Effects of Supplemental Iron and Copper on Lipid Oxidation in Milk. 2. Comparison of Metal Complexes in Heated and Pasteurized Milk

Jack Hegenauer,* Paul Saltman, and Diane Ludwig

The preceding paper showed that lipid oxidation in iron- and copper-supplemented dairy products can be reduced by the use of chelated forms of iron(III) and copper(II). Prolonged batch heating increased the susceptibility of raw milk to oxidation by chelated iron(III) but decreased oxidation by ferrous salts. Oxidation produced by a given metal complex was about the same whether the metal was added before or after heat treatment. Oxidation was affected very little by brief heating or by lengthening the interval between addition of iron and pasteurization by a high-temperature/short-time process. With any of the heating variables tested, the iron(III) chelates of nitrilotriacetate and lactobionate catalyzed less oxidation than ferrous salts. In order to minimize reactions leading to oxidative deterioration in fortified milk, the data indicate that the addition of chelated iron(III) and copper(II) should be made after homogenization (to emulsify and protect the milk fat fraction) but before pasteurization by a high-temperature/short-time process.

In the preceding paper on lipid peroxidation in iron- and copper-supplemented milk, we showed that lipid oxidation reactions—by inference, those leading to "oxidized" flavor—can be reduced when skim and homogenized milks are supplemented rationally with newer chelated forms of these trace elements (Hegenauer et al., 1979). Understanding how metals catalyze oxidative rancidity and cause organoleptic deterioration is of great importance in achieving the widest possible acceptance of iron- and copper-supplemented dairy products which will help reduce the high incidence of anemia among young children, adolescents, and women of menstrual age. The rationale of the test systems employed in these studies has been described in the preceding paper. We focus here on the effects of temperature on the thiobarbituric acid (TBA) reactivity of supplemented milk in an effort to predict deterioration of flavor in pasteurized dairy products. The results show that the chemical nature of the metal supplement strongly influences the degree of oxidation encountered in high-temperature batch processing but that high-temperature/short-time treatment can minimize the deleterious effects of metals, particularly if chelated iron and copper complexes are employed.

MATERIALS AND METHODS

Sources of milk, preparation of iron complexes, and measurement of lipid peroxidation by the TBA assay are described in the previous paper (Hegenauer et al., 1979). Experiments using 30-min heat treatments ("batch heating") were performed in an air or N_2 atmosphere, as indicated, in a thermostated circulating water bath. Time required for most samples (~5 mL) to reach temperature equilibrium was <30 s; 50-mL samples required about 60 s of warm-up.

A low-volume, continuous-flow system was constructed specifically to determine whether the effects of pasteurization on milk flavor depended on the length of time iron and milk were in contact before high-temperature/short-time treatment. Milk and iron solutions were pumped separately through polyvinyl chloride tubing and mixed at a "Y" connection; combined flow rate was 5.0 mL/min. When tubing of 0.094 in. i.d. and 0.025 in. i.d. was stretched around the rollers of a Buchler peristaltic pump, we determined the mixing ratio of milk/iron (as measured by radioisotopic dilution) to be 10.24:1. The combined milk/iron stream was then fed sequentially through coils of thin-walled Teflon tubing (0.049 in. i.d. = 18 guage) of sufficient length to achieve the desired temperature for an appropriate dwell time in (1) a delay coil (0–180 s) maintained at room temperature, (2) a pasteurizer coil placed in a 72 ± 0.1 °C circulating water bath, and (3) a cooling coil (15 s) placed in an ice bath. A thermistor probe placed in the pasteurizer coil 0.5 s downstream assured that temperature equilibration was achieved very rapidly. The length of the pasteurizer coil was adjusted to give a full 15 s of delay time at the desired temperature. The first and last 10% of the effluent from the cooling coil was discarded.

Table I. Effect of Batch Heating on Susceptibility of Raw Milk to Oxidation by Iron and Copper as Measured by TBA Reactivity

supplement	metal concn, mM	A_{532nm} 1 cm[a,e] raw milk	A_{532nm} 1 cm[a,e] heated milk[b]
Fe NTA[c]	2	0.914 ± 0.030	0.976 ± 0.037[d]
Cu NTA	2	0.168 ± 0.014	0.175 ± 0.009[d]

[a] TBA reactivity of milk incubated with iron or copper and pH 6.8 Hepes buffer (20 mM) for 3 h at 25 °C. [b] Raw milk (50 mL) not homogenized before heating under nitrogen at 60 °C for 30 min. [c] NTA, nitrilotriacetate. [d] Significantly greater ($P < 0.05$) than oxidation of raw milk by same metal complex. [e] Results expressed as mean (of triplicate samples from each of three incubation mixtures) ± standard deviation. Number of data values = 9.

EXPERIMENTS AND RESULTS

Effect of Heat on Susceptibility of Milk to Oxidation by Iron and Copper. Batch heat treatment slightly increased the oxidizability of raw milk by chelated forms of ferric and cupric ion. Raw milk that was heated (without homogenization) for 30 min at 60 °C under nitrogen showed a slight, but statistically significant, increase in TBA reactivity after supplementation with 2 mM Fe or 2 mM Cu (as the NTA chelates) compared to unheated milk (Table I).

Whether heating increased or decreased the oxidizability of milk by iron, however, depended on the chemical nature of the supplement. After raw milk was incubated aerobically for 30 min at different temperatures, TBA re-

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

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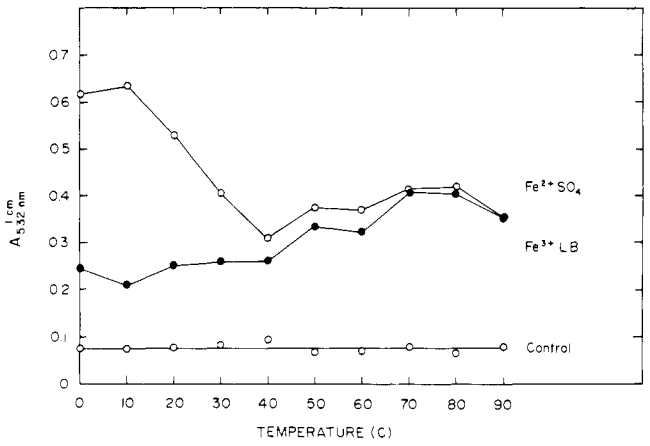

Figure 1. Temperature dependence of oxidation in raw milk supplemented with ferrous sulfate or ferric lactobionate (Fe^{3+} LB). Temperature was held aerobically for 30 min after addition of iron. Final reaction conditions: 0.5 mM Fe; 20 mM Hepes (pH 6.8); 5 mL. "Control" refers to unsupplemented raw milk. Datum points are averages of three incubation mixtures. TBA reactivity of the oxidized product is indicated by A_{532nm}.

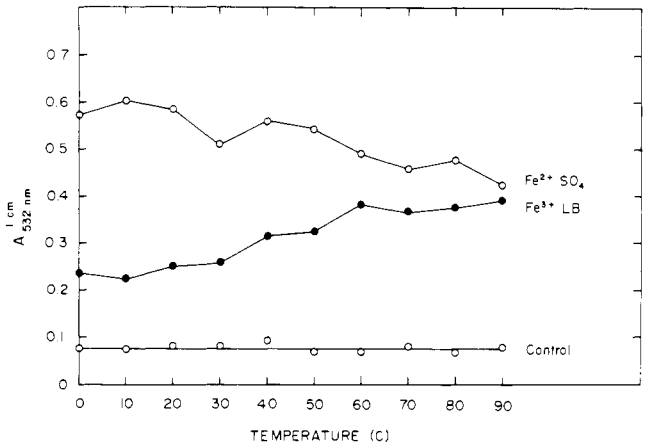

Figure 2. Effect of temperature on oxidizability of raw milk by iron added after heat treatment. Raw milk (5 mL) containing 20 mM Hepes (pH 6.8) was heated aerobically for 30 min at the indicated temperature. Supplemental iron (0.5 mM) as ferrous sulfate or ferric lactobionate (Fe^{3+} LB) was then added, and the iron-supplemented milk was incubated for another 30 min at 2 °C. The "control" curve is reproduced from Figure 1, which should be consulted for further details.

activity catalyzed by added ferric lactobionate increased steadily with increasing temperature, whereas heating caused a decline in initial TBA reactivity with the ferrous sulfate supplement (Figure 1). Although ferrous sulfate produced about twice as much TBA reactivity as ferric lactobionate when incubations were conducted at or below room temperature (cf. Hegenauer et al., 1979), TBA reactivities observed with these two iron supplements tended to converge at the highest temperatures employed (90 °C).

Effect of Heat on Oxidation in Iron- and Copper-Supplemented Milk. *Iron.* The relationship between TBA reactivity and temperature was very similar whether iron was added before or after the heating period. TBA reactivity catalyzed by chelated (ferric lactobionate) or ionic (ferrous sulfate) iron was about the same when milk was heated aerobically for 30 min at temperatures above 40 °C (Figure 2). As we observed previously (Hegenauer et al., 1979), ferrous salt added at the pediatric level (0.5 mM) was more catalytically active than chelated ferric iron at incubation temperatures below 40 °C (Figure 2). Comparison of Figures 1 and 2 shows that the activity of

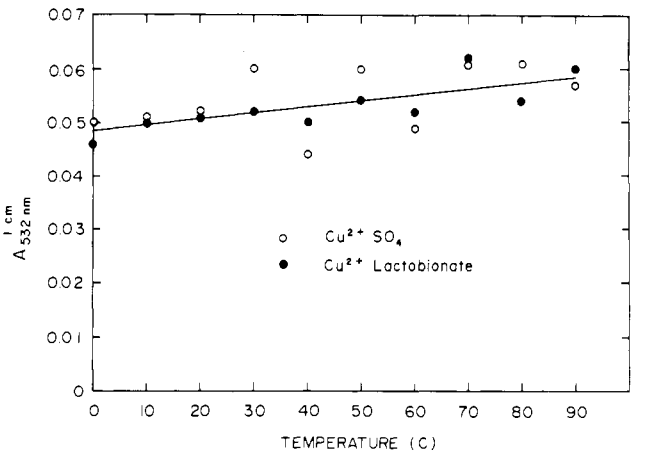

Figure 3. Effect of temperature on oxidation in raw milk supplemented with cupric sulfate or cupric lactobionate (Cu^{2+} LB). Temperature was held for 30 min after addition of copper. Final reaction conditions: 0.05 mM Cu; 20 mM Hepes (pH 6.8); 5 mL. Refer to legend of Figure 1 for further details. Datum points for unsupplemented "control" overlapped the experimental points and have been omitted for clarity.

ferrous sulfate in lipid peroxidation depended greatly on whether iron was present during heating. The ferric lactobionate chelate, on the other hand, showed no such difference. Heating did not cause increased oxidation in unsupplemented milk (Figure 1).

Copper. Aerobic heating very slightly increased TBA reactivity in copper-supplemented milk. We observed no difference between cupric sulfate and cupric lactobionate added at the pediatric level (0.05 mM) (Figure 3). Because of batch-to-batch variation, TBA reactivity in the unsupplemented control for this experiment was less than that observed in the experiments involving iron supplementation (Figure 1). "Control" oxidations considerably overlapped the "copper" oxidations, however. In addition, the slope of the linear regression of temperature on TBA reactivity in copper-supplemented milk was not significantly different from the slope obtained with unsupplemented milk, so a specific increase due to copper is problematic.

TBA Reactivity of Heated Milk: Time Course of Oxidizability by Iron. Although raising the temperature of aerobic batch heating significantly increased oxidation of milk after addition of iron (Figure 2), oxidizability was affected relatively little during the course of heating at a constant temperature when ferric lactobionate was used as the supplement (Figure 4). Thus, the lability of certain lipids to iron-catalyzed peroxidation may be increased by high temperatures, but length of heating does not appear to be a significant variable in promoting oxidative rancidity.

TBA Reactivity of Iron-Supplemented Pasteurized Milk: Effect of Varying Supplementation-to-Pasteurization Interval. As discussed above, heating milk at "pasteurization" temperatures appeared to increase its susceptibility to lipid oxidation catalyzed by ferric chelates (Table I; Figures 1 and 2). High-temperature/short-time treatment following homogenization is now the most frequent pasteurization procedure in dairy processing. It is thus conceivable that, in the production of milk fortified with trace elements, iron/copper supplements would be added continuously to the processing stream only a short time before pasteurization. We wished to determine whether the binding of iron to casein phosphoproteins (Carmichael et al., 1975) was a time-dependent process that could modulate the catalytic activity of iron for lipid

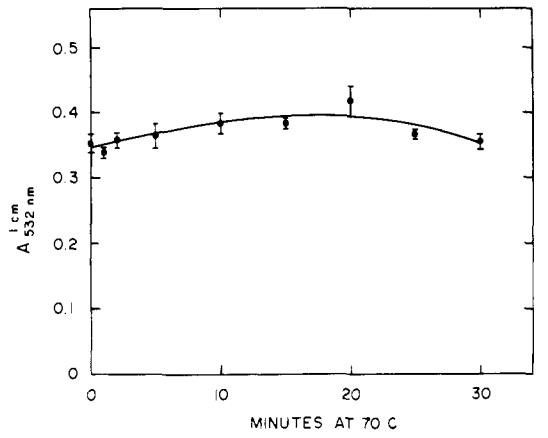

Figure 4. Time course of oxidizability in heated raw milk by added iron. Raw milk (5 mL) containing 20 mM Hepes (pH 6.8) was incubated aerobically at 70 °C for the indicated time. Supplemental ferric lactobionate (0.5 mM) was then added, and the iron-supplemented milk was incubated for another 30 min at 2 °C. Datum points and error bars are the average and standard deviation of ten incubation mixtures. TBA reactivity of the oxidized product is indicated by A_{532nm}.

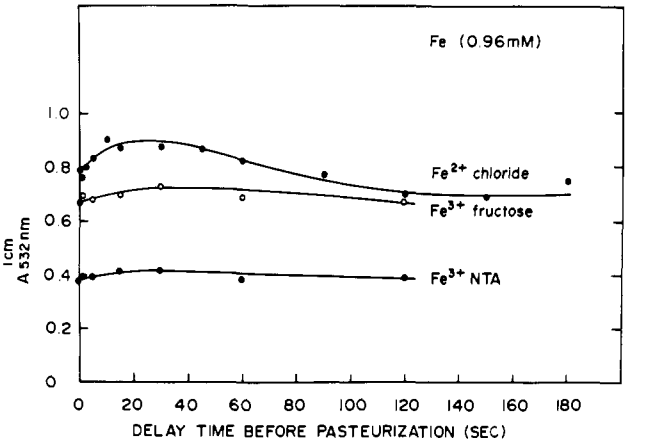

Figure 5. Effect of delay time between addition of iron and pasteurization on milk oxidation. Raw milk and iron solutions were mixed in a continuous stream as described in the text, held in a delay coil at room temperature for up to 3 min, and pasteurized at 72 °C for 15 s. Datum points are averages of five samples from only one pasteurized mixture. TBA reactivity of the oxidized product is indicated by A_{532nm}.

oxidation at pasteurization temperatures. We attempted to simulate this situation in the laboratory by using a small-scale pasteurizer that combined milk and iron solutions in a continuous stream and allowed them to "react" in a delay, or holding, coil for varying times before the stream was subjected to pasteurization temperatures. Except that raw milk is not pasteurized in commercial practice without homogenization, we believe that our heating conditions in this experiment met the legal requirements for pasteurization; we detected no alkaline phosphatase activity in this pasteurized milk using *p*-nitrophenyl phosphate as substrate.

As shown in Figure 5, the time raw milk and iron were allowed to react prior to pasteurization had relatively little effect on lipid oxidation. At a concentration of about 1 mM, ferric NTA ranked in the expected order (Hegenauer et al., 1979) relative to ferrous chloride in catalyzing TBA reactivity in this system. There was, for three iron compounds, a very slight apparent maximum in TBA reactivity when reaction (or delay) time was extended to about 30 s before pasteurization (Figure 5). The precision of the experiment was not sufficient to determine whether the increase in TBA reactivity observed from 0–30 s was statistically significant. Variations in iron concentration in the milk subjected to pasteurization, as determined by radioisotopic dilution, were small. Mean iron concentration in all pasteurized samples of iron-supplemented milk was 0.960 ± 0.011 mM (N = 39); in addition, there was no statistical correlation between iron concentration and TBA reactivity for any of the pasteurized samples.

DISCUSSION

Variables Affecting Oxidation in Heat-Treated Milk. *Temperature.* Increasing the temperature of batch heating may promote or inhibit metal-catalyzed oxidation reactions, depending on the chemical nature of the supplement. As the temperature was raised, TBA reactivity increased when chelated iron(III) was used but decreased when iron(II) was added (Figures 1 and 2). Differences between the two iron compounds disappeared at very high temperatures. Surprisingly, the relationship between temperature and TBA reactivity was about the same whether iron was added before or after the heating period. Heat must therefore primarily affect the lability of oxidizable lipid and may not necessarily increase the activity of trace metals in catalyzing lipid peroxidation. Oxidation by ferric chelates illustrates this effect most clearly, because oxidation increases steadily with increasing temperature regardless of whether the iron is added before or after heating (Figures 1 and 2). Heat could also affect the accessibility of oxidizable lipid to metals, but it is difficult to account for both increased and decreased oxidizability on this basis. Paradoxically, the catalytic activity of ferrous ion declined more rapidly with increased temperature if iron was added before, rather than after, heating. Heating may diminish the catalytic activity of ferrous salts by oxidizing them to the more stable iron(III) compounds which may be sequestered by casein phosphoproteins (Hegenauer et al., 1979). Oxidation of iron could come about through the activity of free-radical oxidants in milk or by removal of reducing substances like ascorbate, thiols, etc. At very high temperatures, oxidation by ferrous and ferric compounds may tend to be about equal (Figures 1 and 2) because both chemical forms have converged on a common physical-chemical state: the iron(III)–phosphoprotein complex.

Time. Batch heating (60 °C, 30 min) increased slightly the susceptibility of raw milk to oxidation by ferric NTA and cupric NTA added after heating (Table I). Although this increase in TBA reactivity was statistically significant, it should be noted that the iron and copper concentrations employed in this experiment (2 mM) were considerably higher than the levels advised for pediatric use. This increase is probably of little practical significance. A larger proportional increase in oxidizability was observed with ferric lactobionate (0.5 mM) added after heating. Using the data in Figure 2, it can be seen that TBA reactivity was higher after batch heating (60 °C, 30 min) than after a "control" incubation at room temperature (20–30 °C, 30 min). A smaller incremental increase was observed in another experiment using ferric lactobionate (Figure 4); in this case, heat treatment for several minutes at 70 °C caused a slight increase in TBA reactivity when iron was added after heating, but there was practically no change within 1–2 min of heating. These results suggest that the addition of ferric chelates to milk—before or after heating—will cause greater lipid peroxidation after batch heating (60 °C, 30 min) than after high-temperature/short-time (72 °C, 15 s) pasteurization. As judged by TBA reactivity, brief heat treatment by itself appears to have

no deleterious effect on oxidative rancidity beyond that caused by the addition of iron.

Supplementation-to-Pasteurization Interval. We observed a slight increase in TBA reactivity associated with lengthening the interval between addition of iron and pasteurization. The increment in TBA reactivity between the minimum (no delay) and maximum oxidation (about a 30-s delay) was greater for ferrous salts and polynuclear iron than for chelated ferric iron (Figure 5). As expected, chelated ferric iron produced less TBA reactivity than other complexes at all delay times tested. The reason for this increased TBA reactivity as pasteurization is delayed after supplementation is not clear from these studies. For iron compounds like ferrous chloride and ferric fructose, the increase in TBA reactivity observed at a 30-s delay may be related to a rapid reaction with a susceptible lipid; the decrease in TBA reactivity as pasteurization is delayed further (up to 120 s) may be associated with complexation of iron by casein phosphorylserine residues, which may remove iron from the environment of the lipid phase. In the pasteurization of iron-fortified milk, there may thus be some advantage to heat treatment immediately after addition of iron, in order to prevent additional oxidation. The relatively small difference between maximum and minimum oxidation observed in this experiment suggests, however, that this interval will not be an important variable for future processing of fortified milk, especially if ferric chelates are used. Similarly, because addition of iron before or after heating seemed to make little difference in oxidizability in our experiments, we would predict little effect of heat pretreatment, or warm-up, before pasteurization.

Pasteurization and Development of "Oxidized" Flavor in Iron-Fortified Milk. In their evaluations of iron-fortified milks, many investigators (Edmondson et al., 1971; Wang and King, 1973; Demott, 1975) have suggested that pasteurization after addition of ferrous or ferric compounds promoted the development of "oxidized" flavor. Wang and King (1973) have commented on the role of homogenization in modulating the development of lipid oxidation and "oxidized" flavor. In their study and those of Edmondson et al. (1971) and Demott (1975), however, fortified milk was homogenized after pasteurization, so presumably much of the protective action of milk fat emulsification (discussed in the preceding paper) was not realized. It is thus not possible from their studies quantitatively to determine the deleterious effect of heat treatment from the protective effect of homogenization. Edmondson et al. (1971) have concluded that oxidation in iron-fortified milks may be reduced by short heat pretreatment of raw milk and by increased temperature of pasteurization of the iron-supplemented product.

Some of these findings may be reinterpreted in the light of our studies. In general, heat treatment of raw milk after addition of iron will tend to increase the susceptibility of labile lipids to peroxidation and thus to promote "oxidized" flavor. The effectiveness of heat pretreatment in reducing oxidation in ferrous sulfate-supplemented milk does not necessarily involve inactivation of a lipase, as proposed by Edmondson et al. (1971). Rather, as we have discussed, heating may accelerate the oxidation of iron(II) to iron(III), which will have reduced catalytic activity due to its sequestration by phosphoproteins.

CONCLUSIONS

Processing Strategies for Iron- and Copper-Fortified Milks. The data presented in this and the preceding paper suggest that, in order to supplement milk with the least amount of lipid oxidation and oxidative deterioration, iron and copper should be added to milk after homogenization (to emulsify and protect the milk fat fraction) and pasteurized without delay (see Figure 5).

We discourage the use of ferrous sulfate and other iron(II) salts as milk supplements; numerous experimental studies and commercial experience have shown that their unfavorable organoleptic properties lead to unacceptable technological costs. Chelated ferric and cupric complexes provide a means of modulating deleterious oxidative reactions by controlling the chemical reactivity of the metal supplements during prolonged storage (Hegenauer et al., 1979). The iron(III) and copper(II) chelates of nitrilotriacetate and lactobionate show excellent bioavailability in animal feeding studies (Hegenauer and Saltman, 1978). Collaborative studies are now in progress to provide organoleptic evaluations of these metal complexes.

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