C. Within each column (raw milk or homogenized milk) means not sharing a common superscript letter are significantly different ($2P \leq 0.05$) based on the two-tailed t test. [b] NTA = nitrilotriacetate. [c] EDTA = ethylenediaminetetraacetate.

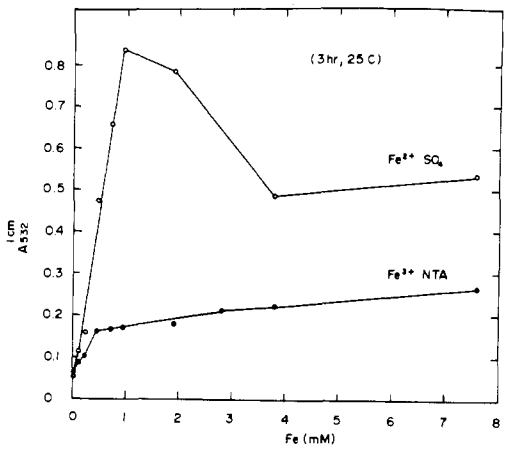

Figure 3. Oxidation of homogenized pasteurized milk as a function of iron concentration, provided by supplemental ferrous sulfate or ferric NTA. Final incubation conditions: 20 mM Hepes (pH 6.8); 25 °C for 3 h. Datum points represent averages of two samples from only one reaction mixture. TBA reactivity of the oxidized product is indicated by A_{532nm}. To permit comparison to unsupplemented milk, absorbances have not been corrected for "control" (= zero iron) level.

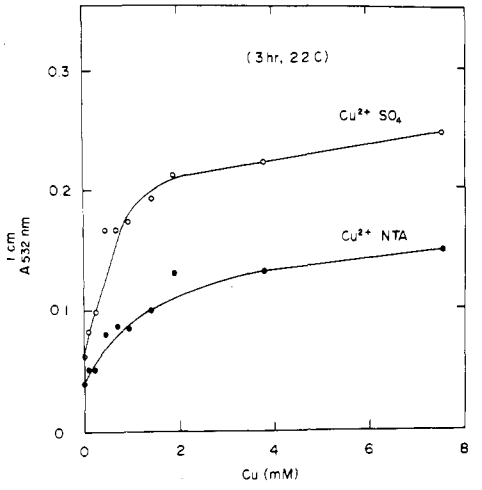

Figure 4. Oxidation of raw milk as a function of copper concentration, provided by supplemental cupric sulfate or cupric NTA. Refer to legend of Figure 3 for further details.

tration. When ferrous sulfate was used as the oxidant, TBA reactivity was roughly a linear function of iron concentration up to about 1 mM (Figure 3). Above 1 mM Fe, TBA reactivity appeared to decrease, perhaps as a result of polynuclear iron formation and side reactions involving the TBA reagent (see Figure 1). Oxidation catalyzed by ferric NTA did not display the "maximum" shown by ferrous sulfate. Ferric NTA caused considerably less oxidation than ferrous sulfate at every iron concentration tested. The level of iron fortification recommended for pediatric use is 0.2–0.3 mM (~12 mg/quart).

Copper. Milk oxidation also increased with increasing supplemental copper. No maxima were found for TBA reactivity catalyzed by copper compounds (Figure 4). In this experiment, raw milk was used in order to increase the sensitivity of the test system. Chelated copper (cupric NTA) produced only about half the TBA reactivity of free cupric ion at every concentration tested. The level of pediatric copper fortification employed in infant formula by some manufacturers is about 6 μM (0.4 mg/quart). At molar concentrations within the practical range of fortification (below 1 mM), the catalytic potential of copper is considerably less than that of iron. When comparing

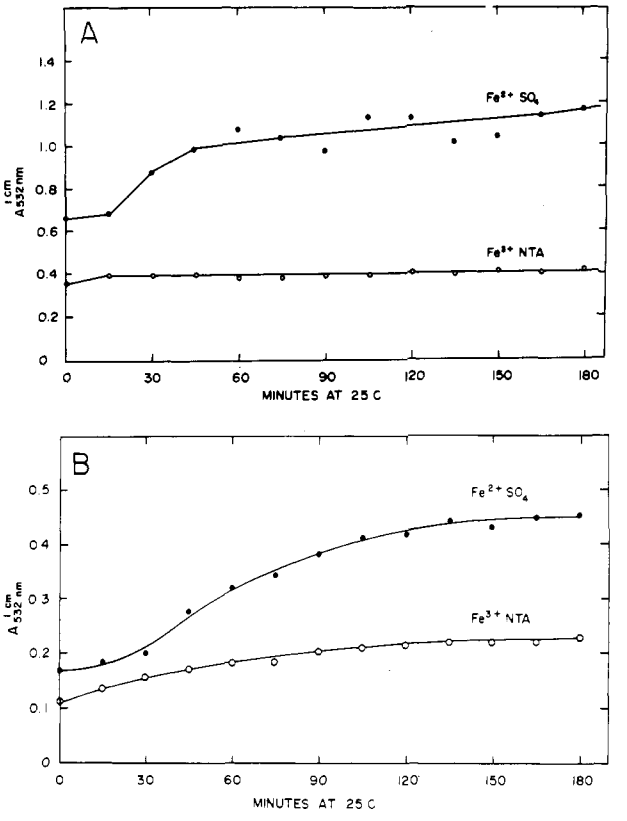

Figure 5. Time course of oxidation in (A) raw milk or (B) homogenized pasteurized milk supplemented with ferrous sulfate or ferric NTA. In A, 250 mL of raw milk was incubated with 0.5 mM Fe and 20 mM Hepes (pH 6.8) at 25 °C. In B, 250 mL of homogenized pasteurized milk was incubated with 1.0 mM Fe and 20 Hepes (pH 6.8) at 25 °C. Datum points are the averages of two samples from each of two incubation mixtures. TBA reactivity of the oxidized product is indicated by A_{532nm}.

the data in Figures 3 and 4, it should be remembered that homogenization and pasteurization has reduced the TBA reactivity of iron to about one-third that observed in raw milk.

Kinetics of Iron-Catalyzed Oxidation: Time Course at Room Temperature. *Raw Milk.* Many iron compounds appear to catalyze lipid oxidation immediately after addition to raw milk, but our TBA assay does not distinguish between oxidation produced during the experimental conditions and that produced during heating with the TBA reagent. We could identify three stages in the oxidation of milk by ferrous ion (Figure 5a): after a brief lag period of about 15 min, oxidation increased rapidly for about 30 min and continued slowly for the remainder of the observation period (3 h). As discussed below, further oxidation could be observed upon prolonged storage. In contrast, oxidation catalyzed by chelates of ferric iron, such as ferric NTA, was essentially unchanged, after the "initial" reaction, over a 3-h period (Figure 5a).

Homogenized Milk. The kinetics of oxidation of raw and homogenized, pasteurized milk have some similarities. Oxidation in homogenized milk supplemented with ferrous sulfate also exhibited a brief lag; oxidation thereafter was approximately linear until a stable plateau was reached after about 2 h at 25 °C (Figure 5b). In both raw and homogenized milk, plateau TBA reactivity catalyzed by ferric NTA was only about half that observed with ferrous sulfate (Figure 5). Note that the total amount of oxidized lipid in raw milk, indicated by TBA reactivity at the plateau, was several times greater than that found in homogenized milk supplemented at the same iron con-

centration. Inspection of Figure 5 shows, however, that maximum TBA reactivity is approached more rapidly in raw milk regardless of the nature of the iron supplement.

Kinetics of Iron- and Copper-Catalyzed Oxidation: Time Course during Cold Storage. In order to simulate a practical situation of commerce in which iron- and copper-fortified milks might be stored for several days before consumption, we surveyed the TBA reactivities of raw and homogenized, pasteurized milks supplemented with iron (0.5 mM) and copper (0.05 mM). We consider these metal concentrations, which are slightly above the presently recommended pediatric level for bottle-fed infants, to be more than adequate to satisfy the nutritional requirements of infants and young children at present levels of milk consumption in the United States. We evaluated four iron complexes that have been shown (Carmichael et al., 1975) to offer equivalent bioavailability: ionic ferrous salts (ferrous sulfate), polynuclear ferric oxyhydroxide (ferric fructose), and chelated ferric ion (ferric lactobionate and ferric nitrilotriacetate). With respect to their potential for catalyzing oxidation in both raw and homogenized milk, these four iron complexes could be sorted into two distinct classes. After storage for 7 days at 10 °C, TBA reactivity in milk supplemented with ferrous sulfate (ionic) and ferric fructose (polynuclear) was several times greater than that observed with either of the chelates ferric lactobionate or ferric NTA (Figure 6).

TBA reactivity in iron- and copper-supplemented raw milk reached a plateau after 3–4 days in the cold (Figure 6a). In homogenized milk (Figure 6b), oxidation catalyzed by ferric fructose and ferrous sulfate was apparently not complete after 7 days since the slopes of the oxidation curves were still strongly positive; oxidation catalyzed by ferric lactobionate and ferric NTA chelates, on the other hand, did not increase significantly after the "initial" reaction. Although not shown in Figure 6, the time course of oxidation was affected very little by the presence of 0.05 mM cupric salt or cupric chelate, which by themselves produced only slightly greater TBA reactivity than the unsupplemented control. Iron was thus the principal oxidant in these reactions, and there is no evidence that iron and copper were synergistic. Although we did not perform bacteriological tests on iron- and copper-supplemented milks subjected to cold storage, we did maintain relative asepsis in order not to contribute to the microbiological burden of the original commercial milk. In addition, final pH (6.6–6.8) was not noticeably different from the initial condition.

The NTA chelates of Fe^{3+} and Cu^{3+} were the least reactive compounds tested in this simulation. The most reactive compound tested was ferric fructose, which catalyzed TBA reactivities in raw and homogenized milk that were greater than ferrous sulfate. Because we did not include appropriate controls, we cannot rule out the oxidation of fructose (Wilbur et al., 1949), which was added as a component of the ferric fructose supplement, as a contributor to the final TBA reactivity observed with this iron complex.

DISCUSSION

Applicability of TBA Reaction to Predict Oxidized Flavor in Fortified Milk. The TBA test has had broad acceptance for determining oxidative deterioration in foods, particularly those containing unsaturated fatty acids. The test has been extensively applied to both milk and milk fat systems, in which the absorbance of TBA reaction products correlates positively with organoleptic evaluation (Patton and Kurtz, 1951; Dunkley and Jennings, 1951; King, 1962; Wang and King, 1973a). The TBA test has been used in one study of chemical and sensory changes in iron-fortified milk and was shown to be useful in predicting the consumer evaluation of such products (Wang and King, 1973a); that study confirmed two earlier reports (Edmondson et al., 1971; Demott, 1975) that "oxidized" flavor developed rapidly in milk fortified with ferrous salts but that milk fortified with ferric chelates was as stable as unfortified milk.

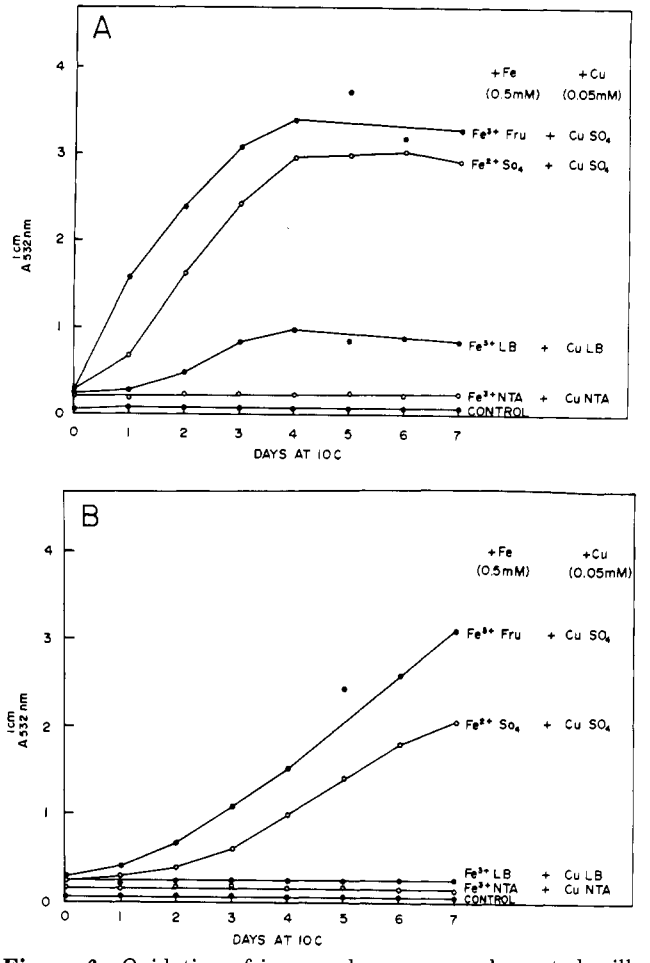

Figure 6. Oxidation of iron- and copper-supplemented milk during cold storage. One-quart cartons of (A) raw milk or (B) homogenized pasteurized milk were fortified by injection of sterile iron and copper solutions. Iron was provided by ferric fructose (Fe Fru), ferrous sulfate ($FeSO_4$), ferric lactobionate (Fe LB), or ferric NTA (Fe NTA). Copper was provided by cupric sulfate ($CuSO_4$), cupric lactobionate (Cu LB), or cupric NTA (Cu NTA). Final reaction conditions: 0.5 mM Fe; 0.05 mM Cu; (unbuffered) pH 6.6–6.8; 10 °C. Datum points are the averages of two samples from each of two cartons of milk. TBA reactivity of the oxidized product is indicated by A_{532nm}.

Oxidized flavor in milk is a consequence of lipid peroxidation in milk fat phosphatides (Shipe et al., 1978). Many workers no longer attempt to interrelate TBA reactivity and flavor, because the compounds that react with TBA to produce the 532-nm pigment are not the same as those responsible for oxidized flavors (Dunkley and Franke, 1967). The TBA test also cannot define qualitative flavor characteristics (Dunkley and Franke, 1967). The relatively low molar activity of copper in catalyzing lipid oxidation in our test system (Figure 4) is a prominent example of the failure of the TBA test to predict oxidized flavor since it is well known in experimental and commercial practice that low concentrations of copper lead rapidly to intense oxidized flavor (Shipe et al., 1978). The TBA test, then, is a complement to organoleptic scores and is a rapid and inexpensive way to screen experimental variables that must

be evaluated in developing an acceptable technology for trace element fortification of dairy products.

Variables Determining Oxidation in Iron- and Copper-Supplemented Milk. *Storage Time.* Oxidation in iron- and copper-supplemented milk increases as a complex function of the susceptibility and accessibility of milk fat lipids. Numerous unsaturated lipids are probably affected by added metal ions. Some particularly sensitive lipids must be responsible for the increase in TBA reactivity immediately following the addition of iron to raw milk, which we observed in a short-term kinetic study at room temperature. Oxidation reaches a relatively stable plateau after a few hours in both raw and homogenized, pasteurized milk supplemented with iron (Figure 5). Oxidation continues at a slow rate during long-term storage in the cold, however, to a level of TBA reactivity that is several times above the "plateau" level of oxidation observed at room temperature (Figure 6). This may be analogous to the observation that oxidized flavor is negatively correlated with temperature (Dunkley and Franke, 1967).

Storage Temperature. The experiments reported here are designed to illuminate features of the kinetics of metal-catalyzed oxidation and to permit comparison of metal complexes that might be suitable for fortifying milk. The data do not apply to the relationship between oxidation and storage temperature. Temperature variables are considered in greater detail in the following paper (Hegenauer et al., 1979). Prolonged storage of milk at temperatures higher than 10 °C was impracticable because of bacterial spoilage.

Homogenization. Homogenized (or emulsified) milk was far less susceptible than raw milk to oxidation by any of the iron or copper complexes investigated in these experiments (Table II; Figures 5 and 6). Our emulsification conditions gave maximum suppression of oxidizability attainable by sonication (Figure 2), but we did not compare the degree of emulsification with that achieved by commercial high-pressure homogenizers. Homogenization (or emulsification) did not alter the relative catalytic activity of any of the iron complexes. We pointed out previously that mononuclear iron chelates (Fe NTA and iron lactobionate) showed catalytic behavior that was clearly different from ferrous ion or polynuclear ferric complexes. This difference is most evident in Figure 6b, which shows that oxidation of commercial homogenized, pasteurized milk by ferric chelates did not increase during cold storage, but that both ferrous sulfate and ferric fructose caused TBA reactivity to increase gradually to the level observed in raw milk fortified with these iron complexes.

If homogenization merely reduced the size rather than the accessibility of the milk fat globules, the increased surface area of lipid might be expected to cause increased oxidation. The effectiveness of homogenization in preventing oxidation must be attributed to emulsification of the milk fat fraction with iron-binding substances (Thurston et al., 1936; Fox et al., 1960). The casein content of the milk fat fraction of homogenized milk is significantly greater than that of raw milk (Hegenauer and Ludwig, 1978), and the iron binding properties of these phosphoproteins are now well documented (Loh and Kaldor, 1974; Carmichael et al., 1975). Homogenization may produce a stable emulsion of milk fat micelles with an outer ion-exchange "membrane" containing sufficient casein phosphoprotein to prevent iron from contacting sensitive lipids. Homogenization is perhaps the single most important physical treatment that can reduce the oxidizability of milk fat by iron and other metal catalysts.

Fat Content. Emulsification protects the milk fat fraction only partially from the effect of metal-catalyzed lipid peroxidation. TBA reactivities for all iron complexes tested were somewhat higher in emulsified raw milk than in raw milk. The significant TBA reactivity of iron-supplemented skim milk with 1% fat (Table II) indicates an appreciable content of oxidizable lipid and, perhaps, carbohydrate. We devoted little attention to skim milk in these studies, because this product is not recommended for early pediatric use (Fomon and Filer, 1974) and would not be a suitable vehicle for supplementing infants.

Selection of Iron and Copper Compounds for Milk Fortification. Chelated forms of iron and copper are clearly preferable to the simple inorganic salts as supplements for milk. Measured by the TBA reaction, iron and copper compounds produce significantly less oxidation on a molar basis when they are chelated to ligands such as lactobionate and NTA (Figures 3 and 4). In our experiments, all iron and copper compounds displayed the same relative catalytic activity in raw, homogenized, raw emulsified, or raw skim milk (Tables II and III). The polynuclear iron complex, ferric fructose, was similar to inorganic ferrous salts in molar catalytic activity (data not shown) and in time course of oxidation in the cold (Figure 6). Its catalytic activity relative to ferrous ion was frequently anomalous, however (cf. Figure 6 and Tables II and III). Such effects are probably attributable to slight differences in age, polymer size, and reactivity among different preparations of ferric fructose (Bates et al., 1973). In one experiment (Table III), the predominantly polymeric "ferric citrate" complex (citrate/Fe = 0.4) catalyzed TBA reactivity in raw and homogenized milk that was intermediate between ferric fructose and several low molecular weight 1:1 ferric chelates (ferric citrate, ferric lactobionate, ferric NTA, and ferric EDTA).

The characteristically high reactivity of such polynuclear iron complexes may reflect the strong affinity of the milk fat fraction for polymeric iron. Ferrous ion is known to be readily oxidized at neutral pH in the presence of oxygen and iron-binding ligands (Harris and Aisen, 1973). Similarities in kinetics of oxidation catalyzed by ferrous salts and ferric fructose (Figure 6) may thus be a consequence of oxidation of ferrous ion with the generation of polymeric iron complexes having a high affinity for susceptible lipids. In addition, ferrous ion may participate in many cyclic oxidation–reduction reactions, so that its potential for catalyzing lipid oxidation may be amplified considerably beyond what would be expected from the same concentration of iron presented as the fully oxidized ferric chelate.

The low catalytic potential of ferric chelates is a striking characteristic of this oxidative system. Ferric NTA, for example, readily exchanges its iron with many iron-binding proteins of biological interest, such as transferrin (Bates and Wernicke, 1971) and casein phosphoproteins (Carmichael et al., 1975), so we may expect most of the iron in supplemented milk to reside in the form of strong ferric-phosphorylserine chelates, which effectively remove this metal from the environment of the lipid fraction. Storage of milk supplemented with ferric lactobionate or ferric NTA does not cause the gradual increase in lipid peroxidation seen with supplements like ferrous ion or polynuclear iron (Figure 6).

CONCLUSIONS

The experiments reported here were designed to test certain options for evaluating metal complexes and processing parameters which may be considered in developing trace element fortification of dairy products. The lipid

peroxidation data obtained by the TBA test in our accelerated test system using raw milk suggest that ferric and cupric chelates of lactobionate and NTA may be promising iron and copper complexes to include in organoleptic evaluations. These data alone are not sufficient to determine the optimum—perhaps least objectionable—complexes or concentrations for fortification.

Homogenization significantly reduced the susceptibility of milk to oxidation by iron. In our experiments, ultrasonically emulsified raw milk was at least as resistant to iron-catalyzed oxidation as commercial homogenized, pasteurized milk.

The TBA reactivity of iron-supplemented milk must be interpreted cautiously, because iron catalyzed a large, nonspecific increase in TBA pigments at the high assay temperature. As measured by the TBA test, the molar catalytic activity of copper was much lower than that of iron in producing lipid oxidation. Since copper affects flavor more adversely than iron in commercial practice, the TBA test cannot supplant flavor scoring of fortified milks. Nevertheless, the TBA test demonstrates significant differences among metal complexes in catalyzing lipid peroxidation in raw and homogenized pasteurized milk during short-term incubations at room temperature or long-term cold storage. Although they do not predict flavor, chemical tests narrow the search for innocuous food additives and provide useful information about processing and storage variables. Organoleptic and further nutritional evaluation of the lactobionate and NTA chelates of iron and copper are in progress.

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