Antioxidative Activity of Urate in Bovine Milk

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The antioxidative effects of urate on peroxidase-induced protein oxidation and light-induced riboflavin degradation and lipid oxidation in whole milk were studied. In addition, experiments using ascorbate were conducted to directly compare the antioxidative activity of urate and ascorbate. The presence of urate and/or ascorbate (10−30 mg/L) lowered peroxidase-induced formation of dityrosine by 44−96% in unpasteurized whole milk. No synergistic effect of urate and ascorbate on peroxidase-induced dityrosine formation was registered, but merely an additive effect. Light exposure of pasteurized whole milk showed that ascorbate was oxidized at the expense of urate, which indicated ascorbate-mediated recycling of the urate radical. Moreover, both urate and ascorbate (30 mg/L) retarded light-induced lipid oxidation in pasteurized whole milk as measured by formation of lipid hydroperoxides with urate being the most effective (28% reduction in lipid hydroperoxides) compared with ascorbate (14%). Finally, addition of urate or ascorbate (300 mg/L) to pasteurized whole milk showed a slight protective effect against light-induced degradation of riboflavin with urate being the most effective.

Keywords: *Uric acid (urate); ascorbic acid (ascorbate); milk; dityrosine; lactoperoxidase; fluorescent light; riboflavin; lipid hydroperoxides*

INTRODUCTION

Milk and dairy products are sensitive to oxidative changes from the time the milk leaves the cow and until the product reaches the consumer. A wide variety of factors can promote oxidative reactions in milk. For example, increased content of soy cakes or toasted soy beans in the feed of the animals will result in milk with a higher content of unsaturated lipid (Mohamed et al., 1988) which decreases the oxidative stability of the milk (Edmondson et al., 1972; Sidhu et al., 1975). Before pasteurization, the milk contains a wide variety of enzymes which may induce oxidative changes of the milk components (Allen and Wrieden, 1982; Hill et al., 1977). Pasteurization eliminates the activity of most of these enzymes, but processing at the dairy plant, content of free metal ions (Ford et al., 1986; Hill et al., 1977), and exposure to fluorescent light during retail display (Bradley, 1980; Cladman et al., 1998) can also promote oxidative changes. The ultimate goal in achieving oxidatively stable milk and dairy products is to eliminate or minimize quality-compromising oxidative reactions in milk from the udder to the final product. In addition to minimizing potential deleterious reactions promoted by the above-mentioned parameters, a high content of endogenous antioxidants in milk will increase the oxidative stability of milk and dairy products. Milk contains a variety of low-molecular antioxidants of which α-tocopherol, carotenoids, and ascorbate are the main ones recognized (Fox and McSweeney, 1998). In contrast, urate, a naturally occurring component in milk and a well-known antioxidant in many biological systems (Ames et al., 1981; Becker, 1993), has to the authors' knowledge not received any attention in relation to preventing oxidative deterioration in milk.

Urate is formed from ruminal breakdown of microbial nucleotides and thereby regulated/influenced by rumen metabolism and feed composition (Giesecke et al., 1994; Johnson et al., 1998). Tiemeyer et al. (1984) have reported a negative correlation between daily milk production and urate content in the milk. These studies indicate that the urate concentration can be controlled by intensity of the milk production and/or feeding regimes promoting high ruminal activity in order to achieve optimal antioxidative activity of this compound in milk.

Here we present data showing that urate is an effective antioxidant in relation to light- and peroxidase-induced oxidation of milk. These data indicate that the antioxidative effect of urate is similar to that of ascorbate.

MATERIALS AND METHODS

Materials. Uric acid and ascorbic acid were purchased from Sigma (Sigma Chemicals, St. Louis, MO). Fresh unpasteurized milk was obtained from our own production herd at the Research Centre Foulum (64 Danish Holstein cows; milk was used within 16 h of milking), whereas pasteurized whole milk was obtained from the local supermarket. All other chemicals were analytical grade, and double-deionized water was used throughout.

Effect of Added Urate and/or Ascorbate on Lactoperoxidase-induced Dityrosine Formation in Unpasteurized Milk. Reaction mixtures were made from 94 vol % pasteurized whole milk and 6 vol % urate and/or ascorbate in 50 mM phosphate buffer (pH 6.6). Final concentrations of urate and/or ascorbate were 5−30 mg/L and controls were made by substituting the urate and/or ascorbate solution with 50 mM phosphate buffer (pH 6.6). Lactoperoxidase in the milk was activated by addition of 1.0 mM H_2O_2, and the solutions were incubated for 4 h in a water bath (25 °C). After the incubation, 3-mL samples were withdrawn and mixed with 3 mL of 0.1 M H_2SO_4 in 1 M NaCl and 12 mL of 2-propanol. The samples

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were vortexed for 3 min, 9 mL of hexane was added, and the samples were vortexed for an additional 3 min. Samples were precipitated by centrifugation (12 000*g* for 5 min). The supernatant was discharged, and the extraction procedure was repeated. The pellet was redissolved in 5 mL of phosphate buffer [pH = 7.4; I = 0.16 (NaCl)], and the protein was precipitated by adding trichloroacetic acid to a final concentration of 10%. The samples were allowed to stand in the hood for 10 min before centrifugation (12 000*g* for 10 min). The pellet was washed with 4 mL of 6 M HCl before an additional centrifugation (12 000*g* for 10 min). The pellet was mixed with 1 mL of 6 M HCl, flushed with argon, and hydrolyzed overnight (105 °C). Samples were neutralized with 6 M NaOH, and 20 μL of the hydrolyzed sample was injected onto a C-18 column (Microsorb 100−5, 250 × 4.6, Varian, Walnut Creek, CA), which was equilibrated with 4% acetonitrile in aqueous 0.10 M citric acid (pH 2.55), with a flow of 1 mL/min as described by Daneshvar et al. (1997). Chromatographic separation was performed on a HPLC system consisting of a Varian 9012 HPLC pump, connected to a Varian 9100 Auto sampler and a Varian 9075 Fluorescence detector (excitation 283 nm; emission, 410 nm) (Varian Chromatographic Systems, Walnut Creek, CA). Samples were spiked with a dityrosine standard for identification and quantified by the use of a standard curve made from the same standard. The dityrosine standard was prepared according to Nomura et al. (1990).

Effect of Light Exposure on the Concentration of Urate and Ascorbate in Whole Pasteurized Milk. Pasteurized milk was exposed to light at 5.0 ± 0.5 °C in 30-mL glass centrifuge. The centrifuge tubes were mounted on a Orbit rotator (Selecta, 08630 Abrera, Spain) which was turning at 12 rpm and tilted at approximately 45 degrees to ensure mixing and equal light exposure (light intensity, approximately 5500 lux). Nonilluminated controls were made by wrapping the tubes in aluminum foil. After 0, 2, 4, and 6 h of incubation, the concentrations of urate and ascorbate were determined by HPLC. Quantification of ascorbate was based on analysis of dehydroascorbate using HPLC separation and fluorescence detection based on a modified method of Tessier et al. (1996). Ascorbate and dehydroascorbate were isolated from the milk by precipitation of the protein by *meta*-phosphoric acid (final concentration, 0.56% w/v). After centrifugation (12 000*g* for 10 min), 150 μL of supernatant was oxidized for 10 min by adding 30 μL of iodine solution (4.92 mM I_2 in 0.16 M KI) to measure the total content of ascorbate and dehydroascorbate. The concentration of dehydroascorbate was measured by substituting the iodine solution with water. Subsequently, solutions were derivatized by adding 150 μL of 1.63 M phosphate buffer (pH 5.4) and 60 μL of dimethyl-*p*-phenyldiamine (DMPD) (1 g/L). (DMPD was solubilized in 1 mL HCl before addition of water.) HPLC analysis was performed by loading 30 μL of derivatized dehydroascorbate on a Hypersil ODS 250 × 4 mm column (Agilent Technologies, Palo Alto, CA) with MeOH/80 mM phosphate buffer, pH 7.4 (1:1 v/v) as mobile phase (0.6 mL/min) and fluorescence detection (excitation, 360 nm; emission, 440 nm) using the Series 1100 HPLC system (Agilent Technologies, Palo Alto, CA). The concentration of ascorbate was calculated as the difference between samples with and without the iodine oxidation step. A standard curve made from oxidized ascorbate was used for quantification. Quantification of urate was also performed by HPLC separation and fluorescence detection. Urate was isolated from the milk by mixing milk, *meta*-phosphoric acid (1.12%), and chloroform (1:1:1 v/v). After centrifugation (12 000*g* for 10 min) the concentration of urate in the water phase was determined by HPLC as described above for the ascorbate analysis except that the mobile phase was 10 mM potassium phosphate (pH 4.0) using a flow of 1.0 mL/min and fluorescence detection (excitation, 285 nm; emission, 450 nm). Quantification of urate was performed by the use of a standard curve.

Effect of Urate and Ascorbate on Light-induced Lipid Hydroperoxide Formation in Pasteurized Whole Milk. Reaction mixtures were made from 94 vol % pasteurized whole milk and 6 vol % urate or ascorbate in 50 mM phosphate buffer (pH 6.6). Final concentrations of urate or ascorbate were 30 mg/l, and controls were made by substituting the urate or ascorbate solutions with 50 mM phosphate buffer (pH 6.6). Samples were exposed to light (5500 lux) at 5.0 ± 0.5 °C in 30-mL glass centrifuge tubes as described above. After 12 h of illumination milk samples were withdrawn and the formation of lipid hydroperoxides was measured by a colorometric reaction using a modification of the method described by Shantha and Decker (1994). Two milliliters of light-exposed milk was mixed with 2 mL of methanol and vortexed. Subsequently, 4 mL of chloroform was added and vortexed for 30 s. Samples were centrifuged for 10 min (12 000*g*) and 1.0 mL of the chloroform phase (lower phase) was transferred to a test tube and mixed with 1 mL of Fe(II)/thiocyanate in methanol:chloroform. [50 mL of 32.7 mM $BaCl_2$ was slowly added to 50 mL of 36 mM $FeSO_4$ under continuous magnetic stirring, 2 mL 10 M HCl was added, and the solution was filtered to remove precipitated $(Ba)_3(PO_4)_2$. Five hundred microliters of this solution and 500 μL of 3.94 M NH_4SCN were added to 49 mL of methanol:chloroform (1:1 v/v).] The reaction mixtures were allowed to react for 5 min at room temperature before the absorbance at 500 nm was read using a HP-8453 diode array spectrophotometer (Agilent Technologies, Palo Alto, CA).

Effect of Urate and Ascorbate on Light-induced Riboflavin Degradation in Pasteurized Whole Milk. Reaction mixtures were made from 80 vol % pasteurized whole milk and 20 vol % urate or ascorbate in 50 mM phosphate buffer (pH 6.6). Final concentrations of urate or ascorbate were 300 mg/L, and controls were made by substituting the urate or ascorbate solutions with 50 mM phosphate buffer (pH 6.6). Reaction mixtures were exposed to light (5500 lux) at 5.0 ± 0.5 °C in 30-mL glass centrifuge tubes as described above. Five milliliter milk samples were withdrawn after 0, 2, 4, 6, and 9 h, and mixed with 500 μL of 2 M sodium acetate and 1.5 mL of 2 M acetic acid. The samples were slowly agitated for 5 min before centrifugation (12 000*g* for 10 min). The supernatant was filtered through a 0.45-μm filter, and the fluorescence was read (excitation 445 nm; emission 520 nm) using a Perkin-Elmer LS 50B fluorescence spectrometer (Perkin-Elmer Ltd., Beaconsfield, Bucks, U.K.). All analytical procedures were conducted using glassware wrapped in aluminum foil to avoid additional riboflavin degradation during sample preparation.

RESULTS

The antioxidative activity of urate was evaluated by addition of different concentrations of this compound to unpasteurized and pasteurized whole milk with subsequent induction of oxidation using hydrogen peroxide-mediated peroxidase activity or fluorescent light. Moreover, experiments were performed substituting urate with ascorbate to make a comparison between these two antioxidants possible.

Activation of peroxidase, mainly lactoperoxidase, in unpasteurized milk can be achieved by addition of H_2O_2, which subsequently promotes oxidation including protein oxidation as measured by formation of dityrosine (Østdal et al., 2000). The presence of additional urate and/or ascorbate retarded the formation of dityrosine by 44−96% in a concentration-dependent manner as illustrated in Figure 1. The addition of both antioxidants showed no synergistic effect between the two, but merely an additive effect.

Fluorescent light exposure of milk promotes degradation of urate and ascorbate which stresses the importance of these antioxidants during light-induced oxidation in milk as illustrated in Figure 2. The figure shows that urate and ascorbate disappeared at different stages

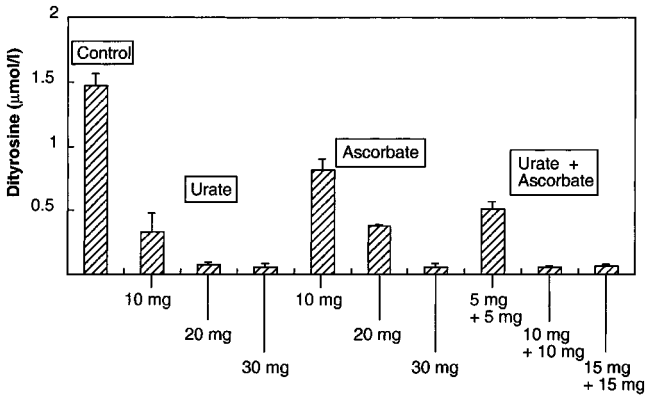

Figure 1. Effect of urate and/or ascorbate on the formation of dityrosine in unpasteurized milk after H_2O_2 activation of endogenous peroxidases. Unpasteurized milk was added H_2O_2 to a final concentration of 1.0 mM and incubated for 4 h at 25 °C. Dityrosine was measured using HPLC after extraction and acid hydrolysis of the milk proteins. All values are the mean of three independent measurements.

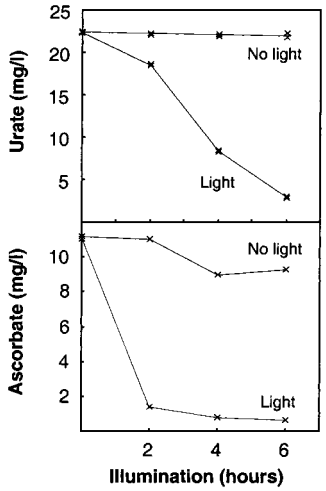

Figure 2. Effect of light exposure at 5 ± 0.5 °C on the concentration of urate and ascorbate in pasteurized whole milk. Control samples (no light) were prepared by wrapping tubes in aluminum foil.

during the course of the oxidation. Ascorbate showed a large decrease during the first 2 h of incubation, whereas urate decreased at the highest rate between 2 and 4 h of incubation. Ascorbate was only determined as single measurements because of the long sample preparation and the low stability of the ascorbate. The experiment showed in Figure 2 was repeated, and the pattern of decrease for the antioxidants was exactly the same (data not shown).

The ability of urate or ascorbate to prevent photoinduced oxidation was verified by measuring formation of lipid hydroperoxides during light exposure of pasteurized milk. Addition of either urate or ascorbate significantly lowered lipid hydroperoxide formation as shown in Figure 3. Urate was twice as effective (28%) in preventing lipid hydroperoxide formation as ascorbate (14%). Moreover, photobleaching of riboflavin was also retarded by the addition of urate or ascorbate as illustrated in Figure 4. All data points for the photobleaching of riboflavin were normalized at $t = 0$ because urate had a small quenching effect. Urate seems to be more effective in retarding the photo bleaching of riboflavin than ascorbate.

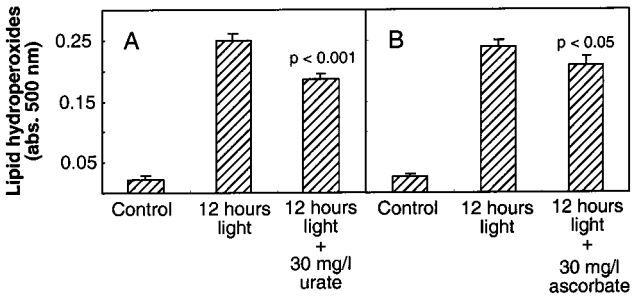

Figure 3. Effect of urate (A) or ascorbate (B) on the formation of lipid hydroperoxides in pasteurized whole milk during light exposure at 5 ± 0.5 °C. All values are the mean of three independent measurements. Differences between samples stored in light with and without added urate or ascorbate were calculated using the student's *t* test. Controls equal aluminum foil wrapped tubes containing milk without added urate or ascorbate.

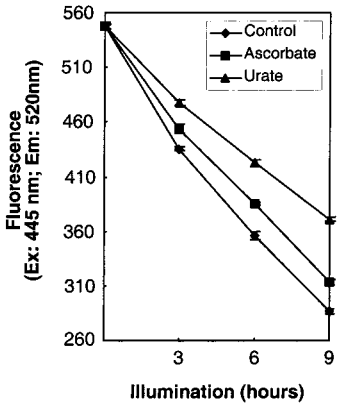

Figure 4. Effect of urate or ascorbate on the riboflavin content in pasteurized whole milk during light exposure at 5 ± 0.5 °C. Riboflavin was measured as fluorescence of milk after precipitation of proteins with acetic acid/acetate. All values are the mean of three independent measurements.

DISCUSSION

Oxidation in milk and dairy products is catalyzed by a variety of pro-oxidants of which enzymes, metal ions, and light exposure generally are considered of major importance depending on the product, processing parameters, and storage conditions (review by Allen and Joseph, 1985). In this study, urate showed an antioxidative effect in relation to protein and lipid oxidation induced by H_2O_2 activation of endogenous peroxidases or light, which leads to the conclusion that urate should be considered as being a general antioxidant in milk. Furthermore, the antioxidative effect of urate was similar to that of ascorbate. However, the basal level of urate in whole pasteurized milk used in this study was approximately twice the level measured for ascorbate, as seen from Figure 2. The antioxidative activities reported in this study therefore reflect the effect of increasing the concentration of these two antioxidants in the milk and not their molar efficiency.

Both urate and ascorbate in milk are degraded during light exposure (Figure 2). However, ascorbate is depleted earlier in the course of oxidation than urate. The standard redox potential of ascorbate (0.28 V) is lower than that of urate (0.59 V), which indicates that urate is regenerated from the urate radical by ascorbate. Thus, increasing concentrations of urate in milk will not preserve ascorbate; moreover, ascorbate will actually

prevent oxidation of urate as long as this antioxidant is present in adequate quantities.

Oxidative changes in milk induced by light are mediated mainly by riboflavin which acts as a photosensitizer (Sweetsur and White, 1975). Riboflavin tends to act more as a type I than a type II sensitizer (reviewed by Bradley and Min, 1992) which means that light-excited forms of riboflavin will have a higher tendency to react directly with other biomolecules rather than transferring the energy to oxygen resulting in formation of singlet oxygen. The reaction between light-excited riboflavin and amino acids leads to subsequent superoxide anion formation (Korycka-Dahl and Richardson, 1978) and thereby gives rise to additionally activated oxygen species. A relatively high concentration of urate or ascorbate was needed to retard photobleaching of riboflavin during light exposure of milk. The effect of ascorbate measured in this study is comparable with what has been reported previously (Lee et al., 1998). Comparison of Figure 3 and Figure 4 shows that the concentration of urate and ascorbate was 10 times higher in the experiment investigating riboflavin degradation than in the experiments measuring lipid hydroperoxide formation. This leads to the conclusion that the main antioxidative effect of urate and ascorbate in relation to lipid hydroperoxide formation is due to quenching of radicals formed after light excitation of riboflavin, and only a minor effect is due to direct quenching of light-excited forms of riboflavin.

According to the present data, an increase in the urate concentration will give milk an increased oxidative stability, but the effect on overall milk quality is unknown. It could be speculated whether spontaneous oxidation in milk is influenced by a low content of urate because it has been difficult to explain the mechanism behind this phenomenon (Barrefors et al., 1995; Granelli et al., 1998). However, urate is like ascorbate, known to be able to reduce transition metal ions making these more pro-oxidative because of lipid hydroperoxide destruction. This means that urate might promote transition metal-catalyzed lipid oxidation (Bagnati et al., 1999). We are currently working on producing milk with different urate contents by the use of different feeding schemes. This will make it possible to evaluate oxidative stability and overall milk quality within the urate concentration span that can be achieved through feeding. Furthermore, such experiments will make it possible to estimate cost/benefit for the practical use of specific feeding schemes to ensure optimal urate levels in milk.

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