Two Types of Radicals in Whole Milk Powder. Effect of Lactose Crystallization, Lipid Oxidation, and Browning Reactions

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Whole milk powder was stored in closed vials at 60 °C to induce crystallization of lactose within a short time scale. After an induction period of 3−4 days simultaneous crystallization of lactose, increase of water activity, formation of browning products, and increase of radical content took place. Radicals detected before lactose crystallization were characterized by a narrow ESR spectrum ($g = 2.006$) and could be depleted by removal of oxygen and therefore were assigned to oxidation processes. Late-stage radicals present after crystallization of lactose gave much wider spectra ($g = 2.0048$) and were independent of oxygen availability and assigned to late-stage Maillard reaction products. The study indicates that the processes of lactose crystallization, browning, and formation of radical species ($g = 2.0048$) are strongly coupled, while lipid oxidation is less dependent on the other processes.

Whole milk powder is produced industrially by spray-drying preconcentrated, heat-treated milk. During this process, water (approximately 87%) is removed, and after drying lactose (approximately 38%) becomes the most abundant component in the dry product together with whey proteins (approximately 4%), caseins (approximately 20%), and milk fat (approximately 26%). As a dry product, the major concern regarding product stability of whole milk powder during storage is chemical and physical processes involving the main components.

On the microscopic level, whole milk powder consists of a continuous mass of amorphous lactose and other low-molecular components in which fat globules and proteins are embedded (*1*). The water activity of milk powder is about 0.2 and thus well below the critical value of 0.4, which is the threshold water activity dividing glassy and nonglassy lactose as dominant form at ambient temperature (*2*). The thermodynamic stable form of lactose in systems with little water is crystalline α-lactose-monohydrate or anhydrous β-lactose depending on temperature and the actual water activity (*3*). The main component in milk powder, lactose, therefore exists in freshly made products in a metastable state as a glass but will eventually be transformed into the stable crystals. As lactose plays the role of solvent diluting potential chemical reactants as well as other components, lactose crystallization is a dramatic event which can be accompanied by an increase in water activity and by initiation of deteriorative chemical reactions. The crystallization rates of amorphous material are highly dependent on temperature (*4, 5*) and on the time scales relevant for handling and storage of

milk powder; crystallization will usually not take place below the glass-transition temperature, T_g (*5*).

Maillard reactions are an important class of deteriorative reactions in milk products (*6*). This type of chemical reaction is initiated by condensation of lactose with the free amino group of lysine in milk proteins. The initial steps of the Maillard reactions are accompanied by loss of the essential amino acid lysine and production of water as a reaction product, whereas the late stages result in formation of a yellow to brown color and flavors usually not acceptable for milk products. On the molecular level, browning is due to production of high molecular weight melanoidins and smaller cyclic compounds (*7*). The volatile compounds formed during the nonenzymatic browning can be divided into three groups: (i) "simple" sugar dehydration/fragmentation products, (ii) "simple" amino acid degradation products, and (iii) volatiles produced by further interactions of these primary degradation products. Many of these volatile compounds are described as having a sweet, coffee-like, tobacco-like, caramel-like, or burnt odor and taste (*8*). The rate of Maillard reactions are strongly dependent on temperature corresponding to activation energies in the range of 70−180 kJ/mol (*9*), and it has been speculated that the high-temperature dependence under some conditions could be attributed to partial immobilization of reactants because of glass formation (*5, 10, 11*).

Beside lactose crystallization and Maillard reactions, lipid oxidation is a third class of deteriorative processes important in whole milk powder. Lipid oxidation involves peroxidation of unsaturated fatty acids, formation of alkoxyl radical by cleavage of hydroperoxides, followed by formation of volatile secondary oxidation products by cleavage of the alkoxyl radicals. Volatile secondary oxidation products are normally considered to be responsible for cardboard-like off-flavor of oxidized whole

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milk powder (*12*). Notably, a correlation between radical content of milk powder and sensory scores for off-flavors and formation of TBA reactive substances, as a measure of secondary lipid oxidation products, has been established (*13*). Such correlations as seen for milk powder and other dried food products are consistent with a free-radical mechanism for lipid oxidation and with radicals being precursors of secondary oxidation products (*14*) and have made ESR spectroscopy a valuable method for detection of early events in lipid oxidation in dry foods (*15*).

Although lipid oxidation and Maillard browning take place in different phases in whole milk powder, both processes might be influenced by the dramatic process of lactose crystallization. As amorphous glassy lactose is a reactant as well as host of the other reactants for Maillard reactions and furthermore serves as encapsulation of the dispersed lipid phase, crystallization of lactose is possibly strongly changing the disposition for both reactions. In the present study, the radical formation in whole milk powder was studied in relation to lactose crystallization, lipid oxidation, and browning reactions to contribute to an understanding of the possible coupling between physical and chemical processes of importance for quality deterioration of whole milk powder.

MATERIALS AND METHODS

Whole Milk Powder. Spray-dried, high-heat whole milk powder Milex was obtained from Arla Foods Ingredients (Arinco, Videbæk, Denmark). The whole milk powder contained 33−42% lactose, 26−29% milk protein, and 26% milk fat according to product specifications and was produced as part of a full-scale standard production. The powder used for this experiment was produced approximately 2 months before the start of the experiments and was stored at 5 °C in a nitrogen atmosphere packed in tins until use.

Sample Preparation. Experiments were performed at mildly accelerated conditions by repackaging approximately 10 g of whole milk powder into 20-mL clear glass headspace vials (Mikrolab, Aarhus, Denmark) sealing with aluminum press caps and incubating the samples at 60 ± 2 °C for up to 7 days. Storage in an oxygen-free atmosphere was performed by applying an oxygen absorber, Ageless, and an oxygen indicator, Ageless-eye (Mitsubishi Gas Chemical, Tokyo, Japan) in the headspace of the vial. Storage in darkness was performed by wrapping the vials in aluminum foil to prevent light from penetrating to the milk powder. Measurement of water activity, water content, surface color, and radical concentration was performed daily throughout the storage period. Two experiments were carried out: In the first experiment, samples were stored without oxygen absorber for 6 days at 60 °C, and the second experiment was carried out using any of the four combinations of light/dark, presence/absence of oxygen absorber.

Water Activity. The water activity of the milk powder was measured at room temperature using an Aqua lab CX-2 (Aqua lab, Pullman, WA) after an appropriate short cooling period. The water activity was determined as the mean of four measurements.

Water Content. The water content of the whole milk powder was determined by incubating samples of 0.500 g whole milk powder in 5.0-mL water-free methanol (max. 0.005% H_2O) for 24 h and subsequently determining the water content of the powder−methanol slurry by Karl Fischer titration (Mettler Toledo DL 18, Schwerzenbach, Switzerland). The water content was determined as the mean of three measurements.

Color Measurements. The surface color was measured with the Hunter L, a, and b color system according to Nielsen et al., (*16*) with a Byk-Gardner Color-guide (Byk-Gardner, Geretsried, Germany). To avoid stray light, the powder was measured in a gray container as follows: Approximately 5 g of powder was spread out in the container and measured three times. The Hunter values were determined as the mean of three measurements.

ESR Spectroscopy Measurements. The radical concentration was measured by electron spin resonance (ESR) spectroscopy. The milk powder was measured according to the method of Stapelfeldt et al.

(*14*) with few modifications. The radical concentration was either measured using an ECS 106 spectrometer (Bruker, Karlsruhe, Germany) or with a JES FR30 Free Radical Monitor (JEOL, Tokyo, Japan). For the Bruker instrument, the parameters were as follows: Sweep width 10 mT, microwave power 2 mW, modulation amplitude 0.79 mT, modulation frequency 100 kHz, conversion time 81.92 s, time constant 20.48, and sweep time 83.89 s. The samples were measured with a weak pitch standard as instrument control and to standardize the sample signal between days. For the JEOL instrument, the parameters were sweep time 2 min, sweep width 5 mT, microwave power 4 mW, modulation width 0.1 mT, and time constant 0.3 s. A built-in manganese (Mn(II)) marker was used as instrument control and to standardize the sample signal between days. The results were expressed as the area under the ESR signal from the powder relative to the area under the signal from the marker and divided by the density of the powder in the tube. These relative radical concentrations were in each case determined as a mean of four measurements.

The *g*-values, which express the ratio between the frequency and magnetic field at resonance, were determined relative to the built-in Mn(II) marker.

RESULTS

The experimental work presented in this paper consists of two parts. The first part was designed to study the interrelation between the development of radicals, water activity, and formation of brown color because of Maillard reactions during lactose crystallization in whole milk powder. For these experiments, the relative radical concentration was measured using the Bruker ESR spectrometer and no action was taken to lower oxygen pressure or to avoid exposure to light. For both experiments, whole milk powder was stored in closed vials at 60 °C to induce crystallization of lactose within a relatively short time scale.

The water content of the whole milk powder, as determined by Karl Fischer titration, remained at approximately 3% during the full period of 6 days. As the powder was stored in sealed containers, this result indicates that within experimental accuracy water neither was consumed nor produced by chemical reactions such as Maillard reactions during the storage period.

Results from measurement of water activity, relative radical concentration, and surface color from the experiment with whole milk powder stored without oxygen absorber for 6 days at 60 °C are shown in **Figure 1**. There is a clear correlation between the results of the three different analyses as all three quantities change dramatically within the same narrow time interval.

The water activity remained constant for the first 3 days (**Figure 1B**), but from day three to five a large increase corresponding to nearly a doubling of the water activity was observed from about 0.22 to 0.4. From day five to six, the water activity was slightly decreasing. As the water content was approximately constant during the storage period, the significant increase of water activity cannot be due to a corresponding water production but must rather be a thermodynamic manifestation of changed interactions between water and the whole milk powder solids. Amorphous materials generally hold more water as compared to the same material on crystalline form. As a result, milk powder stored under conditions of constant water activity loose water upon crystallization of lactose (*2*). In the present study, the system under investigation can be considered as closed and the change of the underlying moisture sorption isotherm following crystallization will in this case result in increase of water activity rather than decrease in water content. The crystallization provoked by the relatively high temperature well above the glass-transition temperature ($T_g \sim$ 40 °C for whole milk powder (*4*)) promotes mobility for molecular reorganization into the crystalline state (*5*). The observation of crystallization in this temperature range and the duration of the induction period
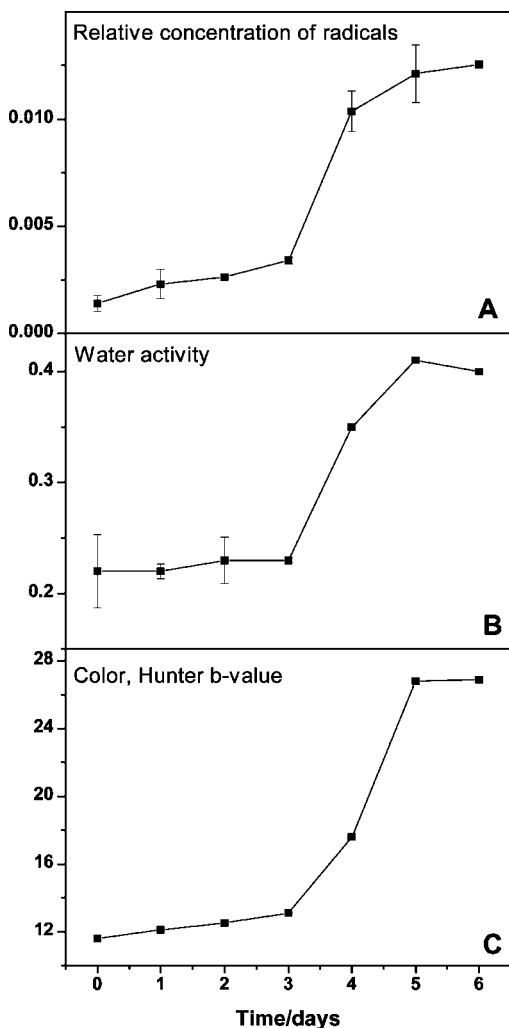
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Figure 1. Concomitant development of radicals, increase in water activity, and browning in whole milk powder stored aerobically at 60 °C.

Figure 2. Initial stages in development of radicals in whole milk powder stored at 60 °C aerobically or anaerobically, exposed to light or protected against light.

is comparable with recent isothermal calorimetric studies on whole milk powder (*4*).

The concentration of radicals was gradually increasing during the first 3 days of storage (**Figure 1A**). After 3−4 days, a steep rise in the concentration of radicals was observed, whereupon the increase was more gradual and seemed to level off after 5−6 days of storage (**Figure 1A**). The surface color of the milk powder, expressed by the Hunter *b* value (yellowness), showed a similar pattern (**Figure 1C**) as during the first 3 days a slow increase was seen. From day three to five, the yellowness increased more significantly as seen from the more than doubling of the Hunter *b* value. This was visually confirmed by a change from white to a brown color of the milk powder. From day five to six, the Hunter *b* value remained constant.

The results presented in **Figure 1** indicate a coupling between lactose crystallization, Maillard reactions, and radical generation in whole milk powder. As lactose crystallization has been reported to initiate lipid oxidation in model systems (*17*) and the radical concentration has been shown to correlate with lipid oxidation in milk powder (*13*), further studies were initiated to provide more insight into the nature of the observed radicals and into the question of an interaction of these radicals with lipid oxidation and Maillard reactions, as evidenced by the present results.

In the second experiment, the effect of light and oxygen was studied using storage at any of the four combinations of
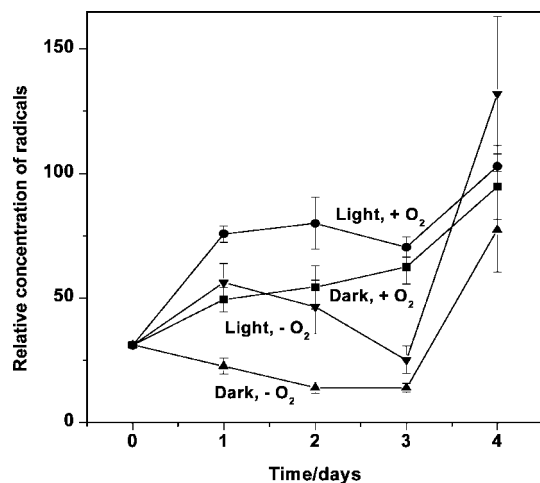
light/dark and presence/absence of oxygen absorber. The ESR spectra were obtained with the JEOL Spectrometer, which because of its internal manganese standards is especially suited for determination of the *g*-values of radicals. Briefly, the *g*-value, which expresses the ratio between the frequency and magnetic field at resonance, is a specific characteristic related to each radical (*18*).

In **Figure 2**, the initial development in radical concentration prior to crystallization is shown for the first 4 days of storage at 60 °C.

For the sample without oxygen and stored in darkness, a gradual decrease in the amount of radicals was observed during the 3 days. After 2 days, it was practically impossible to detect any ESR signals as radical formation apparently had stopped and the radical present initially may have recombined to form nonradical products. However, after 3−4 days, a rather steep rise was observed as an indication of a reinitiation of radical formation. A rather similar pattern was observed for the sample also stored without oxygen but exposed to light. However, for these samples, a rise in radical formation was observed initially during the first day, whereupon a gradual decrease was seen between day one and three. These observations are in contrast to behavior seen for the samples stored in ambient atmosphere, where a gradual rise during all 4 days prior to crystallization was observed and most pronounced in the samples exposed to light.

In **Figure 3**, the results of measurement of water activity, relative radical concentration, the *g*-value, and color from the same experiment are shown on the slightly longer time scale of 7 days, which include the event of lactose crystallization. After the first initial developments in the radical concentration as shown in **Figure 2**, the powder stored in oxygen-free atmosphere underwent crystallization of lactose. As observed in the first experiment, this process was accompanied by major changes in the ESR spectra of the powder, water activity, and color. Thus, a large increase in radical concentration was observed between 4 and 6 days of storage for the samples stored anaerobically. After 6 days of storage, a small decrease in the radical concentration was further observed. The dramatic change in radical concentration for samples stored anaerobically was seen to be independent of exposure to light as both types of samples showed the described behavior.

The development in *g*-values of the radicals detected in the milk powder stored anaerobically is shown in **Figure 3B**. During
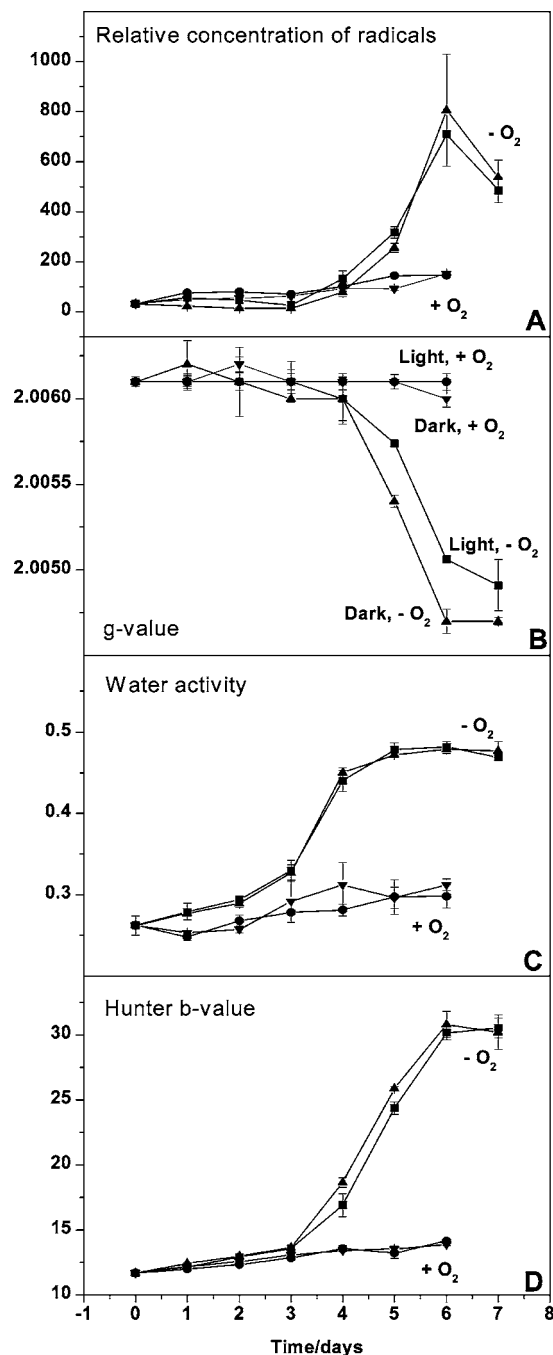
Figure 3. Development in radical concentration, changes in *g*-value for radicals as detected by ESR spectroscopy, increase in water activity, and browning in whole milk powder stored at 60 °C aerobically or anaerobically, exposed to light or protected against light: ▲, dark in absence of O_2; ■, exposed to light in absence of O_2; ▼, dark in the presence of O_2; ●, exposed to light in the presence of O_2. Initial stages in development of radicals (A) see **Figure 2**.

Figure 4. ESR spectra of whole milk powder stored at 60 °C protected against light. Narrow spectrum with *g*-value 2.0060 recorded after 6 days of storage in the presence of O_2 at which time the powder appeared white. Broad spectrum with *g*-value 2.0048 recorded after 6 days of storage in the absence of O_2 at which time the powder had turned brown.

zero to day three. However, the water activity changed by a steep rise from day three to four, after which it remained at an almost constant value of 0.47 for the rest of the storage period. The water content was constant at approximately 4% throughout the storage period as also seen in the first experiment.

In **Figure 3D**, the results from the color measurements expressed as the Hunter *b*-value are shown. For the first 3 days of the experiment, all four samples were similar and only a very slow increase in the *b*-value could be observed during this period, but between day three to six the *b*-value was more than doubled for the samples stored in absence of oxygen. After 6 days of storage, the *b*-value remained constant for these samples. The change in the *b*-value corresponded to a gradual visual change of the powder from white (day zero to three) through light brown (day four to five) into almost cocoa brown (day six to seven).

The samples stored aerobically (without oxygen absorber) showed a somewhat different behavior as crystallization apparently did not take place within the time scale of 7 days. Thus, for these samples, no dramatic change in water activity, radical concentration, *g*-values, or color was observed. Instead, a much smaller and gradual increase in radical content, water activity, and Hunter *b*-values (**Figure 3A**, **C**, and **D**, respectively) and a constant *g*-value of about 2.006 (**Figure 3B**) were observed. Also for these samples, exposure to light did not make any difference during the late stages of the experiments.

Figure 4 shows examples of ESR spectra of whole milk powder stored for 6 days protected against light, with or without oxygen present. In the sample stored aerobically, no crystallization of lactose occurred within 6 days of storage and the powder was white and showed a relatively narrow ESR spectrum with a high *g*-value of about 2.0060. This spectrum only extends over about 20 G. This "white" spectrum is qualitatively very different from the "brown" spectrum appearing in the sample stored anaerobically where crystallization and extended browning have taken place within 6 days. The brown spectrum is shifted to higher magnetic fields and has correspondingly a lower *g*-value of about 2.0048. This spectrum extends over about 60 G. On the basis of the significant differences in *g*-value and spectral width, it is concluded that the two types of spectra originate from at least two distinctly different chemical species. Considering the complexity of whole milk powder being a multicomponent system distributed into at least two phases, it

the first 4 days, the *g*-value remained constant ($\sim$2.0060) for all samples regardless of storage conditions. However, after 4 days of storage, a dramatic decrease of the *g*-values to a value between 2.0048 and 2.0050 was observed for the samples stored anaerobically. It was observed that for samples exposed to light, the change in the *g*-value for the radical is occurring somewhat slower.

In **Figure 3C**, the results from water activity measurements during the same period are shown. For the samples containing no oxygen, a slow rise in water activity was observed from day
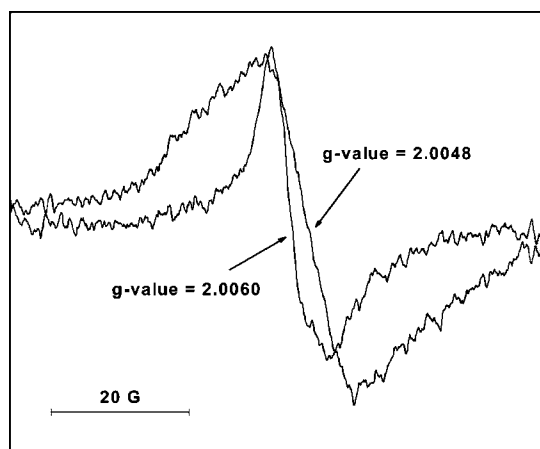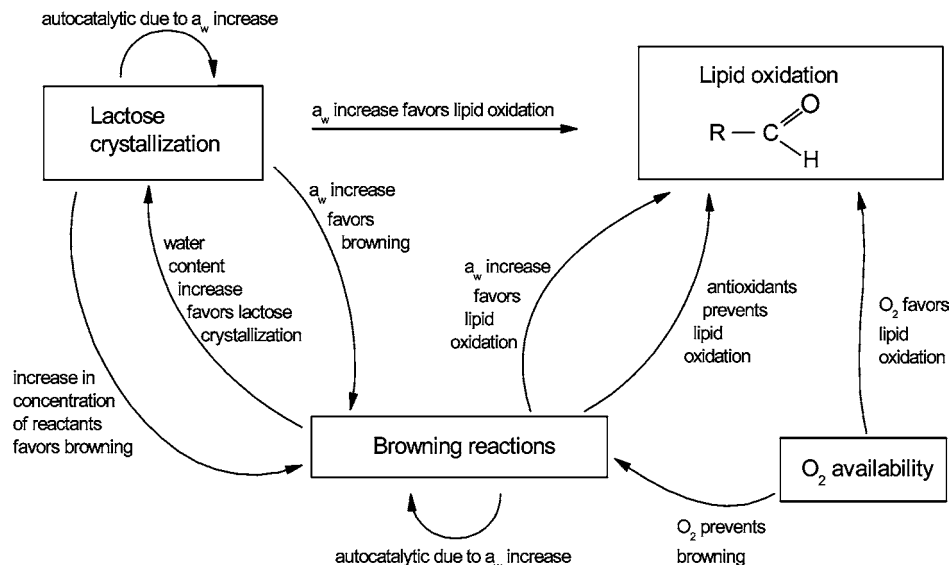
Figure 5. Interrelation between deteriorative reactions in whole milk powder observed in this study and described in the text.

cannot be ruled out that each of the two observed spectra are superpositions of two or more underlying spectra corresponding to more than one radical species.

DISCUSSION

Detection of radicals in milk powder has been associated with lipid oxidation. Stapelfeldt et al. (*19*) found a correlation between lipid oxidation as measured by formation of TBA reactive substances and radical formation in whole milk powder. Furthermore, an inverse correlation between radical formation in whole milk powder and sensory score of the reconstituted milk has been observed (*13*). This correlation was interpreted as radicals being precursors of the secondary oxidation products, which were detected by sensory analysis as an off-flavor. In the present study, two types of radicals have been observed in whole milk powder during storage. The first type of radical, the white radical, has a relatively high *g*-value of 2.0060 and the concentration of this radical depends on the availability of oxygen. This finding suggests that the white ESR signal originates from radicals that are either directly involved in the oxidation processes or formed by appropriate side reactions and is thus consistent with the earlier work on radicals in milk powder (*13*, *19*) and further strengthens the connection between the observed radicals and oxidation in whole milk powder prior to browning and crystallization of lactose. However, much more work is needed to fully understand the chemical nature and the reaction pathway for formation of the white radicals. Clarification of some of the unresolved questions is expected by variation of the chemical compositions of the milk powder under investigation. Measurements of radicals in fat-free skim milk powder will answer the fundamental questions of whether the white radicals depend on the presence of milk fat and if the radicals are formed by other oxidation processes in parallel to lipid oxidation. Work on model systems, where the chemical composition can further be controlled, is expected to point toward the chemical nature of the observed white radicals.

In contrast to the early radicals, the brown ESR signal, which has a completely different appearance and is only formed during the late stages of the process, is not dependent on the availability of oxygen and can be characterized by a much lower *g*-value and a broader spectral width. On the basis of these results, it seems unlikely that the radical or radicals giving rise to the brown ESR spectra are directly involved in oxidation processes

but rather are involved in the late stage of Maillard reaction, where melanoidins are formed. Melanoidins contain nitrogen per definition and are rather inactive chemical substances although little is known about the exact structure of these brown substances (*6*, *20*). Reaction products of the later stages of Maillard reaction possess antioxidative properties and are able to decrease the rate of lipid oxidation; however, neither the structure of the compounds nor the mechanisms responsible for this effect have been completely identified (*21*). Furthermore, radicals have been detected in material containing late-stage Maillard reaction products. Most of these radicals give rise to single-peak ESR spectra with peak width varying from 6.4 to 150 G and *g*-values around 2.004 (*22*).

Concerning the question of the radicals giving rise to both kinds of ESR spectra, it is unfortunate that the observation of single-peak ESR spectra, from which coupling constants cannot be derived, leaves only the *g*-value and the peak-to-peak width as the characteristics of the spectrum, which is not sufficient for identification of radicals involved. It is possible that the nuclear/electron coupling patterns observed by combined ESR/NMR techniques such as ENDOR and TRIPLE-resonance, which excites electron spins and various nuclear spins simultaneously, can provide more information about the chemical nature of the radicals present in milk powder.

The abrupt changes in radical concentration, surface color, and the water activity of the whole milk powder occurring simultaneously suggest that the different physical and chemical processes occurring in the product are strongly interrelated. **Figure 5** summarizes the interrelation between the processes on the basis of the findings of this study and on the available literature.

Increase of the water activity has previously been observed to increase lipid oxidation in milk powder (*19*) and infant formula (*23*). Crystallization of lactose under conditions of constant water content (closed systems) increases the water activity substantially (*24*), as also observed in this study. Maillard reactions in dry systems are generally reported to progress faster at increased water activities (*9*). During the early stage of Maillard reaction, water molecules are produced (*25*), which again will influence water activity. Furthermore, water is an efficient plasticizer making amorphous/glassy food material more prone to crystallization (*26*). Accordingly, because of changes in water activity and production of water, both lactose

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crystallization and Maillard reactions are likely to be autocatalytic as well as catalyzing each other. Furthermore, as lactose is the main diluent in which possible reactants are distributed, crystallization of this main compound is expected to concentrate possible reactants and thereby favor browning reactions. Thus, without accounting for the detailed mechanisms, we put forward that the above-described catalytic interrelations may give rise to the observed abrupt and simultaneous behavior of the measured properties of the product.

From a comparison of **Figures 1** and **3**, it is seen that in whole milk powder stored anaerobically initially a more rapid increase in water activity is observed together with a higher final water activity after crystallization of approximately 0.05 units. These observations together suggest an influence of oxygen on the interrelated Maillard reactions, crystallization, and radical formation. It has previously been found that low oxygen pressures favor Maillard reaction in infant formula (*27*), whole milk powder, and skim milk powder (*28*) at slightly elevated temperatures. It was suggested that the effect of oxygen could be due to oxidation of aldehydes formed from the Amadori compound and consequently less polymerization into brown pigments (*28*), as also included in **Figure 5**. Furthermore, it is possible that antioxidant formed by browning reactions (*21*) affects the progress of lipid oxidation as indicated in **Figure 5**.

In summary, three types of deteriorative processes take place during storage of whole milk powder under accelerated conditions, lactose crystallization, browning reactions, and lipid oxidation. The three processes are strongly coupled through development of water, change of water activity, concentration of reactants, production of antioxidants, and action of oxygen. In addition, it is found that at least two distinctly different radical species are involved in the processes. One, the "white radical", is dependent on the availability of oxygen and is involved in the lipid oxidation process. The other "brown radical" is independent of oxygen availability and is related to the late stages of the browning processes.

ABBREVIATIONS USED

a_w, water activity; ESR, electron spin resonance.

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