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Characterization of Peroxides Formed by Riboflavin and Light Exposure of Milk. Detection of Urate Hydroperoxide as a Novel Oxidation Product

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Characterization of peroxides by size exclusion chromatography (SEC) of milk following exposure to riboflavin and light showed that hydrogen peroxide was the most abundant peroxide formed since it could be removed by catalase. Formation of peroxides after separation by SEC showed that hydrogen peroxide formation was primarily increased in the presence of caseins and ascorbate, although whey proteins also were found to contribute. Caseins and β-lactoglobulin also formed catalase-resistant peroxides, presumably protein hydroperoxides. A catalase-resistant and unstable peroxide was observed in fractions containing urate. Experiments performed with pure urate suggested that urate radicals reacted further with superoxide leading to a urate hydroperoxide. Electron paramagnetic resonance spectroscopy using spin-traps showed that the presence of oxygen was required for urate radical formation, which could be assigned as nitrogen-centered radicals. These results suggest a new route during light-induced oxidation sensitized by flavins, in effect making urate pro-oxidative.

KEYWORDS: Milk oxidation; peroxides; proteins; urate; electron paramagnetic resonance

INTRODUCTION

Milk is an important source of riboflavin (vitamin B_2), but this compound is activated when exposed to blue light and becomes a photosensitizer for oxidation processes in milk. The complex chemical reactions following this activation of riboflavin are often deleterious when occurring in milk and other foods due to formation of volatile oxidation products from lipids and proteins (*1*). Additionally, proteins may become dysfunctional due to cross-linking, precipitation, and/or changes in tertiary structure (*2*, *3*). These oxidative modifications may be a result of reactions directly with excited triplet riboflavin (^{3}Rib*) (Type I mechanism) (*4*, *5*) or indirectly via singlet oxygen ($^1O_2$) formed through reaction of ^{3}Rib* with ground state molecular oxygen (Type II mechanism). The significance of the Type II reaction in milk has been described in several studies (*6*−*8*), whereas Type I reactions in milk have received less attention.

Although formation of peroxides may result from both types of reactions, protein hydroperoxides are well-known products of Type II reactions with histidine, cysteine, tyrosine, and tryptophan residues (*9*). Superoxide ($O_2^{\bullet-}$) initially formed during Type I photooxidation subsequently forms H_2O_2 through dismutation or protein hydroperoxides through reaction with tyrosyl or tryptophanyl radicals (*10*, *11*).

Considering the high concentration of protein in milk, proteins are likely targets of singlet oxygen-mediated oxidation. The resulting peroxides are probably unstable in milk due to the presence of transition metal ions and may fragment into radical species, such as alkoxyl or peroxyl radicals. These can act as chain-propagating agents, in effect leading to extensive oxidative damage (*12*). To contribute to a better understanding of the role of protein hydroperoxides in the oxidative degradation of milk products, we have studied the formation of peroxides during riboflavin-mediated photooxidation of milk using an experimental approach that combines size exclusion chromatography (SEC) and peroxide detection with the ferrous oxidation xylenol orange (FOX) assay. We recently used SEC for detection of major radical scavengers in milk (*13*), and protein hydroperoxides have moreover recently been characterized in cells using similar techniques (*14*). In addition, we have included electron paramagnetic resonance (EPR) spectroscopy to identify radical intermediates related to the low molecular weight (LMW) fractions of milk.

MATERIALS AND METHODS

Chemicals. Riboflavin, flavin mononucleotide (FMN), 5,5′-dimethylpyrroline-*N*-oxide (DMPO), 2-methyl-2-nitrosopropane (MNP), hydrogen peroxide, xylenol orange, catalase, urate, and sodium azide were purchased from Sigma Aldrich (Steinheim, Germany). Sodium dithionite was purchased from Honeywell Riedel-de Häen (Seelze, Germany), guanidinium hydrochloride from Invitrogen A/S (Taastrup, Denmark), superoxide dismutase (SOD) from Fluka (Steinheim, Germany), and $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ from Applichem (Darmstadt, Germany).

Sample Preparation. Fresh bulk milk was collected from the herd of Holstein-Friesian dairy cows at Foulum, Aarhus University. Skim milk

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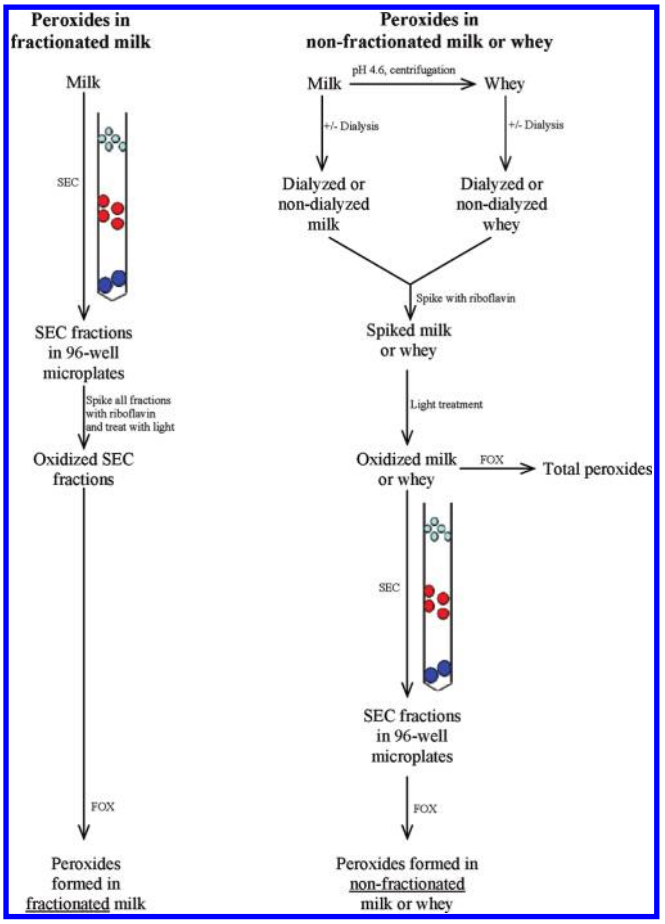


Figure 1. Flow diagram of the experimental setup. SEC, size exclusion chromatography; FOX, ferrous oxidation xylenol orange assay.

was prepared by centrifugation (10 000*g*, 5 min at 4 °C). Whey was prepared from skim milk by acidification to pH 4.6 with lactic acid, incubation for 30 min at room temperature, centrifugation (as above), and readjustment of pH of the supernatant with NaOH to 6.7.

The effect of LMW compounds on the formation of peroxides was assessed by dialysis (3.5 kDa cutoff) of milk and whey against deionized water (15 mL sample to 1.5 L of water). Water was changed twice after 1 h of dialysis and then followed by overnight dialysis. Nondialyzed samples were diluted to the same extent as the dialyzed samples (approximately 1.4 fold (v/v)).

Experimental Setup. Total peroxides were quantified upon riboflavin and light exposure in nondialyzed and dialyzed milk and whey. To further characterize the formed peroxides, nondialyzed samples were subjected to SEC, and the amount of peroxides in the various SEC fractions were determined. This approach is termed peroxide formation in *nonfractionated* samples.

An alternative approach was to perform SEC prior to treatment with riboflavin and fluorescent light. This was done for skimmed milk and is termed peroxide formation in *fractionated* samples. The experimental setup is summarized in **Figure 1**, and further experimental details are given in the following sections.

Size Exclusion Chromatography. SEC was performed as described recently (*13*). In brief, samples (milk or whey, 0.5 mL) were applied to a Superdex 200 10/300GL column using an Äkta FPLC system, both from Amersham Pharmacia Biotech (Uppsala, Sweden). The flow rate was 0.5 mL min^{-1} using a 10 mM phosphate buffer, pH 6.7, with 0.15 M NaCl, and fractions of 0.30 mL were collected.

Detection of Peroxides. Peroxides were determined directly in the microplates using the FOX assay with 125 μM $Fe(NH_4)_2(SO_4)_2$ and xylenol orange in 25 mM H_2SO_4 containing 2.5 M guanidinium hydrochloride. After incubation for 30 min at room temperature in the dark, the absorbance was read at 560 and 700 nm on a $PowerWave_X$ microplate scanning spectrophotometer from Bio-Tek Instruments, Inc. (Winooski, VT, USA) with the KC4 software for data analysis. A sample treated with 2 mM dithionite was used as blank. For estimation of the amount of hydrogen peroxide, total and catalase-resistant peroxides were determined using 0.01 mg mL^{-1} catalase and incubation for 10 min. This was shown in preliminary experiments to completely remove hydrogen peroxide. Hydrogen peroxide was used as standard for quantification in the FOX assay.

Riboflavin and Light-Induced Hydroperoxide Formation. Riboflavin (22 μM final, corresponding to 5 times the endogenous content of riboflavin in milk) was added from a 220 μM stock solution prepared in 1 mM sodium phosphate (ε_{450nm} = 12 200 L mol^{-1} cm^{-1} (*15*)) to all samples. Nonfractionated samples (3 mL) were contained in 10 mL pyrex tubes and placed in a rotor under fluorescent light (1200 Lux) as described (*2*). For fractionated milk samples, riboflavin was added to 96-well microplates containing all fractions from SEC. Samples were covered with transparent polyester sealing tape (Nunc, Roskilde, Denmark) and exposed to fluorescent light as described above. There was no formation of peroxides in controls wrapped in aluminum foil.

Hydroperoxide Formation with Urate. Stock solutions of urate (60 mM) were prepared in 0.5 M NaOH. Reactions between urate and triplet excited riboflavin were performed in microplates using different concentrations of urate (0−50 μM) and 22 μM riboflavin under the same conditions as described above. The effect of the superoxide anion was evaluated by addition of SOD in the reaction mixture together with controls without added urate.

EPR Spectroscopy. Solutions of urate (120 mM) in 0.5 M NaOH and FMN (12.5 mM) in 100 mM phosphate buffer (pH 7.0) were mixed and diluted with phosphate buffer to final concentrations of 5 mM urate and 250 μM FMN. DMPO (100 mM in water) or MNP (100 mM in acetonitrile) were added as spin traps to a final concentration of 10 mM. Solutions for experiments in the presence or absence of oxygen were purged with air or nitrogen (99.998%) and transferred to a flat quartz cell. Irradiation was performed directly in the sample cavity using blue light (center wavelength ~440 nm) generated by a Photonics Polychrome II unit (TILL Photonics, Gräfelfing, Germany). A Bruker ECS 106 spectrometer (Bruker, Karlsruhe, Germany) was used with the following settings: center field, 3480 G; sweepwidth, 80 G; microwave power, 10 mW; modulation frequency, 100 kHz; modulation amplitude, 1.0 G; conversion time, 40.96 ms; time constant, 20.48 ms; sweep time, 42 s. Four repeated scans were accumulated and averaged for each spectrum, and simulations were performed using the PEST WinSIM program (*16*).

RESULTS AND DISCUSSION

LMW Compounds. The impact of LMW compounds on peroxide formation after addition of riboflavin and exposure to light for 16−48 h was investigated in dialyzed milk and compared to nondialyzed samples. Further, we characterized the peroxides as either catalase-sensitive (H_2O_2) or -resistant. The dialyzed samples had a peroxide content of only 44 ± 3% (n = 4) compared with nondialyzed samples. This was entirely due to lower amounts of hydrogen peroxide (36 ± 10%), while there was no effect of dialysis on formation of catalase-resistant peroxides (104 ± 27%).

Bearing in mind the relatively rapid decomposition of ascorbate and urate occurring during light-induced oxidation of milk, this result implies that hydrogen peroxide consuming activities of endogenous lactoperoxidase and catalase were inadequate to deplete hydrogen peroxide formed under our reaction conditions (5 times the endogenous riboflavin content).

Hydroperoxide Formation in Nonfractionated and Fractionated Samples. We then compared peroxide formation in milk before or after separation of the milk components by SEC as illustrated in **Figure 1**. **Figure 2** shows representative results from both types of experiments, where identification of individual milk components in the SEC profiles is based on our previous results (*13*).

When oxidation was performed after separation of milk components by SEC (**Figure 2A**), the most prominent increase was observed in fractions containing caseins, whereas fractions

containing β-lactoglobulin (β-lg), α-lactalbumin (α-la), ascorbate, and urate increased peroxide formation to a minor extent. Catalase treatment removed most of the peroxides that were formed in the various fractions, showing that hydrogen peroxide was the major peroxide formed. Catalase-resistant peroxide concentrations in high molecular weight (HMW) fractions were highest in the β-lg and α-la peaks, whereas caseins formed lower amounts of peroxides. The peroxides formed in the LMW fractions were characterized according to their resistance toward catalase: Peroxides formed by ascorbate were almost exclusively hydrogen peroxide, whereas urate produced much smaller amounts of hydrogen peroxide, since approximately 80% of the peroxides formed was resistant to catalase.

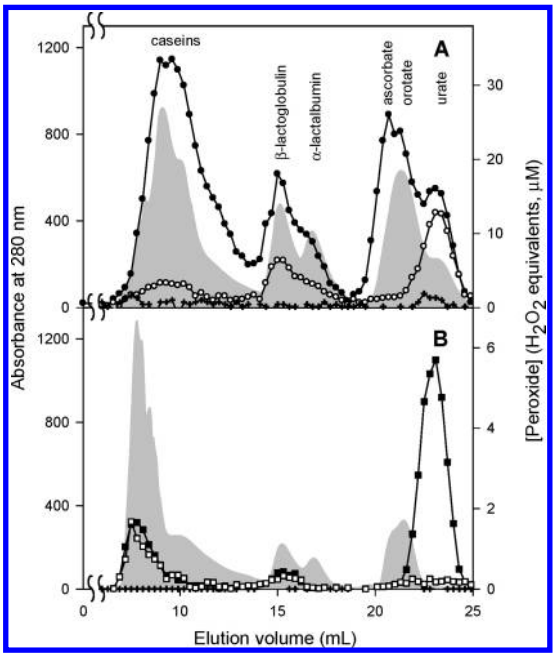


Figure 2. (**A**) Formation of peroxides in fractionated milk after 2 h of light exposure in the presence of 22 μM riboflavin. The background peroxide formation induced by riboflavin alone was calculated from 12 fractions eluting before the void volume. For each fraction, this background was used to calculate the peroxide formation induced by the components present. Total peroxides (●), catalase-resistant peroxides (○), and dark control (+). (**B**) Detection of peroxides formed in nonfractionated milk after 16 h of light exposure in the presence of 22 μM riboflavin. Light-exposed milk was applied to SEC, and peroxides in the flow through were detected using the FOX assay. Total peroxides (■), catalase-resistant peroxides (□), and dark control (+). Absorption profiles at 280 nm are shown in gray, and representative plots from at least five independent experiments are shown.

Singlet oxygen in milk is quenched by ascorbate, urate, carotenoids, milk fat, and riboflavin. Proteins probably also take part in the antioxidative defense of milk (*13*), and protein hydroperoxides have been shown to be formed during $^1O_2$-mediated oxidation (*14*). Despite the increasing evidence of the formation of protein hydroperoxides in vivo during photooxidation (*17*, *18*) and their potential influence on the onset of lipid oxidation in milk, this aspect of milk oxidation has not been studied in any detail. The results presented in **Figure 2A** indicate that the most significant pathway for peroxide formation includes inactivation of triplet excited riboflavin by proteins, ascorbate, and urate, which results in H_2O_2 formation (*19*), and not the singlet oxygen pathway, which would be expected to result in formation of protein hydroperoxides.

The major peroxide formed in nonfractionated milk was also hydrogen peroxide, confirming the results obtained with the fractionated milk components (**Figure 2B**). The β-lg hydroperoxide was the major catalase-resistant HMW peroxide that could be identified, whereas α-la formed very little or no hydroperoxide. An HMW hydroperoxide was observed in the void volume but could not be assigned any single component. As shown by the absorption trace at 280 nm, aggregates were formed in milk, probably consisting of caseins, while both ascorbate and urate disappeared during photooxidation as expected (*20*) (**Figure 2B**). Interestingly, the catalase-resistant peroxide formed by urate in the fractionated milk samples was absent after oxidation of nonfractionated milk.

Rate of Hydroperoxide Formation. Peroxide formation in fractionated milk samples was determined after different time intervals (**Figure 3**). Casein, ascorbate, and urate fractions showed rapid initial peroxide formation. Whereas caseins continued to form peroxides for at least 5 h, ascorbate-induced peroxide formation reached a final level within 30 min and then remained constant for at least 5 h. This was in contrast to urate-induced formation of peroxides, where the peroxides disappeared

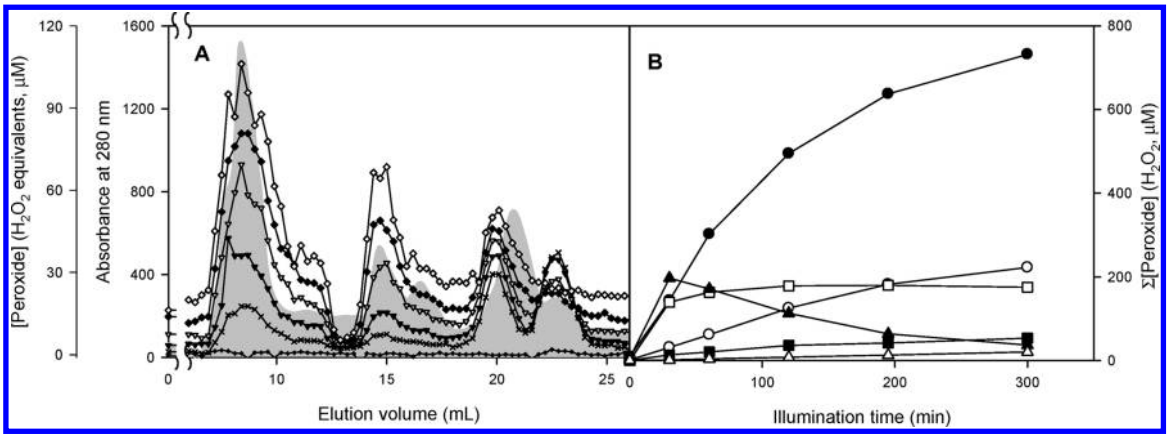


Figure 3. (**A**) Total peroxide formation in fractionated milk after 0 (+), 30 (×), 60 (▼), 120 (▽), 200 (♦), and 300 (◇) min reaction, under the same conditions as in **Figure 2A**. (**B**) Rate of total peroxide formation of individual components calculated from the data in (**A**). The background peroxide formation induced by riboflavin was subtracted as described in **Figure 2A**. The sum of total peroxides was then calculated for fractions containing a given component, caseins (●), β-lg (○), α-la (■), ascorbate (□), and urate (▲). For comparison, the peroxide content in wells only containing riboflavin is also shown (△). Representative plots of three independent experiments. Standard deviation between these experiments was <10% in the protein fractions and <33% in the LMW fractions.

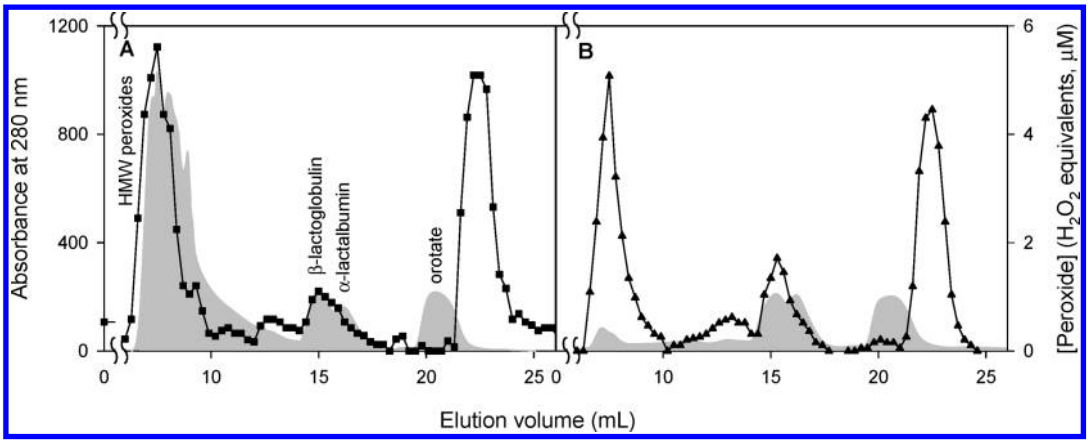


Figure 4. Detection of hydroperoxides formed in nonfractionated milk (**A**, ■) and whey (**B**, ▲) exposed to fluorescent light for 48 h in the presence of 22 μM riboflavin. Light-exposed samples were applied to SEC, and peroxides in the flow through were detected using the FOX assay.

slowly. These results show that the ability of ascorbate and urate to induce peroxide formation was rapidly exhausted, probably due to complete consumption of both compounds, as also indicated by their disappearance from the UV-trace (compare **Figures 3** and **4**).

Whereas caseins continued to generate hydrogen peroxide at a high, albeit declining, rate within the first 5 h, β-lg-induced peroxide formation was considerably slower, and α-la-induced formation was only slightly above the level of background peroxide formation by riboflavin. The apparent difference in peroxide generating ability between caseins and β-lg may be ascribed almost completely to the difference in protein concentration, as estimated from the absorbance profile. Noticeably, peroxide formation at or even below background was observed in fractions corresponding to an elution volume between 12 and 13 mL. Although this effect varied between experiments (c.f. **Figure 2A** and **Figure 3A**), additional experiments indicated that this suppression could be assigned to lactoperoxidase, which eluted at this position (results not shown).

The rate of peroxide formation in nonfractionated milk samples was much slower compared to fractionated milk. The former required at least 4−6 h of light exposure, although longer exposure times (16−48 h) were frequently necessary. This probably reflects the considerable variation in activity of lactoperoxidase (and possibly in concentrations of antioxidants) between batches of milk.

Furthermore, it should be emphasized that an elevated concentration of riboflavin was required to obtain a detectable amount of hydrogen peroxide and organic hydroperoxides and may in fact reflect that under normal conditions (∼4−5 μM riboflavin) the endogenous antioxidant systems of milk either keep the level of peroxides below the detection limit of our assay or prevent their formation.

Comparison of Milk and Whey. As shown in **Figure 2B**, an unknown peroxide was observed in the void volume of our SEC experiment. A simple interpretation is that caseins, which are excluded from the SEC column when in micellar form, were the primary site of hydroperoxide formation. However, as shown in **Figure 4**, quantitatively similar peroxide profiles were obtained for milk and whey, while the UV−absorption profiles indicated a much higher content of proteins eluting in the void for photooxidized milk than for whey. These results indicate that the peroxides detected in the void volume from the gelfiltration of milk may consist primarily of aggregated whey protein hydroperoxides, probably due to polymerized β-lg as native β-lg decreases after exposure to light. Further, aggregation and cross-linking of caseins by light-induced oxidation probably either has occurred prior to our analysis or is independent of formation of peroxides.

Proteins react with $^1O_2$, primarily through amino acids exposed to solvent on the surface of the native structure, whereas reactivity equals that of their constituent amino acids under denaturing conditions (*21*). Thus, it seems reasonable to assume that the loose structure of caseins makes them primary targets for reaction with $^1O_2$ or excited riboflavin during photooxidation of milk. In fact, we have previously shown that caseins exhibit the same radical scavenging activity in the native and denatured form (*13*). However, the results of **Figure 4**, where we primarily observed β-lg hydroperoxides, could be a result of poorer stability of the casein hydroperoxides. Several reasons for this can be speculated: micellar casein is known to bind metal ions (*22*), and the presence of redox-active iron or copper ions could account for an increased instability of casein hydroperoxides. Ascorbate has been shown to catalyze decay of protein hydroperoxides and scavenge radicals derived from their decomposition (*23*). Hence, in our experiments, improved accessibility of LMW compounds to the peroxides formed on the caseins and radicals derived from their decomposition could account for our observations. In analogy with this, Østdal et al. (*24*) showed that radicals formed on caseins were much shorter lived and more prone to cross-linking (dityrosine formation) than β-lg radicals. Finally, the open structure of the caseins is known to promote oxidation of tryptophan residues as compared to the globular whey proteins, whereas oxidation of methionine and histidine was not significantly different (*25*). These oxidation products are likely formed through peroxide intermediates (*26*), reinforcing the need for a characterization of protein peroxides and establishment of a link between the ability of milk proteins to form peroxides and the formation of stable oxidation products.

Effects of Catalase and SOD. Further insight into the mechanism(s) was obtained by including catalase or SOD during peroxide formation in fractionated milk (**Figure 5**). Inclusion of SOD doubled the formation of peroxides formed in fractions containing caseins and β-lg. In contrast, inclusion of catalase almost completely eliminated peroxide formation, indicating formation of primarily hydrogen peroxide and only very limited protein hydroperoxide formation.

SOD had no or very little effect on total hydroperoxide formation in the LMW fractions. Our results show that, unlike ascorbate, peroxides in the urate fractions were resistant to catalase, and as seen in **Figure 5** (inset), formation of the catalase-resistant hydroperoxide decreased in the presence of

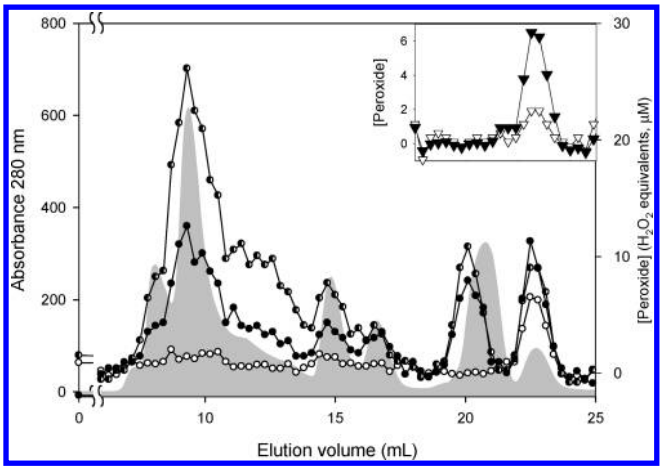


Figure 5. Peroxide formation in fractionated milk after 1 h exposure to fluorescent light in the presence of 22 μM riboflavin. Reactions were performed in the presence of 0.01 mg mL^{-1} catalase (open circles), 0.01 mg mL^{-1} SOD (semi-filled circles), or water as control (closed circles). The inset shows the formation of catalase-resistant peroxides in the presence (open triangles) or absence of SOD (filled triangles) (only 18−25 mL are shown) and shows that formation of the catalase-resistant peroxide is dependent on the presence of superoxide.

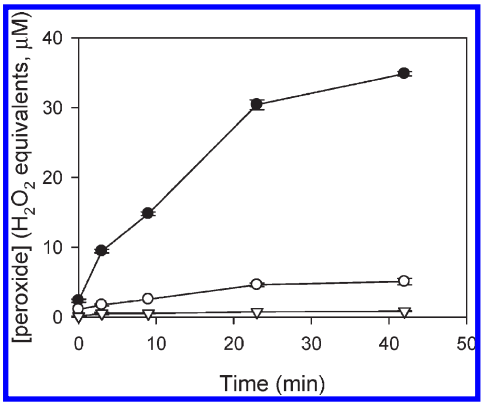


Figure 6. Riboflavin-induced formation of catalase-resistant peroxides with urate in the presence (○) or absence (●) of SOD (0.38 mg/mL) and hydrogen peroxide formation in the absence of SOD (▽). Concentrations were 60 μM urate and 20 μM riboflavin. Doubling the amount of SOD did not result in further inhibition of catalase-resistant peroxide formation.

SOD, arguing that SOD and urate oxidation products compete for superoxide.

Urate Hydroperoxide. Experiments performed with pure urate and riboflavin showed that these compounds formed a catalase-resistant peroxide (**Figure 6**), presumably a urate hydroperoxide, as no catalase-resistant peroxide was detected in the absence of urate. Under our reaction conditions, the results indicated a yield of urate hydroperoxide $>50\%$, and its formation was almost completely inhibited by SOD (**Figure 6**). These results indicate that $O_2^{\bullet-}$ must be involved in the formation of the urate hydroperoxide (**Scheme 1**): One electron oxidation of urate results in the formation of urate radical anions in neutral solutions (*27*, *28*). The urate anion radical does not react with oxygen (*28*), but Santus et al. (*29*) showed that the reaction between the urate radical anion and the superoxide anion occurs in a second-order reaction with a rate constant of 8×10^8 M^{-1} s^{-1}. Consistent with this and the low rate constant for dismutation of superoxide in alkaline solution (<0.3 M^{-1} s^{-1}) (*30*), very little hydrogen peroxide was observed in the absence of SOD (**Figure 6**), and we

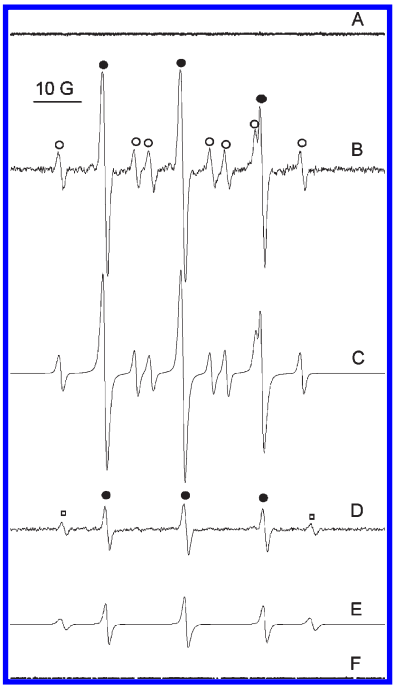
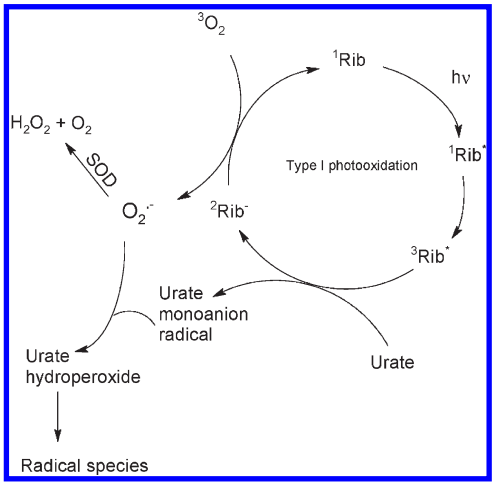


Figure 7. EPR spectra obtained during FMN (125 μM) mediated light-induced oxidation of 5 mM urate, pH 7, in the presence of MNP and atmospheric air. **A**, Dark; **B**, Light, 6 min illumination in the presence and, **D**, in the absence of urate; **C** and **E**, Simulations of spectra in **B** and **D**, respectively; **F**, As **B** but flushed with N_2. Lines marked ● are assigned to di-*tert*-butyl nitroxyl radical adducts. Lines marked ○ are assigned to trapping of a nitrogen radical (two of the lines are hidden under the *tert*-butyl-MNP signal). Adducts assigned to trapping of $O_2^{\bullet-}$ are marked with □.

Scheme 1



accordingly propose that the reactions presented in **Scheme 1** account for the formation of the urate hydroperoxide. EPR spectroscopy (**Figure 7**) was used to investigate the radical species formed during photooxidation. Oxidation of urate was carried out directly in the EPR cavity in the presence of photosensitizer FMN, which is more water-soluble than riboflavin, and a spin trap (DMPO or MNP). Two spin adducts were detected with MNP (**Figure 7B**), whereas no adducts were trapped with DMPO (not shown). The reaction was dependent on light (**Figure 7A** and **B**) and the presence of oxygen (**Figure 7B** and **F**) as no radicals were trapped in the control kept in the dark or the sample flushed with nitrogen. The EPR spectrum in the presence of MNP could

be simulated with 70% di-*tert*-butyl nitroxyl radical (aN = 16.9 G), which is attributed to decay of the spin trap. The other 30% was simulated with aN = 16.1 G and a second nitrogen coupling, aN = 9.6 G, which is consistent with that of a nitrogen-centered radical (**Figure 7B** and **C**). The latter adduct, which readily disappeared when irradiation was interrupted, was very unstable and produced only low spectral intensity. This species most likely referred to a urate radical, as its formation was not observed in the absence of urate (**Figure 7D**). Instead, two lines appeared with aN = 26.7 G, which infers trapping of an oxygen-centered radical, possibly superoxide (*31*) (**Figure 7D** and **E**). Nitrogen-centered urate radicals were previously detected in enzymatic urate oxidation (*27*), and likewise, it was expected to result from electron transfer to triplet-excited FMN upon irradiation. However, the absolute dependence on oxygen for observation of radicals by EPR argues that the radical we observe is not the urate monoanion radical, but rather radicals formed from decay of urate oxidation products.

These results indicate that urate mediates transfer of radicals to other species, though the exact nature of the radicals could not be elucidated. This is consistent with the results of Kittridge and Willson (*32*), who found that urate substantially enhanced radical-induced inactivation of alcohol dehydrogenase. Nevertheless, the relevance of the urate peroxide in actual milk is less certain as we were not able to detect it herein (**Figure 2**). Sufficiently high concentrations of superoxide and urate radical are needed to produce detectable amounts of the peroxide. Korycka-Dahl and Richardson (*33*) measured up to ~20 μM superoxide anion in milk serum during illumination, and the urate radical is probably relatively stable due to resonance stabilization. However, reducing agents present in milk, such as ascorbate (*27*), will rapidly regenerate urate in a reaction competing with superoxide. Furthermore, the instability of the urate peroxide indicated by the results in **Figure 3** may result in very low peroxide concentrations.

For the first time, riboflavin-mediated formation of protein hydroperoxides has been investigated in milk. The distinction between peroxides formed before or after separation of milk components turned out to be a valuable approach for investigation of the complex reactions occurring in milk during photooxidation. In addition, our approach allowed us to detect urate hydroperoxide during photooxidation of fractionated milk, adding a new dimension to the possible pro-oxidative effects of urate.

ABBREVIATIONS USED

DMPO, 5,5-dimethylpyrroline-*N*-oxide; EPR, electron paramagnetic resonance; FMN, flavin mononucleotide; FOX, ferrous oxidation xylenol orange; HMW, high molecular weight; α-la, α-lactalbumin; β-lg, β-lactoglobulin; LMW, low molecular weight; MNP, 2-methyl-2-nitrosopropane; ^{3}Rib*, triplet excited riboflavin; SEC, size exclusion chromatography; SOD, superoxide dismutase.

LITERATURE CITED

(1) Skibsted, L. H. Light-induced changes in dairy products. *Bull. IDF* **2000**, *346*, 4–9.

(2) Dalsgaard, T. K.; Otzen, D.; Nielsen, J. H.; Larsen, L. B. Changes in structures of milk proteins upon photo-oxidation. *J. Agric. Food Chem.* **2007**, *55*, 10968–10976.

(3) Goosey, J. D.; Zigler, J. S.; Kinoshita, J. H. Cross-linking of lens crystallins in a photodynamic system - a process mediated by singlet oxygen. *Science* **1980**, *208*, 1278–1280.

(4) Cardoso, D. R.; Homem-De-Mello, P.; Olsen, K.; Da Silva, A. B. F.; Franco, D. W.; Skibsted, L. H. Deactivation of triplet-excited riboflavin by purine derivatives: Important role of uric acid in light-induced oxidation of milk sensitized by riboflavin. *J. Agric. Food Chem.* **2005**, *53*, 3679–3684.

(5) Cardoso, D. R.; Franco, D. W.; Olsen, K.; Andersen, M. L.; Skibsted, L. H. Reactivity of bovine whey proteins, peptides, and amino acids toward triplet riboflavin as studied by laser flash photolysis. *J. Agric. Food Chem.* **2004**, *52*, 6602–6606.

(6) Jung, M. Y.; Yoon, S. H.; Lee, H. O.; Min, D. B. Singlet oxygen and ascorbic acid effects on dimethyl disulfide and off-flavor in skim milk exposed to light. *J. Food Sci.* **1998**, *63*, 408–412.

(7) Bradley, D. G.; Kim, H. J.; Min, D. B. Effects, quenching mechanisms, and kinetics of water soluble compounds in riboflavin photosensitized oxidation of milk. *J. Agric. Food Chem.* **2006**, *54*, 6016–6020.

(8) Bradley, D. G.; Lee, H. O.; Min, D. B. Singlet oxygen detection in skim milk by electron spin resonance spectroscopy. *J. Food Sci.* **2003**, *68*, 491–494.

(9) Davies, M. J. Reactive species formed on proteins exposed to singlet oxygen. *Photochem. Photobiol. Sci.* **2004**, *3*, 17–25.

(10) Winterbourn, C. C.; Parsons-Mair, H. N.; Gebicki, S.; Gebicki, J. M.; Davies, M. J. Requirements for superoxide-dependent tyrosine hydroperoxide formation in peptides. *Biochem. J.* **2004**, *381*, 241–248.

(11) Fang, X. W.; Jin, F. M.; Jin, H. F.; von Sonntag, C. Reaction of the superoxide radical with the N-centered radical derived from N-acetyltryptophan methyl ester. *J. Chem. Soc., Perkin Trans. 2* **1998**, 259–263.

(12) Gebicki, J. M. Protein hydroperoxides as new reactive oxygen species. *Redox Rep.* **1997**, *3*, 99–110.

(13) Clausen, M. R.; Skibsted, L. H.; Stagsted, J. Characterization of major radical scavenger species in bovine milk through size exclusion chromatography and functional assays. *J. Agric. Food Chem.* **2009**, *57*, 2912–2919.

(14) Morgan, P. E.; Pattison, D. I.; Hawkins, C. L.; Davies, M. J. Separation, detection, and quantification of hydroperoxides formed at side-chain and backbone sites on amino acids, peptides, and proteins. *Free Radical Biol. Med.* **2008**, *45*, 1279–1289.

(15) Kenney, W. C.; Walker, W. H.; Singer, T. P. Studies on Succinate Dehydrogenase. XX. Amino Acid Sequence Around the Flavin Site. *J. Biol. Chem.* **1972**, *247*, 4510–4513.

(16) Duling, D. R. Simulation of multiple isotropic spin-trap EPR spectra. *J. Magn. Reson., Ser. B* **1994**, *104*, 105–110.

(17) Gieseg, S.; Duggan, S.; Gebicki, J. M. Peroxidation of proteins before lipids in U937 cells exposed to peroxyl radicals. *Biochem. J.* **2000**, *350*, 215–218.

(18) Wright, A.; Hawkins, C. L.; Davies, M. J. Photo-oxidation of cells generates long-lived intracellular protein peroxides. *Free Radical Biol. Med.* **2003**, *34*, 637–647.

(19) Görner, H. Oxygen uptake after electron transfer from amines, amino acids and ascorbic acid to triplet flavins in air-saturated aqueous solution. *J. Photochem. Photobiol. B* **2007**, *87*, 73–80.

(20) Østdal, H.; Andersen, H. J.; Nielsen, J. H. Antioxidative activity of urate in bovine milk. *J. Agric. Food Chem.* **2000**, *48*, 5588–5592.

(21) Michaeli, A.; Feitelson, J. Reactivity of singlet oxygen toward proteins: the effect of structure in basic pancreatic trypsin inhibitor and in ribonuclease A. *Photochem. Photobiol.* **1997**, *65*, 309–315.

(22) Aulakh, J. S.; Stine, C. M. Binding of copper by certain milk proteins as measured by equilibrium dialysis. *J. Dairy Sci.* **1971**, *54*, 1605–1608.

(23) Morgan, P. E.; Dean, R. T.; Davies, M. J. Protective mechanisms against peptide and protein peroxides generated by singlet oxygen. *Free Radical Biol. Med.* **2004**, *36*, 484–496.

(24) Østdal, H.; Bjerrum, M. J.; Pedersen, J. A.; Andersen, H. J. Lactoperoxidase-induced protein oxidation in milk. *J. Agric. Food Chem.* **2000**, *48*, 3939–3944.

(25) Dalsgaard, T. K.; Larsen, L. B. Effect of photo-oxidation of major milk proteins on protein structure and hydrolysis by chymosin. *Int. Dairy J.* **2009**, *19*, 362–371.

(26) Berlett, B. S.; Stadtman, E. R. Protein Oxidation in Aging, Disease, and Oxidative Stress. *J. Biol. Chem.* **1997**, *272*, 20313–20316.

(27) Maples, K. R.; Mason, R. P. Free radical metabolite of uric acid. *J. Biol. Chem.* **1988**, *263*, 1709–1712.

(28) Simic, M. G.; Jovanovic, S. V. Antioxidation mechanisms of uric acid. *J. Am. Chem. Soc.* **1989**, *111*, 5778–5782.

(29) Santus, R.; Patterson, L. K.; Filipe, P.; Morliere, P.; Hug, G. L.; Fernandes, A.; Maziere, J. C. Redox reactions of the urate radical/urate couple with the superoxide radical anion, the tryptophan neutral radical and selected flavonoids in neutral aqueous solutions. *Free Radical Res*. **2001**, *35*, 129–136.

(30) von Sonntag, C. *The chemical basis of radiation biology*; Taylor & Francis: London, 1987.

(31) Forrester, A. R.; Purushotham, V. Reactions of ozonate and superoxide radical anions. *J. Chem. Soc., Faraday Trans. 1* **1987**, *83*, 211–217.

(32) Kittridge, K. J.; Willson, R. L. Uric acid substantially enhances the free radical-induced inactivation of alcohol dehydrogenase. *FEBS Lett*. **1984**, *170*, 162–164.

(33) Korycka-Dahl, M.; Richardson, T. Photogeneration of Superoxide Anion in Serum of Bovine Milk and in Model Systems Containing Riboflavin and Amino Acids. *J. Dairy Sci*. **1978**, *61*, 400–407.

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