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Impact of Whey Protein Emulsifiers on the Oxidative Stability of Salmon Oil-in-Water Emulsions

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To obtain a better understanding of how the interfacial region of emulsion droplets influences lipid oxidation, the oxidative stability of salmon oil-in-water emulsions stabilized by whey protein isolate (WPI), sweet whey (SW), β-lactoglobulin (β-Lg), or α-lactalbumin (α-La) was evaluated. Studies on the influence of pH on lipid oxidation in WPI-stabilized emulsions showed that formation of lipid hydroperoxides and headspace propanal was much lower at pH values below the protein's isoelectric point (p*I*), at which the emulsion droplets were positively charged, compared to that at pH values above the p*I*, at which the emulsion droplets were negatively charged. This effect was likely due to the ability of positively charged emulsion droplets to repel cationic iron. In a comparison of lipid oxidation rates of WPI-, SW-, β-Lg-, and α-La-stabilized emulsions at pH 3, the oxidative stability was in the order of β-Lg $\geq$ SW $>$ α-La $\geq$ WPI. The result indicated that it was possible to engineer emulsions with greater oxidative stability by using proteins as emulsifier, thereby reducing or eliminating the need for exogenous food antioxidants.

KEYWORDS: Whey proteins; emulsions; fish oils; omega-3 fatty acids; lipid oxidation; antioxidants

INTRODUCTION

The susceptibility of lipids to oxidation is a major cause of quality deterioration in food emulsions (*1*). Such food quality deterioration includes undesirable changes of flavor, texture, shelf life, appearance, and nutritional profile. Recently, a great deal of research has been focused on oxidation in oil-in-water emulsions rather than bulk lipids due to the former being more commonly encountered in actual food products (*1*−*3*). In many of the studies of oxidation in oil-in-water emulsions, model emulsions have been prepared with synthetic surfactants [e.g., sodium dodecyl sulfate (SDS), dodecyltrimethylammonium bromide (DTAB), Brij, and Tween], even though not all of these surfactants (e.g., SDS, DTAB, and Brij) are approved for food use. Proteins represent generally regarded as safe (GRAS) food additives that can form physically stable emulsions while altering the properties of the emulsion droplet interface in a manner that increases oxidative stability.

The emulsifying properties of whey proteins have been studied for years. The polymerizations of whey proteins in whey protein-stabilized emulsions (*4*, *5*), the interactions between whey proteins and commonly used surfactants at the emulsion droplet surface (*6*−*8*), and the influence of pH, $CaCl_2$, sucrose, heat treatment, and protein concentration on the stability of whey protein-stabilized emulsions (*9*−*13*) have been reported. Whey proteins have been found to inhibit lipid oxidation in oil-in-water emulsions when they are either at the emulsion droplet surface or in the aqueous phase. The antioxidant mechanisms of whey proteins have been attributed to their ability to (1) form cationic charges on the surface of emulsion droplets, which repel transition metals; (2) form thick viscoelastic films at emulsion droplet interfaces, which physically minimize lipid hydroperoxide−transition metal interactions; (3) chelate prooxidative metals; and (4) inactivate free radicals through their sulfhydryl groups and other amino acids (*14*−*20*). A better understanding of how whey protein can influence oxidative reactions could help in the development of new antioxidant strategies for oil-in-water emulsions.

The objective of this study was to utilize salmon oil-in-water emulsions stabilized by whey protein isolate (WPI), sweet whey (SW), β-lactoglobulin (β-Lg), or α-lactalbumin (α-La) in an attempt to better understand how absorbed proteins influence lipid oxidation chemistry. The effect of pH and temperature on the oxidative stability of whey protein-stabilized emulsions was also evaluated.

MATERIALS AND METHODS

Materials. Salmon fillets were purchased from a local grocery. WPI, β-Lg, and α-La were obtained from Davisco Food International, Inc. (Eden Praire, MN), and SW was obtained from Kraft Foods (Tarrytown, NY). Proteins were used without further purification. The protein contents of WPI and SW were 97.6 and 12.1 wt %, respectively. The major protein components of WPI were 55−61% β-Lg, 19−22% α-La, and 6−8% bovine serum albumin. The α-lactalbumin content of α-La powder was 90.6 wt % and the β-lactoglobulin content of β-Lg powder 95.0 wt %. All protein concentrations and composition data cited were obtained from the respective manufacturers. Imidazole, sodium acetate, *N*-ethylmaleimide (NEM), ferrous sulfate, and cumene hydroperoxide

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were purchased form Sigma Chemical Co. (St. Louis, MO). All other reagents were of analytical grade or purer.

Methods. *Preparation of Salmon Oil.* Fresh salmon fillets were hand chopped, minced, and centrifuged at 15000*g* for 20 min at 5 °C. The resulting oil was decanted and stored at −80 °C until use. The salmon oil contained ≥99% triacylglycerols as determined by thin-layer chromotography (*21*).

Preparation and Characterization of Emulsions. An oil-in-water emulsion was prepared using 5.0 wt % salmon oil, 0.2% protein (WPI, SW, α-La, or β-Lg), and 94.8% 5 mM acetate−imidizole buffer (pH 3.0). In experiments where the role of sulfhydryls in WPI was investigated, WPI was reacted with NEM (3.45 mmol of NEM/g of protein) for 15 min at 25 °C. Excess NEM was removed by dialysis of the WPI solution against the 5 mM acetate−imidizole buffer (pH 3.0; WPI solution to buffer 1:100) with 6000−8000 molecular weight cutoff dialysis tubing a total of two times after 6 and 12 h with constant stirring at 4 °C. Oil-in-water emulsions were made by blending the lipid and aqueous phases for 2 min using a hand-held homogenizer (M133/1281-0, Biospec Products, Inc., Bartlesville, OK). The coarse emulsion was then homogenized four times at 5000 psi through a high-pressure valve, a two-stage APV Lab 1000 homogenizer (Albertslund, Denmark). The pH (3−7) of the emulsion was adjusted with 0.1 M HCl and/or NaOH after homogenization. The particle size distribution of the emulsions was measured using a Coulter LS 230 laser light scattering instrument (Coulter Corp., Miami, FL). The final emulsion mean particle diameters ranged from 0.73 to 0.80 μm. Droplet size distributions were checked periodically to monitor emulsion stability. Emulsion droplet charge (zeta potential, ζ) was measured by directly injecting diluted (1:1000) oil-in-water emulsions into the measurement chamber of a ZEM5003 Zetamaster (Malvern Instruments, Worchester, U.K.). The ζ-potential measurements are reported as the average of two separate injections, with five readings made per injection.

Measurements of Lipid Oxidation. Emulsions (5 mL) were placed in lightly sealed screw-cap test tubes and allowed to oxidize at different temperatures (4.0, 20.0, and 37.0 °C). Lipid hydroperoxides were measured (*22*) by mixing 0.3 mL of emulsion with 1.5 mL of isooctane/2-propanol (3:1, v/v) by vortexing (10 s, three times) and isolation of the organic solvent phase by centrifugation at 1000*g* for 2 min. The organic solvent phase (200 μL) was added to 2.8 mL of methanol/1-butanol (2:1, v/v), followed by 15 μL of 3.94 M ammonium thiocyanate and 15 μL of ferrous iron solution (prepared by mixing 0.132 M $BaCl_2$ and 0.144 M $FeSO_4$). The absorbance of the solution was measured at 510 nm, 20 min after the addition of the iron. Hydroperoxide concentrations were determined using a standard curve made form cumene hydroperoxide (Sigma Chemical Co.).

For headspace analysis, emulsion samples (1 mL) were placed into 10 mL headspace vials and sealed with poly(tetrafluoroethylene)/butyl rubber septa. Headspace propanal was determined using a Shimadzu 17A gas chromatograph equipped with a Hewlett-Packard 19395A headspace sampler (*23*). The headspace conditions were as follows: sample temperature, 40 °C; sample loop and transfer line temperature, 110 °C; pressurization, 10 s; venting, 10 s; injection, 1.0 min. The aldehydes were separated isothermally at 70 °C on an HP methyl silicone (DB-1) fused silica capillary column (50 m, 0.31 mm i.d., 1.03 μm film thickness). The splitless injector temperature was 180 °C, and the eluted compounds were detected with a flame ionization detector at 200 °C. Concentrations were determined from peak areas using a standard curve made from authentic propanal.

Statistical Analysis. Assays were measured in triplicate. Statistical analysis was preformed using the Student *t* test (*24*).

RESULTS AND DISCUSSION

Previous experiments (*18*) showed that 0.2 wt % WPI was suitable for stabilizing 5 wt % oil-in-water emulsions that had monomodal droplet size distributions and minimal detectable aqueous phase protein. Therefore, these protein and lipid concentrations were used for the following experiments.

WPI solubilized in water has a p*I* of 5.1 (*25*). When WPI is absorbed onto the surface of a lipid droplet during the formation of an oil-in-water emulsion, it is possible that the p*I* would change as the protein conformation changes and the amino acids are exposed to new environments. To determine if the p*I* of the WPI had been altered by its absorption onto lipid droplets in an oil-in-water emulsion, the electrical charge of the droplets (ζ-potential) was measured as a function of pH. The p*I* of the WPI on the emulsion droplets was found to be ∼4.8 (**Figure 1**). This means that at pH >4.8 the emulsion droplets stabilized with WPI are negatively charged, whereas at pH <4.8 the droplets are positively charged.

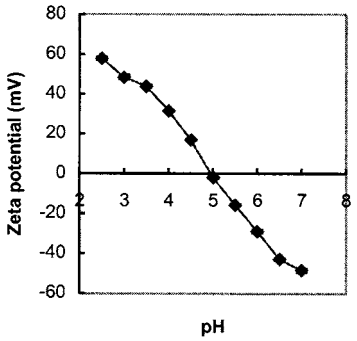

Figure 1. Influence of pH on the ζ-potential of 5% salmon oil-in-water emulsions stabilized by 0.2% whey protein isolate. Data points represent means ($n = 5$) ± standard deviations. Some error bars are within the data points.

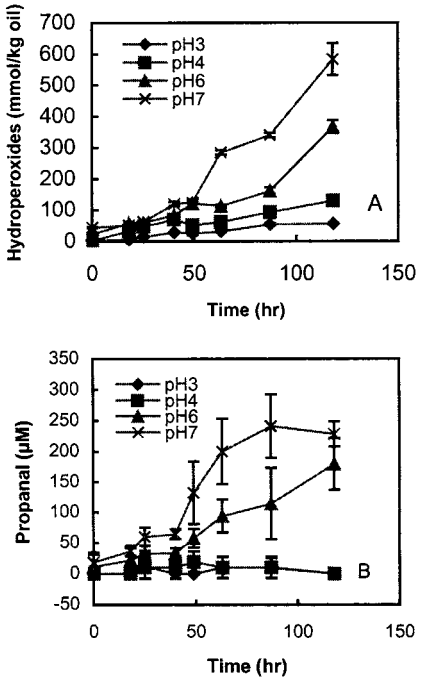

Figure 2. Influence of pH on the formation of lipid hydroperoxides (A) and headspace propanal (B) in 5% salmon oil-in-water emulsions stabilized by 0.2% whey protein isolate at 20 °C. Data points represent means ($n = 3$) ± standard deviations. Some error bars are within the data points.

Effect of pH on Lipid Oxidation Rates in WPI-Stabilized Oil-in-Water Emulsions. Figure 2 shows that the formation of lipid hydroperoxides and propanal in WPI-stabilized salmon oil-in-water emulsions increased with increasing pH. Measurements were not made at pH 5.0 because the emulsions were highly unstable to droplet flocculation because the low net charge on the droplet meant that electrostatic repulsion was not sufficient to overcome attractive interactions (*8*, *9*). Lipid oxidation rates were significantly lower at pH values below the p*I* of the WPI. At pH 3.0 and 4.0, lipid hydroperoxide

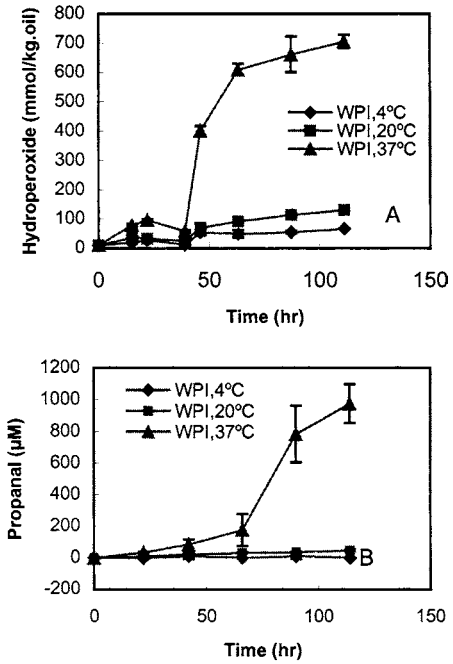

Figure 3. Influence of temperature on the formation of lipid hydroperoxides (A) and headspace propanal (B) in 5% salmon oil-in-water emulsions stabilized by 0.2% whey protein isolate. Data points represent means ($n = 3$) ± standard deviations. Some error bars are within the data points.

concentrations increased from 2 to 56 and 93 mmol/kg of oil, respectively, after 87 h of incubation. This compares to pH 6.0 and 7.0 when initial hydroperoxides concentrations were high (22 and 44 mmol/kg of oil, respectively), and hydroperoxide concentrations after 87 h were 161 and 340 mmol/kg of oil, respectively (**Figure 2A**). Propanal formation was also lower at pH values below the p*I* of WPI with concentrations remaining below 10 μM during the entire incubation period for emulsions at both pH 3.0 and 4.0. This compares to emulsions at high pH values where propanal concentrations were 114 and 240 μM at pH 6.0 and 7.0 after 87 h of incubation. The low oxidation rates at pH values below the p*I* of the WPI-stabilized emulsion droplets are likely due to the ability of positively charged emulsion droplets to repel prooxidant iron and thus inhibit oxidation, whereas negatively charged emulsion droplets attracted iron and accelerated oxidation. Acceleration of lipid oxidation by anionic emulsion droplets and inhibition by cationic emulsion droplets is in agreement with the findings of Mei et al. (*21*), who reported that negatively charged emulsion droplets stabilized by SDS were able to bind iron and thus accelerate oxidation, whereas positively charged emulsion droplets stabilized by dodecyltrimethylammonium bromide (DTAB) did not bind iron and were more oxidatively stable. As the difference between the pH and the p*I* of the WPI-stabilized emulsion droplet was increased, the inhibition (pH 3.0 compared to pH 4.0) or the acceleration (for pH 7.0 compared to pH 6.0) of lipid oxidation was increased.

Storage temperature had a significant impact on the oxidative stability of salmon oil-in-water emulsions stabilized with WPI or sweet whey (SW); with increasing storage temperature (4−37 °C) was found increasing lipid oxidation at pH 3.0 (**Figures 3** and **4**). The effect of temperature on oxidative stability became quite obvious at 37 °C with lipid hydroperoxide concentrations being 5- and 6-fold greater at 37 °C than at 20 °C for WPI and SW, respectively, after 5 days of storage (**Figures 3A** and **4A**). Propanal concentrations were approximately 20- and 9-fold

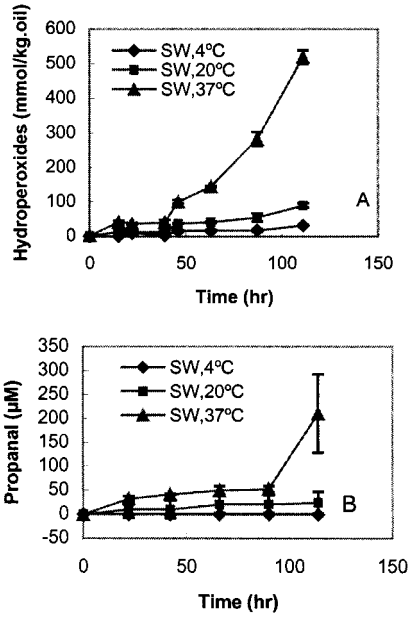

Figure 4. Influence of temperature on the formation of lipid hydroperoxides (A) and headspace propanal (B) in 5% salmon oil-in-water emulsions stabilized by sweet whey powder at 0.2% protein. Data points represent means ($n = 3$) ± standard deviations. Some error bars are within the data points.

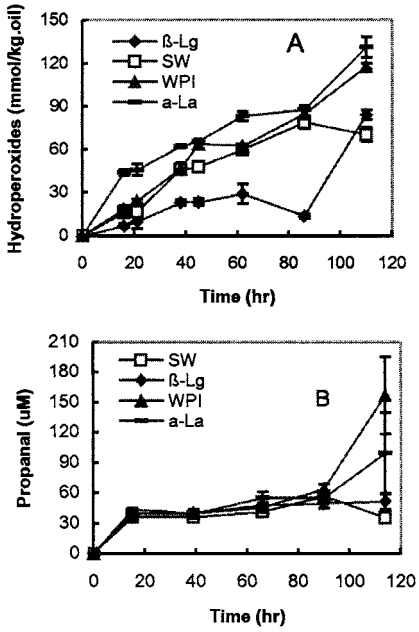

Figure 5. Formation of lipid hydroperoxides (A) and headspace propanal (B) in 5% salmon oil-in-water emulsions stabilized by 0.2% protein from whey protein isolate (WPI), sweet whey (SW), β-lactoglobulin (β-Lg), or α-lactalbumin (a-La) at 37 °C. Data points represent means ($n = 3$) ± standard deviations. Some error bars are within the data points.

greater at 37.0 °C than at 20 °C for WPI and SW, respectively, after 5 days of storage (**Figures 3B** and **4B**).

Comparison of Differences in Oxidative Stability of Emulsions Stabilized by Different Sources of Whey Proteins. **Figure 5** shows the formation of lipid hydroperoxides and headspace propanal in salmon oil-in-water emulsions stabilized with WPI, SW, β-Lg, and α-La. All emulsions were prepared with 0.2% protein and 5.0% salmon oil. Oxidation rates increased with increasing pH in emulsions stabilized with all four proteins with strong inhibition of lipid oxidation occurring

Table 1. ζ-Potentials of Salmon Oil-in-Water Emulsions Stabilized by Whey Protein Isolate, Sweet Whey, α-Lactalbumin, and β-Lactoglobulin at pH 3 and 20.0 °C

	WPI	SW	α-La	β-Lg
zeta potential (mV)	51.2 ± 1.2	33.3 ± 0.6	57.8 ± 1.1	62.4 ± 0.2

at pH values (3.0 and 4.0) below the p*I* values of the proteins (data not shown). Because production of cationic protein-stabilized emulsion droplets is a potential strategy for the production of oxidatively stable emulsions, comparisons between different whey protein-stabilized emulsions were determined at pH 3.0. These emulsions were incubated at 37 °C to accelerate oxidation rates, thus making it easier to determine differences in the oxidative stability of the samples at pH 3.0. After 45 h of incubation, hydroperoxide concentrations (**Figure 5A**) were statistically different between the treatments ($p < 0.05$) and were in the order of β-Lg (23.4 ± 2.4 mmol/kg of oil) < SW (47.8 ± 1.6 mmol/kg of oil) < α-La (65.5 ± 1.2 mmol/kg of oil) < WPI (63.9 ± 3.1 mmol/kg of oil). After 39 h of incubation, there were no statistical differences in headspace propanal concentrations (**Figure 5B**) between the samples. However, after 114 h of incubation, headspace propanal concentrations were different between samples with SW (35.7 ± 0.5 μM)^a < β-Lg (51.7 ± 7.6 μM)^a < α-La (99.4 ± 40.2 μM)^{ab} < WPI (156.9 ± 35.7 μM)^b (numbers with different superscripts are significantly different, $p < 0.05$). SW would contain numerous components not found in the purified WPI such as minerals, citrate, phosphate, lactose, and low molecular weight peptides. The greater oxidative stability of the SW-stabilized emulsion could be due to the ability of these components to alter oxidation. For example, citrate, phosphate, and casein phosphopeptides are known to chelate iron and potentially inhibit lipid oxidation. However, it should be noted that the emulsion made with SW had lower physical stability as evidenced by the formation of a clear serum layer after several days of storage.

The ability of the whey proteins to alter oxidation rates in protein-stabilized emulsions was not solely related to their ability to produce cationic emulsion droplets because the positive charge of the emulsions droplets was in the order of β-Lg > α-La > WPI > SW (**Table 1**), whereas inhibition of lipid oxidation was in the order of β-Lg ≥ SW > α-La ≥ WPI. This suggests that other factors are also influencing the ability of the absorbed proteins to inhibit lipid oxidation. Free sulfhydryl groups have been associated with the antioxidant activity of whey proteins due to their ability to scavenge free radicals (*15*, *19*). This could explain why emulsions stabilized with β-Lg (which has one free sulfhydryl) were more oxidatively stable than emulsions stabilized with α-La (no free sulfhydryls).

To evaluate whether free sulfhydryls in the whey proteins could be involved in the inhibition of lipid oxidation in the emulsions, WPI was incubated with NEM to block the sulfhydryls. Corn oil-in-water emulsions were then prepared with WPI, and NEM-treated WPI and oxidation rates were compared (**Figure 6**). As can be seen, the formation of lipid hydroperoxides was not significantly different for the NEM-treated WPI compared to untreated WPI (**Figure 6A**). Formation of headspace hexanal was lower in the presence of NEM (**Figure 6B**). If the WPI sulfhydryls were inhibiting oxidation, the NEM-treated samples would have been expected to oxidized more quickly because NEM blocks the antioxidant activity of cysteine. Therefore, these data suggest that the free sulfhydryls did not play a major role in oxidation rates. Whey proteins can also scavenge free radicals through non-sulfhydryl pathways (e.g., tyrosine), a mechanism also influenced by the thermal denaturation of the whey proteins (*17*, *19*). Therefore, differences in the oxidation rates of the emulsions stabilized by the different whey protein sources could be due to differences in amino acid compositions or differences in exposure of amino acids if the protein sources were heat processed to different degrees. Alternately, the different whey protein sources could produce emulsions with interfacial layers of varying thickness. Previous research has shown that emulsions made with surfactants that produce thick interfacial regions will exhibit slower oxidation rates (*26*). Therefore, if the different whey protein sources produced emulsions with varying interfacial thickness, this may explain the differences in oxidation rates.

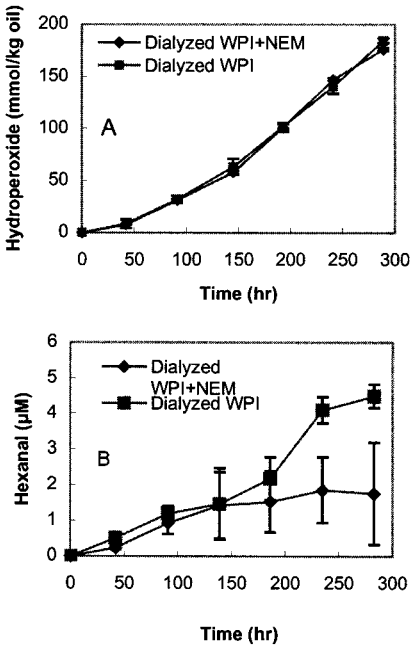

Figure 6. Influence of *N*-ethylmaleimide (NEM) on the formation of lipid hydroperoxides (A) and headspace propanal (B) in 5% salmon oil-in-water emulsions stabilized by 0.2% whey protein isolate (WPI). WPI was reacted with NEM and dialyzed prior to formation of the emulsion. Data points represent means ($n = 3$) ± standard deviations. Some error bars are within the data points.

Conclusions. The oxidative stability of salmon oil-in-water emulsions stabilized by whey proteins was greatest at pH values below the p*I* of the proteins. Presumably, these emulsions decrease lipid oxidation rates through the electrical repulsion of metal ions away from the emulsion droplet, which reduces contact between prooxidants (e.g., iron) and the oxidation substrate (e.g., unsaturated fatty acids and lipid hydroperoxides). However, the emulsion droplet charge was not the only factor influencing the oxidative stability of the lipids because the surface charge was in the order of β-Lg > α-La > WPI > SW, whereas oxidative stability was in the order of β-Lg ≥ SW > α-La ≥ WPI. Other factors that could be responsible for differences between the oxidative stability of emulsions stabilized by the different whey proteins include differences in amino acids that can scavenge free radicals or differences in how the proteins impact the thickness or packing of the emulsion droplet interface with thicker interfacial regions potentially inhibiting lipid oxidation. These results indicate that it was possible to engineer emulsions with improved oxidative stability by producing protein-stabilized emulsions under acidic conditions. This technology could dramatically increase the utilization of oils high in $\omega-3$ fatty acids in foods.

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