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Polyunsaturated Fatty Acids, Riboflavin and Vitamin C: Effects of Different Storage Conditions of Human Milk

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Abstract

This study addresses a matter of importance for: healthy infants; sick infants in the Neonatal Intensive Care Units; infants fed expressed human milk and infants who receive milk from Human Milk Banks. Current storage parameters for freezing of mother's milk are not well established and are often contradictory. Pooled fresh human milk was stored raw, in nitrogen gas and following Holder pasteurization for 6 days at 4°C and for 6 months at -20°C and at -80°C. Contents of linoleic (LA), α−linolenic (ALA), arachidonic (ARA) and docosahexaenoic (DHA) acids, riboflavin and total vitamin C were analyzed under these conditions and during these storage times as they are sensitive to oxidation. The results of this study confirm the general appropriateness of freezing human milk at either -20°C or at -80°C, for preservation of two vitamins and four fatty acids. These storage recommendations are applicable also for the storage of human milk which underwent Holder pasteurization. Both the exclusion of oxygen and freezing at -80°C may be redundant for nutrient preservation and in some cases even detrimental. Recommendations for human milk storage while based on bacteriological safety appear to be appropriate for preservation of vitamins C and riboflavin and LA, ALA, DHA and ARA. As vitamin C is highly susceptible to oxidation, its addition to human milk or direct supplementation of the infant is recommended, if the milk had been frozen for longer than 2 weeks or had been pasteurized. We recommend supplementation of the infant with vitamin C at the Adequate Intake (AI) level in these cases.

Keywords: Human milk; storage; Fatty acids; Vitamin C; Riboflavin; Holder pasteurization

Abbreviations: LA: Linoleic Acid (18:2n-6); ALA: α-linolenic Acid (18:3n-3); DHA: Docosahexaenoic Acid (22:6n-3); ARA: Arachidonic Acid (20:4n-6)

Introduction

Human milk provides numerous advantages for infants, mothers, families and society [1]. Human milk is sufficient to support optimal growth and development of the infant for approximately the first 6 months of life. Therefore, many health organizations recommend exclusive breastfeeding for this period, defined as the consumption of human milk alone, with the exception of vitamins, minerals and medications [2,3]. The World Health Organization (WHO) recommends continued partial breastfeeding into the second year [3]. When breastfeeding is chosen, some babies may be fed mechanically or hand expressed milk from a bottle, rather than directly from the breast. Mothers express their milk when they have sore nipples, postpartum breast engorgement, in order to increase milk supply, to leave the milk if they are away from their baby and also in situations of adoption or surrogacy [4-10]. Expressed breast milk, often supplemented with nutrients, is used in hospitals for the feeding of premature, small for gestational age and normal term infants who cannot suckle [11]. Breast milk can be donated to human milk banks, which provide pasteurized donor human milk for infants with medical conditions, for adopted infants and for those who are not able to receive their own mother's milk [12]. Heat treated breast milk is recommended for the feeding of infants of HIV-positive mothers in developing countries in order to prevent transmission of the virus through milk [13]. The American Academy of Paediatrics has stated that data on prolonged storage of human milk needs updating [14].

The purpose of human milk pasteurization is to destroy pathogenic microorganisms [15]. Commonly used conditions are 62.5°C for 30 minutes ("Holder pasteurization"). Holder pasteurization has been shown to destroy the pathogens in milk, does not affect key nutritional factors, but may have an adverse effect on some of the milk nutritional, functional, immunologic and anti-infective components. Holder pasteurization achieves a sufficient compromise between microbiological safety and biological and nutritional integrity of human milk [16].

Immediately after expression, human milk becomes susceptible to food degradation and its quality may decrease during storage [17]. Several methods are used to extend the shelf life of food, including reducing storage temperature and restricting oxygen availability. Low-temperature storage slows down microbial growth and delays changes in the physiochemical character of milk [15]. Reducing the availability of oxygen using vacuum packaging or nitrogen gas flushing slows down oxidative reactions and reduces microbial growth [18]. Freezing at -70°C or -80°C is considered the gold standard for conservation of human milk for extended periods of time [16] and is a common temperature for storage of other human tissues [19].

Many protocols have been published in an attempt to establish conditions for storage of human milk. Depending on the study, recommended refrigeration storage (4°C) length varies from 2 days to 8 days [20-22]; frozen storage at -15°C from 2 weeks [22-24] to 1 month [25]; storage at -18°C from 3 months [20] to 12 months [21]; storage at -20°C to 12 months [21,22,24,25]. Pasteurized human milk has been recommended to be stored at a temperature -20°C, with no established length of time [16]. These guidelines pose 2 problems: firstly, the variation in recommendations makes it unclear which guidelines should be followed. Secondly, they are aimed primarily at avoiding

bacterial growth, while not considering loss of nutritional properties during storage and handling [26,27].

Human milk provides the normative standard for infant nutrition. Nevertheless, many micronutrients vary in human milk depending on maternal diet and body stores [28]. The essential fatty acids including the n-6 linoleic acid (18:2n-6, LA) series and n-3 α-linolenic acid (18:3n-3, ALA) series, are required in the diet to avoid fatty-acid deficiency in both mother and infant [29]. LA and ALA are the essential long-chain polyunsaturated fatty acids (LC-PUFA), which are found in cell membrane phospholipids, plasma lipids, storage lipids and in intracellular cholesterol esters [30,31]. Docosahexaenoic acid (DHA, 22:6n-3) and arachidonic acid (ARA, 20:4n-6), the metabolites of ALA and LA, respectively, are the major LC-PUFA components in membranes phospholipids of the brain and retina [30,32]. Preterm infants require DHA and ARA for rapid brain and body growth [30]. The most potent of the n-3 fatty acids is DHA, which has a beneficial anti-inflammatory activity. Another important biological effect of DHA pertains to brain development and cognition [33]. Two hundred milligrams of DHA as a supplement, for the first 4 months of breastfeeding, results in higher infant Bayley Psychomotor Development Scores at 30 months of age [34] and better performance on tests of sustained attention. This suggests that DHA intake during early infancy confers long-term benefits on specific aspects of neurodevelopment [33]. Vitamin C is essential for infant growth and development [35,36]. Its functions are based primarily on its properties as a reversible biological antioxidant [37]. The term vitamin C is the generic descriptor for all compounds which have the qualitative biological activity of ascorbic acid (AA). The principal natural compound, L-ascorbic acid, is oxidized to dehydroascorbic acid (DHAA) in the body. This reaction is reversible; therefore DHAA exhibits full biological activity. Riboflavin (vitamin B_2) is a precursor of the flavin coenzymes, flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), which participate in oxidation–reduction reactions in many metabolic pathways [37]. Riboflavin is central to energy production. Its other major functions include drug and steroid metabolism and lipid metabolism [38]. Previous studies have been conducted to follow the changes in the content of LA, ALA, ARA, DHA, vitamin C and riboflavin during storage of human milk at -20°C and -80°C [27,39-41] and following Holder pasteurization [16,42-44]. However, the reasons we selected these 6 nutrients is because from the literature the two water soluble vitamins are very sensitive to oxidation. Therefore we wished to see if we could find the optimum method to of milk storage to preserve these vitamins. Fatty acids were chosen because they are so important to growth and development and liable to oxidation. Oxidized lipids may make the milk undesirable to the infant! We could have chosen many more nutrients but these ones appear to be the most sensitive and representative of milk quality. However, to the best of our knowledge, no studies have assessed the effects of oxygen restriction on the nutrient integrity of human milk after storage at various temperatures, and no studies have analyzed the nutrient content of pasteurized human milk after storage at various temperatures. Our study reports the effects of different storage parameters on the nutrient integrity of mother's milk.

Materials and Methods

We stored human milk for 6 days at 4°C and for 6 months at both -20°C and -80°C. Milk was stored raw, in nitrogen gas (to limit oxygen availability) and following Holder pasteurization. Contents of LA, ALA, DHA, ARA, riboflavin and total vitamin C were analyzed during storage under the differing experimental conditions.

Sample collection and study design

Five mothers (age range 25-35, healthy) of full-term infants donated their mature breast milk (lactation established for at least 1 month) for a total volume of 300 ml collected by breast pump or hand expression. Milk was delivered to the laboratory on ice. Subsequently, it was heated to 38°C and to achieve sufficient volume, was pooled with 400 ml of milk from a single donor of mature milk (age unknown). This pooled milk was apportioned into opaque, hard, polypropylene tubes with tight-fitting lids [45]. Nitrogen gas flushing was applied for 3 sec. Holder pasteurization was carried out by holding tubes with human milk in a shaking water bath at 62.5°C for 30 min with subsequent rapid cooling under tap water at 8°C [16,46,47]. Control, nitrogen flushed and pasteurized samples were placed for refrigerated storage (at 4°C) or for frozen storage (at -20°C or -80°C). All treatments were completed within 2 hours of delivery of the milk to our laboratory. Human milk was shielded from light at all times. Three pooled samples of each treatment were analyzed at each analysis point. Refrigerated samples were analyzed for fatty acid and vitamin content every 48 hours for 6 days. Frozen samples (-20°C or -80°C) were analyzed at week 2, week 3, week 4 and then every calendar month for a total of 6 months. This study was approved by the University of Manitoba Ethics Committee, Bannatyne Campus Research Ethics Boards, and Ethics Reference Number: H2008:217.

Determination of riboflavin and total vitamin C

Riboflavin and vitamin C were analyzed according to Zafra-Gomez et al. [48]. Prior to the initialization of the procedure, aliquots of human milk were warmed in water (40°C) until the contents reached a temperature of 38°C, in order to liquidize milk fat and to dissolve lipids adhered to the walls of the containers [49]. Dithiothreitol (DTT) (Sigma-Aldrich) was used for the reduction of DHAA to AA [27,32]. Peak separation was carried out in the Agilent 1100 series HPLC (Agilent Technologies, Waldbronn, Germany). Waters Spherisorb ODS2, C_{18}, 250×4.6 mm I.D. A 5 μm analytical column was used, protected with a Waters Spherisorb ODS2, C_{18}, 10×4.6 mm I.D. 5 μm guard cartridge. Gradient elution was: initial values 98% A and 2% B; then a decrease of A to 10% over 8 min; hold for 10 min; an increase of A to 98% and a hold for 6 min. A constant flow rate was set at 1.0 ml/min. The UV detector was set at 245 nm for ascorbic acid; the fluorescence detector was set at 400/520 nm (excitation/emission) for riboflavin. Peak areas were calculated by the Agilent ChemStation for LC 3D Rev. B.01.03 [204] system. Values are expressed in mg/L (vitamin C) and ug/L (riboflavin). In order to determine vitamin retention times, a standard curve was generated immediately before each analysis of human milk samples using solutions with known concentrations of vitamin C (3.125 μg/ml-200 μg/ml), riboflavin (0.078 μg/ml-5 μg/ml) and DTT (1.5 mg/ml) in 2.4% acetic acid. A linear relationship was observed between concentration and signal at these ranges. The method was validated by calculating concentrations of vitamins C and riboflavin at every analysis point in freshly prepared Standard Reference Material (SRM) 1849 – Infant/Adult Nutritional Formula powder (National Institute of Standards & Technology, Gaithersburd, MD), diluted 15-fold with double distilled water (ddH$_2$O). Results obtained at 3 months were not included in the analysis due to analytical uncertainty.

Determination of LA, ALA, DHA and ARA

Fatty acids were analyzed by gas chromatograph (Varian 450-GC, Agilent Technologies) with split injection and a flame ionization detector (GC-FID). The one-step transesterification method of Masood et al. [50] was used for preparation of fatty acid methyl esters (FAMEs). The method was validated at every analysis point using SRM 1849, diluted 15-fold with ddH$_2$O. Heptadecanoic acid (C17:0) (Nu-Chek

Prep. Inc., USA) was used as the internal standard [51]. An Agilent J&W DB-225MS column was used for FAMEs separation (30 m, 250 μm I.D., and film thickness 0.25 μm). Hydrogen was the carrier gas at a flow rate of 1.3 ml/min with nitrogen as a make-up flow at 25ml/min. Hydrogen (30 ml/min) and air (300 ml/min) were used for combustion. The initial temperature of the 1 μL injection (10:1 split ratio) was 270°C. The column oven initial temperature was 70°C for 2 min, increased to 180°C at 30°C/min and held for 1 min, increased to 200°C at 10°C/min and held for 2 min, increased to 220°C at 2°C/min and held for 4 min and finally increased to 240°C at 20°C/min and held for 5 min. The detector temperature was 290°C. FAME peaks were identified by comparison with GLC Reference Standard 461 (Nu-Chek Prep, Inc. USA). Peak areas were determined by Galaxie Software and expressed as "composition %" of total fatty acids.

Statistical analysis

Statistical analysis was performed using Graph Pad Prism version 5.00 for Windows, GraphPad Software, and San Diego California USA. Statistical significance was assigned to $P<0.05$. For the analysis of a compound concentration change over time, one-way ANOVA with Post-Hoc Tukey's Multiple Comparison Test was performed. Two-way Repeated Measures ANOVA with Post-Hoc Bonferroni Test was used for the comparison of concentrations by treatment at the same time points.

Results and Discussion

Vitamin C content change in refrigerated human milk

The vitamin C content of all treatments (control, pasteurized and unheated milk stored with nitrogen gas) remained stable from the 2nd until the 6th day of storage (Figure 1). No statistical difference between treatments or treatments over time was observed. After the second day, the unpasteurized milk content was no higher than that of the heated milk. It may be that a major loss of vitamin C in human milk occurs with pasteurization [15,42,43] or occurs during the first 2 days of storage if not heat-treated, with no appreciable change thereafter. Restriction of oxygen with nitrogen flushing did not affect the vitamin C content.

Riboflavin content change in refrigerated human milk

The riboflavin content was not affected by oxygen restriction or pasteurization and did not change over the 6 days of storage in all human milk samples (Figure 2). There was no statistical difference between treatments or treatments over time. A decrease may have been expected based on a study of photo degradation of several vitamins in cow's milk as reported by Fanelli et al. [52]. However, our observed heat stability of riboflavin was in agreement with Ball, [37] who reported that riboflavin is generally stable during heat treatment if light is absent.

Vitamin C content change in frozen human milk

There was a rapid and consistent decrease in the vitamin C level for all treatments during the first month of storage (Table 1). Statistical analysis indicated that the vitamin C content of all treatments (control, pasteurized and unheated milk stored with nitrogen gas) decreased during the first month of storage at -20°C and -80°C followed by a 5-month period of stability. Notably, the vitamin C content of the control milk stored at -80°C dropped to an undetectable level during the first month, while control milk stored at -20°C retained some of the vitamin as seen by others [27,51]. A lower storage temperature (-80°C) did not provide increased stability for vitamin C preservation in pasteurized milk. Therefore, it may be that a -20°C temperature is
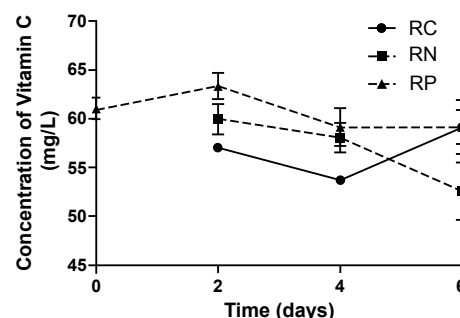

Figure 1: Vitamin C content of refrigerated (4°C) human milk for 6 days of storage. RC - control (untreated) human milk; RN - human milk stored in nitrogen gas environment; RP - pasteurized human milk.

Figure 2: Riboflavin content of refrigerated (4°C) human milk for 6 days of storage. RC - control (untreated) human milk; RN - human milk stored in nitrogen gas environment; RP - pasteurized human milk.
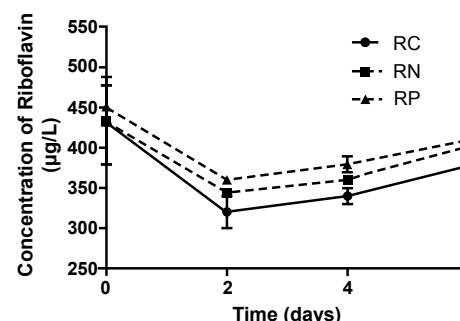
Duration	Treatment (mg/L)					
Month	FC	FN	FP	SC	SN	SP
1st	18.29 ± 1.73	14.23 ± 3.11	4.93 ± 2.21	0	14.20 ± 2.20	13.28 ± 1.91
2nd	18.15 ± 1.87	10.36 ± 1.34	18.35 ± 1.56	0	14.15 ± 2.22	12.57 ± 0.64
3rd	2.87 ± 0.70	3.88 ± 0.79	3.56 ± 0.15	0	3.98 ± 0.00	2.99 ± 1.06
4th	16.71 ± 0.62	20.89 ± 0.62	9.40 ± 1.89	2.10 ± 0.12	4.30 ± 1.92	4.67 ± 0.85
5th	13.34 ± 0.54	18.05 ± 1.15	6.54 ± 0.94	6.12 ± 0.48	7.31 ± 0.58	8.46 ± 0.91
6th	20.81 ± 1.41	26.22 ± 1.44	11.16 ± 0.83	4.82 ± 0.43	5.67 ± 0.53	5.95 ± 0.12

Table 1: Vitamin C content of frozen human milk (-20°C and -80°C) for 6 months of storage. FC – control (untreated) human milk stored at -20°C; FN - human milk stored at -20°C in a nitrogen gas environment; FP - pasteurized human milk stored at -20°C; SC – control (untreated) human milk stored at -80°C; SN - human milk stored at -80°C in a nitrogen gas environment; SP - pasteurized human milk stored at -80°C. The concentrations are expressed as mg/L for vitamin C, mean +/- SE.

preferable over -80°C for optimal storage of human milk. Flushing with nitrogen improved the preservation of vitamin C in unpasteurized human milk compared to controls stored at both temperatures. However, the small changes do not appear to justify the expenses involved in oxygen removal.

Riboflavin content change in frozen human milk

Control human milk samples retained their riboflavin content for 6 months of storage at both temperatures (-20°C and -80°C), as reported by others [53], with no advantage to either (Table 2). A nitrogen gas environment lowered riboflavin levels compared to controls in contrast to the expected preservation effect of oxygen restriction [18]. In the stored pasteurized samples, riboflavin levels decreased at both temperatures. At the end of the storage period of 6 months, all samples retained appreciable levels of riboflavin compared with initial concentrations.

LA, ALA, DHA and ARA content change in refrigerated human milk

Statistical analysis of the four fatty acids in the 3 types of human milk (LA only, Figure 3) revealed only non-significant fluctuations in their concentrations from one analysis point to another and between different treatments. Overall, the 4 FAs remained stable during the 6 days of storage at 4°C, in agreement with Slutzah et al. [54] (storage for 4 days) and Tacken et al. [41] (storage for 2 days), and were not affected by Holder pasteurization, as supported by other studies [15,44,55,56]. For LA, ALA, DHA and ARA, during refrigeration (at 4°C) for 6 days, there was no advantage or detrimental effect of oxygen restriction.

LA, ALA, DHA and ARA content change in frozen human milk

All four fatty acids remained stable independent of treatment for 6 months of storage at -20°C and at -80°C. Therefore, results are presented for LA (Table 3) only. These results were in agreement with other reports that indicate stability of human milk fat stored in similar conditions [39,41,56]. The observed stability of the four fatty acids with all treatments during 6 months of storage at -20°C and at -80°C suggests that a lower storage temperature of -80°C may not be necessary for FA preservation and that oxygen restriction adds no advantage. The stability of the fatty acids is not a novel finding [54,55], although polyunsaturated fatty acids are sensitive to light, oxygen exposure and high temperature. In milk they are incorporated into fat globules that are membrane bound. As long as milk is carefully handled, the globule membranes protect fatty acids [55].

Conclusions

The study's objective was the evaluation of the shelf life of expressed human milk under a variety of storage conditions. The results of

Duration	Treatment (composition %)					
Month	FC	FN	FP	SC	SN	SP
1st	13.69 ± 0.23	13.60 ± 0.03	13.53 ± 0.15	13.48 ± 0.04	13.55 ± 0.08	13.54 ± 0.17
2nd	14.70 ± 0.04	14.62 ± 0.02	14.64 ± 0.02	14.67 ± 0.00	14.61 ± 0.02	14.58 ± 0.01
3rd	14.65 ± 0.04	14.86 ± 0.03	14.81 ± 0.06	14.71 ± 0.05	14.87 ± 0.00	14.80 ± 0.07
4th	15.47 ± 0.02	15.39 ± 0.07	15.30 ± 0.09	15.44 ± 0.03	15.46 ± 0.10	15.28 ± 0.02
5th	15.47 ± 0.05	15.31 ± 0.09	15.29 ± 0.07	15.36 ± 0.11	15.41 ± 0.02	15.34 ± 0.01
6th	15.56 ± 0.10	15.45 ± 0.09	15.47 ± 0.03	15.46 ± 0.07	15.38 ± 0.07	15.56 ± 0.06

Table 3: LA acids content of frozen human milk (-20°C and -80°C) for 6 months of storage. Results for the analysis of LA concentration in human milk stored in the freezer at -20°C and at -80°C for 6 months not treated (FC for -20°C; SC for -80°C), in nitrogen gas environment (FN for -20°C; SN for -80°C) or pasteurized prior to storage (FP for -20°C; SP for -80°C). The concentrations are expressed as "composition (%)" for LA, mean ± SEM.

this study showed that out of the 6 nutrients analyzed, the limiting nutrient for stored human milk is vitamin C. The 4 fatty acids and the vitamin riboflavin maintained general stability under all experimental conditions. These results were in a general agreement with previous reports. The results of this study for vitamin C, riboflavin, LA, ALA, ARA and DHA support most storage recommendations [2,16,20-25], which are based on avoiding bacterial growth [27]. Our results also showed that raw human milk storage can be extended with Holder pasteurization. This study also showed that the exclusion of oxygen and a freezing temperature of -80°C, which are widely acceptable means of shelf life extension of stored food products [15,18] and other human tissues provide no advantage for human milk storage over at least 6 months. Thus additional means and expensive equipment for the optimal preservation of expressed human milk in the hospital or at home are not required. For growth and development, infants need an optimum supply of ascorbic acid [35,36]. We recommend adding vitamin C to human milk at the adequate index level before feeding, if milk had been frozen for longer than 2 weeks, as previously suggested by Romeu-Nadal et al. [40].

Duration	Treatment (µg/L)					
Month	FC	FN	FP	SC	SN	SP
1st	317.50 ± 3.82	282.50 ± 0.00	320.00 ± 2.50	322.50 ± 2.89	-	304.17 ± 3.00
2nd	392.50 ± 2.89	355.83 ± 2.20	392.50 ± 2.89	355.83 ± 2.20	403.33 ± 5.46	404.17 ± 1.67
3rd	470.83 ± 1.67	431.67 ± 0.75	470.83 ± 1.67	431.67 ± 2.20	475.00 ± 2.50	477.50 ± 1.44
4th	374.17 ± 3.63	339.17 ± 0.83	374.17 ± 3.63	339.17 ± 0.83	382.50 ± 1.44	386.67 ± 0.83
5th	416.67 ± 3.63	370.83 ± 6.51	416.67 ± 3.63	370.83 ± 6.51	417.50 ± 1.44	420.83 ± 3.33
6th	385.00 ± 0.00	324.17 ± 7.41	389.17 ± 2.21	324.17 ± 7.41	389.17 ± 2.20	397.50 ± 6.61

Table 2: Riboflavin content of frozen human milk (-20°C and -80°C) for 6 months of storage. FC – control (untreated) human milk stored at -20°C; FN - human milk stored at -20°C in a nitrogen gas environment; FP - pasteurized human milk stored at -20°C; SC – control (untreated) human milk stored at -80°C; SN - human milk stored at -80°C in a nitrogen gas environment; SP - pasteurized human milk stored at -80°C. The concentrations are expressed as µg/L for riboflavin, mean ± SEM.
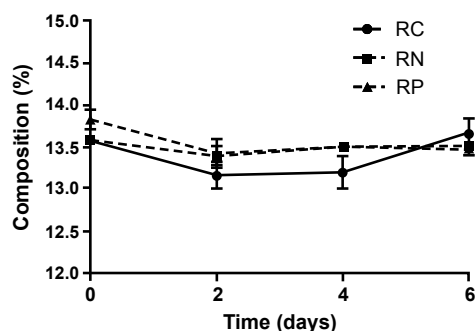

Figure 3: LA content of refrigerated human milk (4°C) for 8 days of storage. . RC - control (untreated) human milk; RN - human milk stored in nitrogen gas environment; RP - pasteurized human milk.

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