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REVIEW ARTICLE

Glycation products in infant formulas: chemical, analytical and physiological aspects

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Abstract Infant formulas are milk-based products, which are adapted to the composition of human milk. To ensure microbiological safety and long shelf life, infant formulas usually undergo rigid heat treatment. As a consequence of the special composition and the heat regimen, infant formulas are more prone to thermally induced degradation reactions than regular milk products. Degradation reactions observed during milk processing comprise lactosylation yielding the Amadori product lactulosyllysine, the formation of advanced glycation end products (AGEs), and protein-free sugar degradation products, as well as protein or lipid oxidation. Several methods have been developed to estimate the heat impact applied during the manufacturing of infant formulas, including indirect methods such as fluorescence analysis as well as the analysis of defined reaction products. Most studies confirm a higher degree of damage in infant formulas compared to regular milk products. Differences between various types of infant formulas, such as liquid, powdered or hypoallergenic formulas depend on the analyzed markers and brands. A considerable portion of protein degradation products in infant formulas can be avoided when process parameters and the quality of the ingredients are carefully controlled. The nutritional consequences of thermal degradation products in infant formulas are largely unknown.

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Introduction

Milk is secreted by the female of all mammalian species to perfectly meet the nutritional requirements of the newborn. In particular in the Western world, humans continue to consume milk beyond infancy, using mainly the milk of cattle, goats and sheep. Most of the milk consumed in the industrialized countries is heat treated. Pasteurization is a relatively mild heat treatment (15 s at a temperature minimum of 71.7°C), which kills pathogenic bacteria without affecting the taste of the final product. Compared to this "fresh" milk, which can be stored in the refrigerator for a few days, ultrahigh-temperature (UHT) treated milk is heated for some seconds up to a temperature of at least 135°C, resulting in a product with a shelf life of a few months. Significantly higher heat load is used for sterilizing milk (10–30 min at 110°C or above) or in the manufacture of evaporated milk, which is an in-vacuo concentrated sterilized milk (The Dairy Council 2010). Whereas pasteurization or even the newly developed process of "high pasteurization" hardly shows any substantial influence on the nutritional quality of the processed milk when compared with raw milk, sterilization as well as drying and long-term storage induce several chemical changes, among which glycation reactions are of outstanding importance (Hegele et al. 2008). The impact of processing on the nutritional quality is of particular importance for infant milk formulas (IFs). IFs are a special type of milk product, made from cow milk-derived constituents (casein or whey proteins, lactose, and butter fat) combined with vegetable oils, starch and other ingredients to meet the dietary requirements of the

newborn or infants of varying age (Nasirpour et al. 2006). Among the IFs, hypoallergenic (HA) infant milk formulas represent a type of IFs, in which the allergenic potential of milk proteins is reduced using partially or extensively hydrolyzed casein or whey proteins as a constituent. The degree of hydrolysis and composition of peptides may greatly vary due to the production and methods of preparation, such as enzymes used for hydrolysis or temperature (Nasirpour et al. 2006; Martin et al. 2008).

The chemistry behind glycation reactions in processed milk

Milk is an ideal matrix for the Maillard reaction. Due to its high content of lactose and proteins on the one hand, and the relatively high heat impact applied during milk processing on the other, a great variety and abundance of reaction products can be expected. Most dominant among the Maillard reaction products in milk is certainly lactulosyllysine, the Amadori product of lactose and lysine side chains of the milk proteins (Erbersdobler et al. 1987). In some milk products, such as skim milk powder, up to 50% of the lysine residues are converted to lactulosyllysine (Finot et al. 1981; Henle et al. 1991). However, some studies analyze lactulosyllysine by indirect methods, such as the furosine method that cannot really differentiate between the Amadori product and lactosylamine, the primary condensation product between lactose and a lysine residue. Therefore, it cannot be ruled out that part of the detected Amadori product should rather be assigned to lactosylamine (Finot et al. 1977).

When higher heat impact is applied during milk processing, the Amadori product can be converted into further reaction products, the so-called advanced glycation end products (AGEs). The best characterized process in this regard is the oxidative degradation of lactulosyllysine yielding N^{ε}-carboxymethyllysine (CML) (Bueser et al. 1987). Non-targeted mass spectrometric analysis suggests that CML is the major AGE in heated milk models (Meltretter et al. 2007). Further AGEs that have been identified in processed milk are N^{ε}-carboxyethyllysine (CEL) (Ahmed et al. 2005), pyrraline (Henle 2003), oxalic acid monolysinylamide (OMA) (Hasenkopf et al. 2001), methylglyoxal-derived hydroimidazolone (Ahmed et al. 2005) and pentosidine (Henle et al. 1997). In addition to the formation of AGEs by the degradation of lactulosyllysine, AGEs can also be generated by the reaction of lactose degradation products, such as methylglyoxal, with the amino acid side chains of proteins (Henle et al. 1994).

Besides early and late glycation products of milk proteins, unbound Maillard reaction products are formed in considerable amounts during milk processing. Lactose is easily transformed into lactulose by a Lobry de Bruyn–van Ekenstein rearrangement (Adachi 1958). Furthermore, the epimerization of lactose to epilactose, its hydrolysis to glucose and galactose, and the further epimerization of galactose to tagatose were observed during milk processing (Olano and Calvo 1989; Troyano et al. 1992a). Upon heat treatment of milk, these sugars are further degraded, for example, to yield 3-deoxypentulose, galactosyl-β-pyranone, furfural or hydroxymethylfurfural (HMF) (Troyano et al. 1992b; Pischetsrieder et al. 1999; Ferrer et al. 2000a; Pellegrino and Cattaneo 2001). Nitrogen-free products may arise either by direct sugar degradation or via Maillard reaction by amine catalysis. Another group of unbound glycation products are AGE-modified amino acids. In processed milk, their concentration is much lower compared to protein-bound AGEs. In raw milk, however, free and protein-bound CML occur in similar concentration ranges (Ahmed et al. 2005; Hegele et al. 2008).

Volatile Maillard reaction products are present in processed milk in lower concentrations. Nevertheless, they may have a major influence on the product quality due to their flavor activity. In UHT milk, for example, 2-acetyl-1-pyrroline, furaneol and furfural were identified as Maillard compounds with a high flavor impact (Colahan-Sederstrom and Peterson 2005).

Although protein oxidation is not the direct consequence of Maillard reaction, it has been shown that the presence of lactose is an important prerequisite for extensive protein oxidation during the thermal treatment of milk models (Meltretter et al. 2007). Two different mechanisms may explain this observation. The presence of sugar degradation products with α-dicarbonyl structure can trigger the formal oxidation of the ε-amino group of lysine yielding aminoadipic semialdehyde by a Strecker-type degradation (Meltretter and Pischetsrieder 2008). The oxidation of other amino acid side chains can be promoted by reactive oxygen species, which are formed in the course of the Maillard reaction (Mossine et al. 1999). The most prevalent protein oxidation product, which is generated during milk processing, is methionine sulfoxide (Baxter et al. 2007; Meltretter et al. 2008). Additionally, oxidation of cysteine to sulfenic acid or oxidation of tryptophan to hydroxytryptophan has been discussed (Puscasu and Birlouez-Aragon 2002; Meltretter et al. 2007). Moreover, protein adducts of the lipid oxidation product hydroxyl-2-nonenal have been identified in processed milk (Fenaille et al. 2005). In a similar way, lipid oxidation products can serve as precursors for CML formation (Lima et al. 2010).

Finally, several protein modifications that occur during milk processing independently from the milk sugars have been described. The protein cross-link products, lysinoalanine and histidinoalanine, are formed by the nucleophilic addition of lysine or histidine to dehydroalanine,

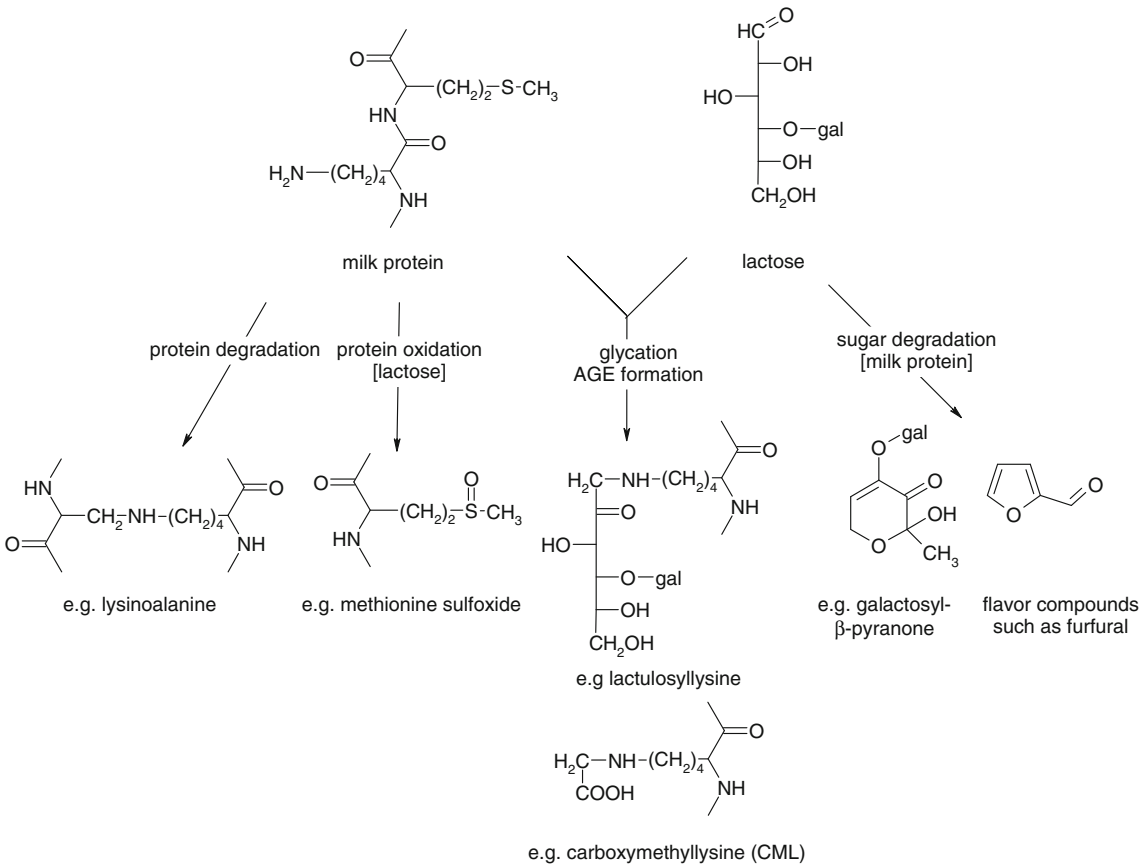

Fig. 1 Major thermal degradation reactions during milk processing

which arises from the elimination of cysteine and serine derivatives (Fritsch and Klostermeyer 1981; Henle et al. 1993). In milk models, cyclization of N-terminal glutamic acid has also been reported (Meltretter et al. 2007).

The different thermally induced reaction pathways, which take place during milk processing are summarized in Fig. 1.

Analysis of heat impact

The general impact of heating on complex food items such as milk products can be assessed by several methods with a varying degree of complexity and, therefore, varying informative value. For instance, the heat load of pasteurized or high-pasteurized versus UHT-treated milk can be compared by the measurement of routine parameters, such as the activity of the endogenous milk enzymes phosphatase and acid peroxidase, or the degree of denaturation of the major whey protein, β-lactoglobulin. Screening methods to analyze the Maillard reaction in milk are based on front-face fluorescence, an interesting approach for routine analysis (Diez et al. 2008). A more profound view of the early Maillard reaction in milk products and the modification of individual amino acid side chains of proteins can be achieved by measuring furosine, a reaction product that is formed from lysine Amadori products such as lactulosyllysine or maltulosyllysine during acid hydrolysis (Resmini et al. 1992; Henle et al. 1995). For the use of furosine and other furoylmethyl amino acids (FMAAs) as indirect parameters for Amadori products, it is necessary to know the quantity of these derivatives that are formed from the corresponding Amadori products during acid hydrolysis (Krause et al. 2003). The conversion rate depends on the sugar precursor of the Amadori product and the hydrolysis conditions. The influence of the matrix on the conversion rate needs to be determined. Furosine and other FMAAs can be quantified via ion-exchange chromatography, reverse phase high performance liquid chromatography (RP-HPLC) with UV detection (Rada-Mendoza et al. 2002) or capillary electrophoresis (Vallejo-Cordoba et al. 2004) and are valuable analytical tools for the sensitive monitoring of Amadori product formation. As another routinely used indicator for heat treatment of milk, CML can be analyzed using gas chromatography–mass spectrometry (GC–MS), RP-HPLC after derivatization with ophthaldialdehyde, liquid chromatography–mass spectrometry (LC–MS) and matrix-assisted laser desorption/ionization time-of-flight

mass spectrometry (MALDI–TOF–MS) (Liardon et al. 1987; Hartkopf et al. 1994; Kislinger et al. 2003). An enzyme-linked immunosorbent assay (ELISA) for the quantification of CML in milk products without need for cleanup has been published (Birlouez-Aragon et al. 2004), which—when compared with GC–MS—gave satisfactory results in powdered but not in liquid formulas (Charissou et al. 2007). CML levels of processed milk and IFs obtained by different methods in several studies have been recently compared (Assar et al. 2009; Delatour et al. 2009). Some discrepancies have been reported between CML concentrations in milk products determined by different methods, and also by different LC–MS protocols (Delatour et al. 2009). The reasons for this observation, however, remain speculative. Finally, relative quantification of protein lactosylation by MALDI–TOF–MS proved to be a very fast and reliable method to monitor the early Maillard reaction during milk processing (Meltretter et al. 2009).

Why are infant formulas particularly prone to glycation reactions?

Cow's milk is the major ingredient of milk-based IFs. To meet the special nutritional requirements of the young consumers, the composition of the products is usually adapted to human milk. The macronutrient composition of cow's milk, human milk and a representative first-stage IF are summarized in Table 1. A major difference between human milk and cow's milk is the lactose concentration (7.1% vs. 4.6%). Therefore, IFs are usually supplemented with lactose. Sometimes, other carbohydrates are added, such as maltose, sucrose, glucose syrup or starch (Nasirpour et al. 2006). For the production of IFs, the protein concentration of cow's milk (~3.2%) is usually adjusted to ~1.5–2.0%, which is more similar to human milk (~0.9–1.6%) (Belitz et al. 2009). To reflect the lower casein to whey ratio of human milk compared to cow's milk (40:60 vs. 82:18), whey proteins can be added to IFs, usually in the form of whey powder (Nasirpour et al. 2006). For infants with an elevated allergy risk, HA formulas have been developed. In these formulas, the potentially allergenic cow milk proteins are extensively or partially hydrolyzed by proteases followed by ultrafiltration and/or heat treatment. As a result, low allergenic peptides with a molecular size <1,500 Da are obtained (Beyer 2007).

Table 1 Macronutrients in cow's milk, human milk and a representative IF (first stage, German market) (Belitz et al. 2009)

	Protein (%)	Sugar (%)	Fat (%)
Cow's milk	3.2	4.6	3.9
Human milk	0.9–1.6	7.1	4.5
IF	1.4	7.1	3.5

Although the fat content of human milk is very similar to cow's milk, there are major differences in the fatty acid composition. To adjust the fatty acid composition of IFs, the milk fat can be (partially) replaced by vegetable oils (Innis 1992). Furthermore, long chain polyunsaturated fatty acids, which seem to be essential for the development of vision and brain in early childhood, can be enriched in formulas, e.g., by the addition of fish oil or egg lipids (Fleith and Clandinin 2005). Finally, supplementation with micronutrients, such as iron and vitamins, is warranted.

The microbiological safety is another crucial point in the production of IFs, for two reasons: a microbial infection can be a major health risk for the infant, and the producers aim at a long shelf life of the formulas. Therefore, IFs are usually heated more rigorously than regular milk. Sterilization can either be achieved by in-bottle sterilization of liquid formulas or by high-temperature, short-time treatment followed by spray drying to obtain powdered formulas. Alternatively, UHT treatment can be applied (Lonnerdal and Hernell 1998). In some cases, different heating regimes are combined (Birlouez-Aragon et al. 2004).

As a consequence of their specific formulation and processing, IFs show higher levels of glycation markers than regular milk products (Birlouez-Aragon et al. 2004). Both the high lactose content and the supplementation with whey proteins promote Maillard reactions in IFs. Whey proteins contain more lysine residues compared to caseins and can, therefore, be more extensively glycated. Furthermore, the ingredient proteins, such as whey powder, which are used for the supplementation of IFs, can already be highly damaged (Contreras-Calderon et al. 2008). Particularly, HA formulas show very high glycation rates, because the proteolysis releases N-terminal α-amino groups, which are targeted by glycation (Penndorf et al. 2007). Moreover, (long chain) polyunsaturated fatty acids can promote the glycation reaction because they are easily oxidized during production. As a result, glycation precursors, such as glyoxal, are released (Lima et al. 2010). The reaction of ascorbic acid with protein side chains (ascorbylation) can lead to AGE formation in a similar way (Pischetsrieder et al. 1997). Particularly in the case of milk fortification with ascorbic acid and iron, enhanced protein damage can be expected resulting from a Fenton-type reaction (Leclère et al. 2002).

Together with the more drastic processing conditions, the composition of IFs, which is adapted to human milk, leads to sensitive products with a higher risk of protein damage and contamination with unwanted thermal degradation products. Therefore, studies are warranted that monitor heat-induced reactions in IFs and investigate technological approaches to reduce these processes.

Glycation products in infant formulas

Several studies investigated glycation products in commercial IFs using different marker compounds. Thus, the assumption could be confirmed that the composition and processing of IFs resulted in elevated concentrations of glycation markers compared to regular milk. Birlouez-Aragon et al. (2004) detected increased levels of lysine loss (sixfold), lactulosyllysine (two to threefold, after conversion into furosine) and AGEs (three to fivefold, CML, OMA and fluorescence of advanced Maillard products and soluble tryptophan (FAST) index). Similar results were obtained by Fenaille et al. (2006) for lactulosyllysine[furosine] and CML. The levels of HMF and furfural were also significantly lower in powdered cow's milk samples compared to powdered IFs (Albala-Hurtado et al. 1997).

The extent of glycation reactions in different types of IFs, i.e., powdered, liquid, HA powdered and HA liquid, cannot be compared so easily. The result depends on the analyzed marker compound and also varies considerably among different samples of the same group. Significantly higher levels of CML, OMA, and FAST index were detected in liquid formulas compared to powdered ones (Birlouez-Aragon et al. 2004). Delatour confirmed this trend for CML (Delatour et al. 2009), whereas in another study, CML levels of the same magnitude were measured in liquid and powdered formulas (Fenaille et al. 2006). However, only two liquid IF samples had been available for the latter study. In contrast, the content of lactulosyllysine[furosine] as well as HMF and furfural were higher in powdered formulas than in liquid products (Albala-Hurtado et al. 1997; Birlouez-Aragon et al. 2004; Fenaille et al. 2006). In both product types, whey-enriched formulas showed a higher lactulosyllysine[furosine] content than regular ones (Birlouez-Aragon et al. 2004). Interestingly, no correlation was observed between protein glycation and oxidation markers in different commercial products (Fenaille et al. 2006).

In HA formulas, higher CML levels were observed, but also a broader dispersion of the values than in regular formulas of the same consistency (liquid or powdered, respectively) (Dittrich et al. 2006; Fenaille et al. 2006; Delatour et al. 2009). In contrast, the lactulosyllysine[furosine] content in powdered HA formulas was even lower than in regular powdered formulas (Fenaille et al. 2006). It has to be noted that the Amadori product formation and carboxymethylation of α-amino groups of amino acids, which should be predominant in HA products, cannot be detected when the glycation is estimated by the furosine method, or when CML is analyzed by GC–MS or LC–MS. Thus, considerably higher glycation rates must be expected in HA formulas, when detection methods are applied that cover the lactosylation or carboxymethylation of the α-amino groups of amino acids, in addition to the modifications of the ε-amino group of lysine (Penndorf et al. 2007). It is likely, however, that various N^{α}-carboxymethylated amino acids are covered when CML is analyzed by ELISA.

Finally, significantly higher lactulose concentrations were reported for whey-enriched IFs compared to milk-based products. In all products, the lactulose levels ranged well below the level recommended for UHT milk by the European Communities Commission (Pereyra Gonzales et al. 2003).

Several studies have been performed to analyze the glycation progress during the storage of IFs. Whereas storage at 20, 30 and 37°C for up to 9 months did not change the reactive lysine content, a time and temperature-dependent increase in HMF and furfural concentrations was observed (Albala-Hurtado et al. 1999; Ferrer et al. 2000b). Additionally, storage at 20 and 37°C for up to 2 years resulted in intensified color (Ferrer et al. 2005).

The studies confirm that the specific formulation and processing conditions applied for IFs is closely associated with an increased risk of protein damage by glycation and oxidation reactions. However, the wide dispersion of glycation parameters, which has been observed for different brands in some of the studies, suggests that the load of thermal degradation products in commercial formulas can be minimized by improving the processing parameters. This hypothesis was investigated by Cattaneo et al. (2009). Experimental samples produced in an industrial plant revealed that 90% of galactosyl-β-pyranone and lactulose were formed during UHT sterilization. Interestingly, the formation of thermal degradation products during UHT sterilization could be halved, when the pH was adjusted from 7.2 to 6.9 prior to heating. A similar improvement could be achieved when the milk re-circulating in the plant was discarded. Another important source of protein degradation products were the ingredients: up to 60% of the Amadori product measured as furosine and 20% of lysinoalanine were already present in the protein ingredients, such as whey powder or whey protein concentrate. These results imply that the load of thermal degradation products, which have to be handled by the infants, can be considerably reduced by a relatively simple adjustment of the processing conditions as well as by the selection of the raw materials.

Nutritional consequences

Finot et al. (1977) were the first to prove that the Amadori product of lysine, namely N^{ε}-fructosyllysine, was not used as a lysine source in vivo. From that time on, the quantification of "lysine blockage" due to the early Maillard reaction was a widely used tool to assess the nutritional

quality of milk products. Heating or storage may induce 5–20% of the initially present lysine to react with lactose in IFs. For adults, this fact should not be too distressing from a nutritional point of view, due to the general overload of protein in human nutrition in industrialized countries. However, to prepare foods processed as minimally as possible, this parameter should serve as a suitable quality index. In IFs, the loss of essential amino acid lysine is generally compensated to guarantee adequate lysine intake. One possible means to increase the lysine content in IFs is to increase the protein concentration of the products (Alexy et al. 1999). However, the protein concentration in IFs must be handled with care, because a high protein intake in the first 2 years of life, for example due to an early switch to cow's milk, has been related to negative effects such as a risk for obesity in later years (Riva et al. 2007).

Studies dealing with the metabolic fate of chemically defined dietary glycation compounds are rare and generally point to either a low bioavailability or rapid elimination. Intervention studies indicate that only 3–10% of peptide-bound Amadori products, such as N^{ε}-fructosyllysine or N^{ε}-lactulosyllysine, are resorbed and rapidly excreted via the kidney, whereas most of the Amadori products are probably fermented by the human microbiota (Finot et al. 1981; Erbersdobler et al. 1991; Henle et al. 2000). Preterm infants excreted 1.3–3.9% of the ingested lactulosyllysine and 6.2–9.3% of the ingested lysinoalanine with urine (Langhendries et al. 1992). Short-term feeding of different types of infant formulas to this group did not result in changes in the kidney function, but in an increase in urinary microprotein levels compared to breast milk feeding. In animal studies, the fast elimination of [18F]-fluorobenzylated CML was found by positron emission tomography (Bergmann et al. 2001). It needs to be confirmed if free CML shows a similar biodistribution. Neither Amadori products nor CML are actively transported in the colon epithelial cells (Grunwald et al. 2006; Hellwig et al. 2009). Reports dealing with the quantification of CML in plasma and urine of infants given CML-containing formulas concluded that some CML was absorbed and excreted in the urine (Dittrich et al. 2006; Sebekova et al. 2008). In very young neonates, CML formed as a consequence of oxidative stress during natural birth seems to overcompensate the nutritional CML intake from infant formulas (Dittrich et al. 2006). During the course of decades, infant nutrition has been constantly modified to comply with the nutritional requirements of infants. Thus, adverse health effects of today's generation of IFs are by far not evident from clinical or experimental studies. However, further studies are necessary that deal with the nutritional consequences of infant formulas, which represent the sole food intake for infants in the first 3 months, particularly with respect to the role of glycation products therein.

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