THE EFFECTS AND INTERRELATIONSHIP OF COPPER, IRON AND PASTEURIZING TEMPERATURE ON THE STABILITY OF ASCORBIC ACID ADDED TO SKIMMILK

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The increasing use of synthetic ascorbic acid in milk and milk products places more emphasis on knowledge of the factors affecting its stability, and a wider use of nutritionally significant quantities of iron and copper in milk products has intensified the problem of metal catalysis of oxidation.

The catalysis of ascorbic acid oxidation by copper is well known. Barron and DeMeio (2) and Marston (15) concluded that copper but not iron catalyzed the oxidation of ascorbic acid, but that iron increased the catalytic power of copper and that in all except very highly purified solutions copper was present in sufficient quantity to make the iron appear to be independently catalytic. Because of the natural occurrence of iron and copper in milk, the effects of the two metals cannot be studied separately at the zero level of either and for practical purposes they both may be considered oxidation catalysts.

In studies on the stability of ascorbic acid in milks during processing, storage and use, numerous investigators have noted the destructive effects of added copper (8, 9, 10, 11, 14, 20, 23, 24), the superiority of high temperature pasteurization over holder pasteurization with regard to the losses of ascorbic acid incurred in pasteurization and in subsequent storage and use (11, 14, 16, 23) and the effects of added iron (23). However, there do not appear to be any comprehensive studies on the effect of the concentration of one of the metals upon the activity of the other and on the effects of temperature of pasteurization as related to copper and iron content.

It was the object of this work to study some of the effects and interrelationships of copper, iron and temperature of pasteurization, upon the stability of ascorbic acid added to skimmilk after pasteurization.

EXPERIMENTAL

Skimmilk was chosen in preference to whole milk because the ascorbic acid oxidation in milk occurs in the aqueous phase and because skimmilk was obtainable from day to day as a more nearly uniform material. The experimental work was designed to determine the comparative effects of the various combinations of the three variables on the stability of ascorbic acid added to the milk after pasteurization during a fixed 16-hr. incubation period under standard experimental conditions. Incubation conditions were chosen to allow sufficient excess of oxygen. The ascorbic acid oxidation in skimmilk under our experi-
mental conditions has been shown in previous work in this laboratory (4) to approximate a first order reaction as in water solutions (13, 22). The levels of copper used were 0, 0.1, 0.2 and 0.4 ppm.; of iron, 0, 2, 5 and 10 ppm. Temperatures of pasteurization were 50, 65, 75, 85 and 95° C. The control unpasteurized samples were held at 35° C. for parallel times. A 30-min. pasteurizing time was used throughout, except when effect of length of heating time was studied.

Materials. Except for the samples milked into glass and contacting only glass throughout the experiments, the milk used was a composite sample of raw skim milk 6 to 18 hr. old, of grade A fluid milk quality obtained daily from a cream separator connected to a 2,000-gal. receiving tank. It had been handled principally in tinned and steel equipment. Milk free from metal contamination was milked into glass, cooled in the receiving flask, centrifuged in glass and the experimental procedure started within 5 hr. of milking time. A composite sample of milks handled entirely in glass contained 0.1 mg. of copper per liter and 0.48 mg. of iron per liter. A composite sample of "receiving plant" milks contained 0.2 mg. of copper per liter and 1.2 mg. of iron per liter.

CP cupric sulfate and U.S.P. ascorbic acid were used. The CP ferrous sulfate used contained 0.01 per cent copper which, at the maximum iron addition of 10 ppm., contributed 0.005 ppm. of copper to the milk.

In the work on milk having no metal contamination, all equipment was Pyrex, washed with nitric acid and finally rinsed with water redistilled from Pyrex. This double-distilled water was used for solutions and for all additions to the milk samples.

Procedure. One metal was added as a concentrated solution to a bulk volume of the milk. For each sample, 490 ml. of this milk then were placed in a 1000 ml. Erlenmeyer flask and, if both metals were being investigated, 5 ml. of a freshly made solution of the other metal were added or 5 ml. of water were used when only one metal was to be added. The sample was heated immediately in a water bath at the required temperature (± 1° C.) with intermittent shaking. After heating, the sample was cooled rapidly to 35° C. in cold water, water was added to replace that lost by evaporation and the flask tightly stoppered and held in a 35° C. incubator in the dark for about 1 hr. to reach equilibrium conditions. Ascorbic acid at a level of 100 mg. per liter, was added as 5 ml. of a freshly made solution.

Fifty ml. were immediately withdrawn for the zero-hour ascorbic acid determination. Three hundred ml. were discarded, 0.5 ml. of toluene added as a preservative to the remaining 150 ml. and the flask tightly stoppered. The sample was incubated in the dark at 35° C. for 16 hr. and the loss of reduced ascorbic acid determined.

Methods of assay. Reduced ascorbic acid was determined by the colorimetric indophenol dye-xylene extraction method of Nelson and Somers (18) with 3 per cent HPO₄ as an extractant. Since the data are intended primarily to be comparative, assay was made for reduced ascorbic acid only without correction for interfering substances.
Iron was determined by the alpha-alpha' dipyridyl method of the A.O.A.C. (1). Copper was determined by the method of Bendix and Grabensetter (3).

RESULTS

Effects of time of pasteurization. Figure 1 shows the effects of heating time at several pasteurizing temperatures, on the stability of reduced ascorbic acid added after pasteurization to milk containing 0.1 ppm., added copper.

![Graph showing effects of pasteurization](image)

**Fig. 1.** Effects of time of pasteurization at several temperatures on the stability of reduced ascorbic acid added to skimmilk after pasteurization. "Receiving plant" milk containing 0.1 ppm. added copper. Reduced ascorbic acid loss in parallel sample not pasteurized was 35.6%.

At 65°C, maximum loss occurred in samples heated for 30 min. and no significant decrease in loss was effected by heating up to 75 min. At 75 and 85°C, a minimum loss was noted for the samples heated for 30 min., although the increase in loss in samples heated for a longer time was not great. At 95°C.
the ascorbic acid was practically as well retained in samples heated 5 min. as in those heated for a longer time. Except in samples heated at 85 and 95° C., the differences in the effects of temperature of heating were considerably greater than the effect of time of heating at a given temperature.

A 30-min. heating period which represents a compromise between adequate pasteurization at low temperatures and avoidance of excessive heating of the milk at high temperatures was chosen for the remainder of the experiments.

Effects of temperature of pasteurization in milks of low copper and iron content. Figure 2 shows the differences in ascorbic acid stability in milk handled in glass and in “receiving plant” milk at various pasteurizing temperatures. The large differences in stability of ascorbic acid added after pasteurization at various temperatures with a minimum stability in samples pasteurized at 65° C. indicate that the heat effects are complex. The destruction of ascorbic acid which occurs in milk handled entirely in glass presumably is due to the copper, iron and other pro-oxidants present in milk as secreted.

The increase in ascorbic acid loss in the “receiving plant” milk, as compared to the milk handled in glass was about constant except for samples pasteurized at 65° C. and probably is due to copper and iron contamination.

Effects of temperature of pasteurization and added copper. The effects of added copper at the various temperatures are shown in figure 3. The ascorbic acid losses due to the added copper in the “receiving plant” milk are assumed to be the differences between losses at each copper level and the losses in parallel samples of “receiving plant” milk containing no added copper.

The ascorbic acid losses caused by the added copper varied considerably
with pasteurizing temperature. Significant is the sharp increase in loss in the 65° C. samples. For milk containing up through 0.2 ppm. added copper, pasteurization at from 75 to 95° C. eliminated substantially the ascorbic acid losses caused by the copper. In the unheated samples (35° C.) and at the lower temperatures, the increases in ascorbic acid losses were roughly proportional to the added copper, but the proportionality did not hold at 85 and 95° C.

Effects of temperature of pasteurization and added iron. Figure 4 shows the effects of added iron. As with added copper, the greatest ascorbic acid losses occurred in the samples heated at 65° C., although the losses in the 50° C. samples were relatively greater in comparison to the 65° C. samples than with copper. As for copper, the loss of ascorbic acid due to the added iron varied with pasteurizing temperature but over a much smaller range, and ascorbic acid losses in the milk pasteurized at the higher temperatures were relatively greater. The 16-hr. ascorbic acid losses in the milk handled in glass and with iron added were much lower than for "receiving plant" milk pasteurized at the same temperature (85° C.), and the losses due to the added iron also were lower.

Effects of temperature of pasteurization and added copper and iron in combination. Figure 5 shows the effects of addition of both copper and iron in a

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**Fig. 3.** Effects of pasteurizing temperature on the stability of reduced ascorbic acid added to "receiving plant" skimmilk after pasteurization, at several levels of added copper: A, reduced ascorbic acid losses at the various pasteurizing temperatures; B, losses assumed to be due to the added copper. Representative trial of 3 trials. 100% loss points indicate oxidation of all reduced ascorbic acid at an unknown time prior to 16 hr.
series of samples in which the added copper was 0.2 ppm. and the added iron was varied. Figure 6 shows parallel data at an added copper level of 0.4 ppm. Incomplete data was obtained, as the 16-hr. incubation proved more than enough time for complete oxidation with the higher concentrations of the metals. The losses due to the added iron are assumed to be the differences between the losses at the given iron level and those for samples at the same added copper level but with no added iron. As the copper content was increased, the effect of added iron decreased at 50 and 65 ° C.; temperatures at which ascorbic acid losses caused by copper were high. Thus, in the 50 ° C. samples, the ascorbic acid losses caused by 5 ppm. of added iron were reduced from 35 to 18 to 0 per cent as the added copper content was increased from 0 ppm. (figure 5) to 0.2 ppm. (figure 6). However, in the high temperature samples in which the ascorbic acid losses caused by copper were low, the effect of the added iron remained more nearly the same at all three levels of added copper. In the 95 ° C. samples losses caused by 5 ppm. added iron in the presence of 0, 0.2 and 0.4 ppm. added copper were 14.5, 17 and 17 per cent, respectively.

**DISCUSSION**

Pro-oxidant activity of copper varies widely with the state of combination of the copper, and complexes of the metal have been prepared that show no pro-
oxidant activity. It has been shown that the rate of oxidation of ascorbic acid by oxygen can be used to estimate the concentration of copper having pro-oxidant activity (4, 6, 10, 19, 22).

Therefore, it may be assumed that the rate of ascorbic acid oxidation in skimmilk under fixed experimental conditions, including a constant composition of the milk, particularly a constant iron content, is an approximate measure of the pro-oxidant activity of the copper present and that the effects of heating milk on the rate of ascorbic acid oxidation are due to formation of complexes of varying pro-oxidant activities. According to these assumptions, the pro-oxidant activity of copper in milk shows marked variation with pasteurizing temperature.

![Graph](image)

**Fig. 5.** Effects of pasteurizing temperature on the stability of reduced ascorbic acid added after pasteurization to 'receiving plant' skimmilk containing 0.2 ppm. added copper, at several levels of added iron: A, reduced ascorbic acid losses at the various pasteurizing temperatures (at 65° C., 100% destruction of the reduced ascorbic acid occurred in all samples); B, losses assumed to be due to the added iron—calculated by subtracting loss for equivalent point in 'receiving plant' milk containing 0.2 ppm. added copper and no added iron. 1 trial.

Native, undenatured proteins have metal complexing properties (21) and both the naturally occurring and added copper of unheated milk is present in complex form (4, 17). Greater ascorbic acid losses were found after heating at 65° C. than at 55° C. or for unheated samples (35° C.) so it may be that heating at this intermediate temperature reduces the concentration of naturally occurring copper complexing substances of milk to a minimum; 65° C. is below the point of significant protein denaturation or formation of sulfhydryl groups (5). Heating at temperatures above 65° C. results in a decreased rate of as-
corbic acid oxidation presumably due to copper inactivation. The principal mechanisms of inactivation of copper at higher temperatures probably are the formation of complexes with protein (4, 7) and with sulfhydryl groups produced by heat (4, 5, 11) which are less active than the copper complexes present in unheated milk. Heating 30 min. at 95 °C. appears to be sufficient to practically inactivate up through 0.2 ppm. of added copper.

Though the behavior of iron is most correctly expressed in terms of its effect on the catalytic power of copper, as a practical matter, in milk of low copper content, it can be expressed as pro-oxidant activity in a manner allowing comp-

**FIG. 6.** Effects of pasteurizing temperature on the stability of reduced ascorbic acid added after pasteurization to "receiving plant" skim milk containing 0.4 ppm. added copper, at several levels of added iron: A, reduced ascorbic acid losses at the various pasteurizing temperatures (at 65 °C., 100% destruction of the reduced ascorbic acid occurred in all samples); B, losses assumed to be due to the added iron—calculated by subtracting loss for equivalent point in "receiving plant" milk containing 0.4 ppm. added copper and no added iron. 1 trial.

parison with the effects of copper. Figure 7 compares the pro-oxidant effects of copper and iron in the "receiving plant" milk at intermediate added levels of each metal (specifically this data represents the effects of added copper in the presence of about 1.2 mg. per liter of iron and the effects of added iron on the pro-oxidant activity of about 0.2 mg. per liter of copper). The pro-oxidant activity of iron was much less than that of copper. In milk stored at 35 °C. the activity of copper was 24 times as great as iron in samples not heated, 55 times as great in samples heated 30 min. at 65 °C. and only 4 times as great in the 95 °C. samples. The activity of iron varied with temperature of heating but showed only a two-fold change throughout the range of pasteurizing tempera-
tures as against a twenty-fold variation for copper. Iron showed a maximum activity after heating at 50 °C instead of at 65 °C and heating up to 95 °C did not appear to cause inactivation. Successive increments of added iron caused successively less increase in ascorbic acid loss, except in the milk heated at the higher temperatures in which the pro-oxidant copper content was very low (figure 8).

Joslyn and Miller (13) have demonstrated a reduction of the pro-oxidant activity of copper by iron in neutral water solutions of ascorbic acid. In water solutions of pH 7.0 buffered with H₃PO₄, containing 50 mg of ascorbic acid per liter, 0.07 ppm copper and 0.07 ppm iron, the rate constant for oxidation of ascorbic acid at -1.1 °C was reduced from 5.19 to 1.46 hr⁻¹ by increasing the iron content to 11.31 ppm. With milk the data suggest, besides the iron reactions which act to increase the catalytic power of copper, reactions acting to prevent the destruction of ascorbic acid. Within the ranges of copper and iron investigated here, the 16-hr. incubation ascorbic acid loss in milk containing a given amount of pro-oxidant copper was always made greater by the addition of iron. The opposing reaction preventing oxidation of ascorbic acid is illustrated in figure 8 in which the 16-hr. ascorbic acid loss caused by the addition of 0.2 ppm copper at zero added iron level is compared with the losses caused by this copper in the presence of 2, 5 and 10 ppm added iron. Here, after heating at 50 and 65 °C where pro-oxidant copper level presumably is high,
the loss caused by the added copper is steadily reduced as the iron content is increased.

As a result of the occurrence of these two opposing reactions the stability of the ascorbic acid could be the same over a range of copper and iron concentrations. This would explain the observation (figure 8) that the increase in ascorbic acid loss caused by addition of 0.2 ppm. copper would (as indicated by the intersection of the curves representing the losses caused by this copper) remain the same in milk containing 0, 2, or 5 ppm. added iron and pasteurized at about 81°C.

![Figure 8: Effect of added iron on the pro-oxidant activity of added copper as measured by the rates of loss of reduced ascorbic acid added after pasteurization to “receiving plant” skimmilk pasteurized at several temperatures. A 16-hr. reduced ascorbic acid loss caused by 0.2 ppm. added copper in the presence of (1) zero added iron; (2) 2 ppm. added iron; (3) 5 ppm. added iron; and (4) 10 ppm. added iron. Calculated by subtracting the ascorbic acid loss occurring at each level of added iron in milk containing no added copper from that occurring with 0.2 ppm. added copper. Data is incomplete at 65°C.]

**SUMMARY**

The effects of copper, iron and temperature of pasteurization on the stability of ascorbic acid added to skimmilk after pasteurization, and the interrelationship of these variables have been investigated.

1. Pasteurization at temperatures up to 65°C decreases the stability of ascorbic acid added after pasteurization. Minimum stability occurs at about 65°C. and the ascorbic acid is markedly more stable in milk pasteurized at 75°C. or higher than at 65°C. and in unheated milk.

2. Ascorbic acid added after pasteurization is significantly more stable in
skimmilk handled entirely in glass than it is in grade A fluid quality skimmilk having known copper and iron contamination. The stability of ascorbic acid varies with pasteurizing temperature in the uncontaminated milk in a manner similar to the variation in skimmilk containing added copper and iron, indicating that part of the ascorbic acid losses in uncontaminated milk are caused by the naturally occurring copper and iron.

3. The effect of added copper in the oxidation of ascorbic acid in milk varies widely with pasteurizing temperature. Heating to 65° C. greatly increases the pro-oxidant activity of copper. It then decreases sharply with increases in temperature, and heating to 85 or 95° C. inactivates up through about 0.3 ppm total copper content.

4. The effect of added iron on the oxidation of ascorbic acid in milk varies with pasteurizing temperature, but over a comparatively smaller range than for copper. The pro-oxidant effect of iron appears to be at a maximum after heating at 50° C. and heating at 95° C. does not decrease the activity greatly.

5. Within the range of added metals investigated, all combinations of added iron and copper caused more rapid ascorbic acid loss than the same level of either metal alone. Under conditions in which the level of pro-oxidant copper presumably was high, however, the ascorbic acid loss caused by each part per million of iron became smaller as the iron content was increased. After heating at 50 and 65° C., the addition of a given amount of copper to milk caused successively smaller increases in ascorbic acid loss rate as the iron content of the milk was increased.

6. The behavior of copper and iron in combination suggests two opposing reactions involving copper, iron and ascorbic acid. The first tends to cause more rapid oxidation of the ascorbic acid, while the second acts to prevent oxidation. The net pro-oxidant effect of the combination of the metals is the resultant of these reactions.

REFERENCES


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