Quantitation of and inter/intra-individual variability in major carotenoids of mature human milk

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The quantitation of carotenoids from milk has been technically challenging due to the small quantities present, insolubility and instability of the carotenoids, and significant inter-and intra-individual variation. Here we report methods for extraction and quantitation by high pressure liquid chromatography of the major carotenoids, β-Cryptoxanthin, lycopene, α-carotene, and β-carotene in a normal population of lactating women. Methodology to evaluate and protocol to accommodate the significant variation in carotenoids of human milk is also presented. Carotenoids present in the highest concentrations in breastmilk of these mothers were lycopene and β-carotene at 31.2 and 45.9 nM respectively. α-carotene and β-carotene concentrations in this population of mothers showed the greatest variation, ranging as much as 20 fold between individuals. The remaining carotenoids varied fourfold to sixfold across individuals. Within individuals, carotenoid concentrations varied from twofold to fivefold when measured on separate days. For an individual on a single day, breastmilk carotenoid concentrations vary as much as fourfold and are strongly correlated with lipid concentrations. For routine field collections, a collection protocol was developed that will approximate 24-hr collections of a population of mothers within 10%. (J. Nutr. Biochem. 5:551–556, 1994.)

Keywords: carotene; breastmilk; inter/intra-individual variability; human milk; lactation; vitamin A; lycopene; cryptoxanthin

Introduction

Carotenoids have important functions in immunity both independently and as provitamin A compounds.[1–3] Thus, carotenoids may contribute significantly to the immunoprotection that breastmilk provides to the nursing infant. In addition, carotenoids are an important source of vitamin A in breastmilk. This is particularly important in developing countries, where a reliable source of preformed vitamin A (retinol) is not consistently available in the mother's diet.[4] Total carotenoids in mature human milk have been estimated previously using spectroscopic[5,6] and high pressure liquid chromatography (HPLC) methods,[7] and in collaboration with others, we have previously identified and quantitated carotenoids of colostrum.[8] We have recently reported methodology for isolating carotenoids of mature human milk.[9] Extreme variation in concentrations of carotenoids in human milk, both within and between individuals has previously been noted;[6] however, these differences have not previously been quantitated. These variations are sufficiently large to impose significant constraints on the design and interpretation of studies of milk carotenoids. Therefore, we present here methods to accurately determine the concentrations of the major carotenoids both for populations and for individuals.

Methods and materials

Subjects

Subjects were healthy mothers in the Tucson metropolitan area, more than 1 month postpartum, and exclusively breast feeding. Mothers were over the age of 18, had no chronic diseases, were not on any routine medication or steroid contraceptives, had children with normal growth patterns, and were non-smokers. Prior to sample collection, subjects signed informed consent forms in accordance with regulations of the University of Arizona Human Subjects Committee.

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Sampling procedures

The complete contents of one breast were collected under subdued lighting by electric breast pump (Ameda Egnell, Cary, IL USA) into sterile polypropylene containers or glass bottles. To insure that residual hind milk did not contaminate breastmilk samples, 2 to 3 hours prior to collection, the baby nursed from the breast to be sampled, and the breast was then completely emptied using the breast pump. For 24-h collections, breastmilk was sampled at every feeding. For other collections, sampling occurred twice in the mid-afternoon at the normal feeding schedule of the mother. Mothers used the same breast for collection of all samples throughout the study.

Sample analysis

Milk samples (4 mL) were saponified by addition of 3 mL 50% KOH (wt/wt) in 5 mL ethanol for 0.5 h at 25° C and extracted twice with hexane as previously described.[8] Extracted samples (50 μL) were injected onto a YMC (Wilmington, NC USA) reversed phase C_{18} column using an IBM auto sampler (Model LC/9050 SE, IBM, Rochester, NY USA). The mobile phase was methanol: tetrahydrofuran (THF), (90:10 vol/vol) containing 0.25 g/L butylated hydroxytoluene (BHT). Samples were eluted isocratically at a flow rate of 1.7 mL/min using a Waters model 510 pump, a Milton Roy programmable detector model SM 4000 and a Waters Maxima 810 version 3.02 system controller (Waters Associates, Milford, MA USA).

Quantitation

As exogenous carotenoids are not uniformly equilibrated in the milk matrix,[9] carotenoids were quantitated using external standards.[10] Recovery was estimated using the method of exhaustive extraction. In five separate experiments, no carotenoids could be detected in the third extraction, indicating that extraction was essentially complete after two extractions. One breastmilk pool was constructed and analyzed daily to standardize the daily HPLC analyses of individual breastmilk samples. To construct the milk pool, freshly collected samples were gently stirred to insure uniform mixing and 4-mL samples were removed and stored at −70° C until analysis.

Analysis of milk lipids

Lipid content of the milk was estimated by "creamatocrit" as described by Lucas et al.[11]

Between and within-person variability

The between and within-subject variability for carotenoids in breastmilk was estimated using a variance components estimation procedure in SAS PC (Cary, IL USA). Data were ln transformed prior to analyses to obtain more normally distributed data. Data were from collections on 2 to 8 separate days from 18 lactating women over a 7-month period between April and November 1992. For nine subjects, milk samples were collected on 2 days. For the remainder of the subjects, collections were made on 3 to 5 days. The daily mean carotenoid concentration per individual was estimated either from the average of 5 to 7 samples collected over a 24-hr period or from the average of two midafternoon breastmilk collections.

Results

Carotenoids of mature human milk resolved by HPLC chromatography in our system are shown in Figure 1. The major carotenoids were lutein (peak 1), β cryptoxanthin (peak 5), *trans*-lycopene (peak 7), α-carotene (peak 10) and β-carotene (peak 11). As in serum, β-carotene and lycopene were the most abundant of the milk carotenoids. Relative to total carotenoid concentrations, (A_{452}) β-carotene and lycopene were present in highest concentrations. As can be seen in *Figure 1*, several unresolved fractions are present that cannot be quantitated using the present method.

Concentrations of the major carotenoids in mature milk from 18 lactating mothers are presented in *Table 1*. To control for within-day variation, as described below, we obtained two samples at midafternoon from each mother on 2 separate days and analyzed each of these samples separately. The four samples were averaged to estimate carotenoid concentrations for individual mothers. Similar to our earlier studies in colostrum,[8] we observed a wide range of variability for the individual carotenoids. The concentrations of most of the carotenoids varied fourfold to sixfold in this population. However, concentrations of α-carotene and β-carotene ranged up to 20 fold in breastmilk of this population of mothers.

Analyses of within and between-person variability for β-cryptoxanthin, lycopene, α-and β-carotene are shown in *Table 2*. For these analyses, samples were collected for 2 to 5 days over a 7-month period. Within-person variability was highest for β-cryptoxanthin and lycopene, while variability between individuals was highest for β-carotene and α-carotene. In addition, within person variation was greater than

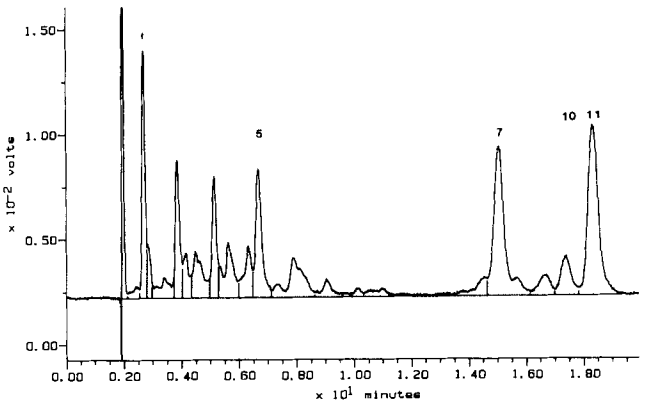

Figure 1 Carotenoids of human milk. Carotenoids were extracted from hydrolyzed samples and analyzed on HPLC as described in Methods and materials. 1 = lutein; 5 = β-Cryptoxanthin; 7 = Lycopene; 10 = α-Carotene; 11 = β-Carotene.

Table 1 Carotenoids of mature human milk

Carotenoid	Range (nM)	Mean (nM)	Std.
β-Cryptoxanthin	7.6–40.6	20.0	7.9
Lycopene	9.9–60.7	31.2	16.9
α-Carotene	2.0–34.4	11.1	8.3
β-Carotene	6.5–127.5	45.9	30.4

Carotenoids were extracted from frozen milk samples and assayed as described in Methods and materials. Two midafternoon samples were collected from each mother on two separate days. Each data point represents the average of two days' midafternoon samples. Samples were analyzed individually prior to averaging to approximate 24-hr concentrations as described in Methods and materials. n = 18 except for α-carotene (n = 17).

Table 2 Within-person and between-person variances for carotenoids of human milk

	Within-person (w)	Between-person (b)	Ratio (w:b)
β-Cryptoxanthin	0.201	0.135	1.48
Lycopene	0.213	0.189	1.13
β-carotene	0.184	0.555	0.33
α-carotene	0.183	0.663	0.25

Between and within-subject variability was estimated using a variance components estimation procedure in SAS PC. Samples were collected on 2 days for nine subjects, on one subject for 3 days, on five subjects for 4 days, and on three subjects for five days. Samples were collected over the 7 months between April and November 1992. Daily mean carotenoid concentrations were calculated from either a 24-hr or two midafternoon breast milk collections as described in Methods and materials. Data are ln transformed. n = 18 except for α-carotene (n = 17).

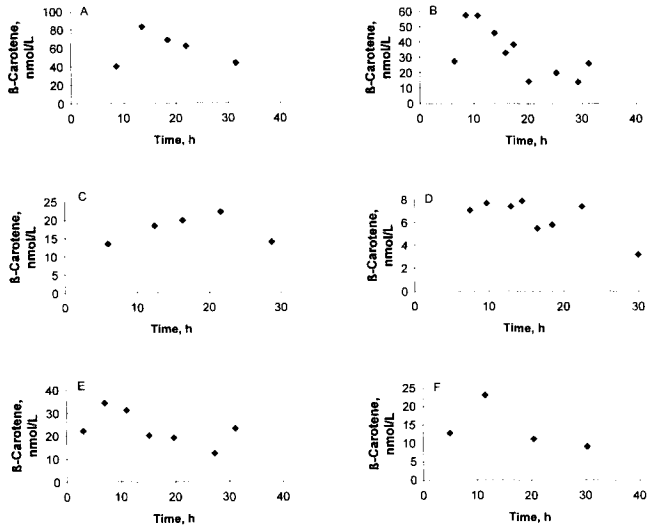

Figure 2 Concentrations of β-carotene in individual mothers over 24 hr. Milk samples were collected at each nursing episode over a 24-hr period in six mothers. Carotenoids were extracted from hydrolyzed samples and analyzed on HPLC as described in Methods and materials. (Time 0 = midnight.)

between person variation for β-cryptoxanthin and lycopene, while the reverse was true for α-carotene and β-carotene.

To measure changes in milk concentration over a 24-hr period, samples were obtained from six mothers at each nursing episode over a 24-hr period. Although all four carotenoids were quantitated, for simplicity, data for β-carotene only is presented in *Figure 2*. Patterns of variation in carotenoid concentrations over 24 hr differ in individual mothers. The maximum concentration of carotenoids occurs in midmorning for four of the six mothers. However, the maximum concentrations occurred in early morning for one mother *(Figure 2E)* and late evening for another *(Figure 2C)*.

As shown in *Figure 3*, for a given mother, all four carotenoids follow essentially the same concentration profile over a 24-hr period. As for *Figure 2*, although all four carotenoids were measured for six mothers, only three representative profiles are presented.

Figure 4 shows concentrations of β-carotene over 24 hr in one mother on 3 days separated by approximately 2 weeks. These data show that concentration profiles of carotenoids can vary significantly from day to day within individual mothers. In this example, the maximum concentration was shifted by approximately 8 hours over this 3-day period.

Collectively, the data in *Figures 2–4* clearly demonstrate that a single midmorning sample cannot be assumed to be

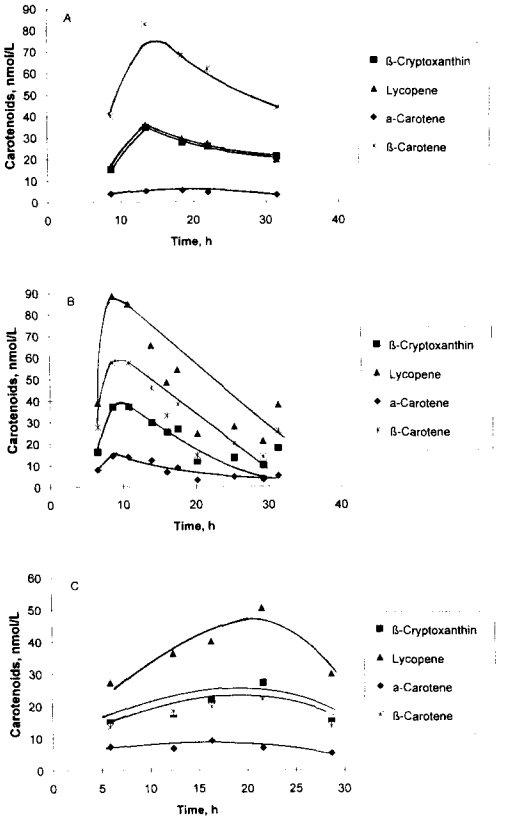

Figure 3 Concentrations of breastmilk carotenoids in three mothers over 24 hr. Milk was collected over 24 hr from three women (panels a, b, and c). Carotenoids were extracted from hydrolyzed milk samples as described in Methods and materials. ■ = Cryptoxanthin; ▲ = Lycopene; ◆ = α-Carotene; * = β-Carotene.

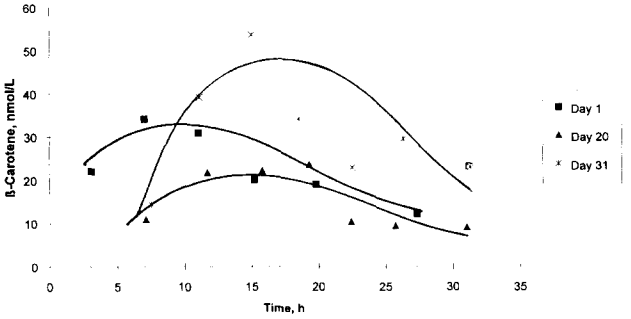

Figure 4 Concentrations of β-carotene in breastmilk of one mother on 3 days. Carotenoids were extracted from hydrolyzed milk samples and analyzed on HPLC as described in Methods and materials. Collections were made over 24 hr on 3 days every other week. ■ = Day 1; ▲ = Day 20; * = Day 31.

representative of an individual mother's total daily carotenoid production. However, in most cases it is impractical to collect breastmilk samples over an entire 24-hr period. Therefore, we designed experiments to develop practical abbreviated protocols to approximate 24-hr carotenoid concentrations for an individual mother. In these experiments, samples were obtained at each feeding over 24 hr, the samples assayed separately, and the results averaged to obtain a 24-hr average for an individual mother's β-carotene concentration. As shown in *Figure 5,* in seven separate experiments, the average concentrations of β-carotene in milk in two midafternoon collections for an individual was strongly correlated with the concentration of that mother's 24-hr average ($r = 0.98$, $P < 0.001$). A single midafternoon collection was only slightly less representative of 24-hr β-carotene concentrations in these mothers ($r = 0.97$), while two midmorning samples ($r = 0.78$) and one midmorning sample ($r = 0.76$) were less well correlated.

Variations in carotenoid concentrations relative to volume and lipid content were evaluated in samples of complete breast emptying collected over a 24-hr period for seven mothers as described above *(Figures 2–4).* Total milk volumes for the 24-hr period were recorded for each mother and β-carotene and total lipid concentrations measured. *Figure 6* shows data for one mother that is representative of the seven we measured. As shown in *Figure 6,* when measured as percent of change from the first sample of the day, variation in the data is greatest when only β-carotene concentrations (nmol/L) are considered. When variability due to changing volumes is eliminated (total nmol), variation is substantially decreased, reflecting the fact that β-carotene concentrations in milk are highest when volumes are lowest. There is the least variation over the 24-hr period when β-carotene concentrations are expressed relative to lipid (nmol β-carotene/g lipid).

Discussion

As reported earlier by us[8,9] and by others,[6] there is significant intra- and inter-individual variability in milk carotenoids *(Tables 1 and 2).* These variations apparently are related

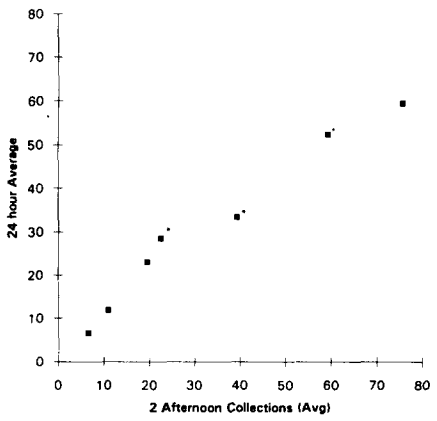

Figure 5 Correlations between two midafternoon samples and 24-hr β-carotene concentrations. Samples were collected as described in Figures 2–4. Points represent the averages for seven individual mothers. * = The same mother sampled on 3 separate days.

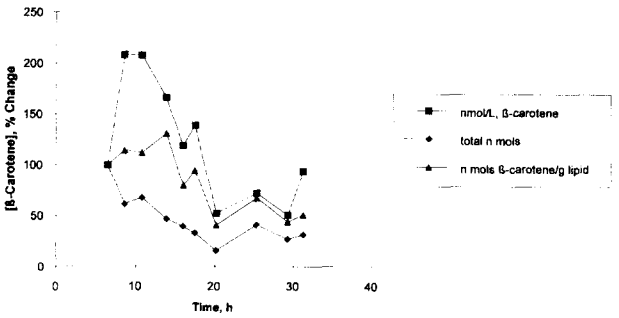

Figure 6 Percent change in breastmilk β-carotene concentrations over 24 hr in one mother, expressed as: (■), nmol/L β-carotene; (◆), total nmol; (▲), nmol β-carotene/g lipid. Complete breast emptyings were collected over a 24-hr period for seven mothers as described in Figures 2–4. Total milk volumes for the 24-hr period were recorded for each mother and β-carotene and total lipid concentrations measured as described in Methods and materials.

to dietary intake of these carotenoids,[12] and vary significantly across populations.[6] In addition, because α-carotene and β-carotene are provitamin A carotenoids, there may be individual variation in bioconversion of these carotenoids to retinoids. β-carotene is typically measured to assess carotenoid status. Given the variability in the concentrations of this carotenoid in milk, particularly when individuals or small populations are being measured, it is recommended that measurements of other carotenoids, or at a minimum total carotenoids, be included.

The distribution of major carotenoids in breast milk was qualitatively similar to those observed us[9] and by others[13–16] in serum. The carotenoids in highest concentration in our population were lycopene and β-carotene. It has previously been reported[6] that the majority of carotenoids in human milk have no vitamin A activity. In contrast, in our mothers, β-carotene was the carotenoid present in highest concentration on average *(Figure 1, Table 1).* In addition, β-cryptoxanthin contributed significantly to total carotenoid concentrations in milks of some mothers. Therefore, we conclude that human breastmilk carotenoids are a good source of vitamin A for the nursing infant.

Breastmilk carotenoid concentrations in our population were 10- to 20-fold lower than those in serum.[13–16] In earlier studies,[5–7,17–19] significantly higher quantities of total milk carotenoids (0.40 to 1.25 μM) were reported. Using the extinction coefficient for β-carotene, we estimate an average concentration of 0.25 μM total carotenoids for this population of mothers. Logistics of milk sampling, particularly where 24-hr samples are collected, are complex. For this reason, most studies, including the present one, have used small sample sizes. As shown in *Table 2,* individual variation undoubtedly explains some of the differences observed by various investigators. In addition, some studies may have included mothers in early stages of lactation, in which case carotenoid concentrations can be as high as 10 fold those in mature milk.[7,8]

In contrast to carotenoids, milk retinol concentrations are essentially the same as those in serum.[4–6] Thus carotenoids and retinoids are handled differently by the mammary gland. Carotenoids are presumed to enter the mammary epithelial

cell in lipoproteins.[20] Although this has not been studied in humans, in monkeys, retinol is apparently delivered to the cell via retinol binding protein.[21] In addition, secretory mechanisms may differ for secretion of carotenoids and retinoids into milk. Further studies are needed to describe the mechanisms regulating the transport of carotenoids and retinoids into the mammary gland and secretion into milk.

As predicted by the data in *Table 1,* there was large variability in breast milk α and β-carotene concentrations between individuals, reflecting the ~20-fold range in concentrations observed in these breastmilk carotenoids. As shown in *Table 2,* of the carotenoids we measured, between-person variance was highest for milk α- and β-carotene and in fact, was threefold to fourfold higher than within-person variance for these carotenoids. These data are consistent with results of a study of between- and-within-person variability of serum β-carotene in adult men.[22] As discussed above, differences in patterns of variability in the carotenoids may be related to variability in dietary intake, bioavailability, or bioconversion of carotenoids in individuals. Additional experiments are needed to more precisely determine the relative contributions of diet, bioconversion, and bioavailability on the concentrations of these carotenoids in breast milk.

The data in *Tables 1 and 2* show that a single experimental protocol may not be appropriate to measure variation in carotenoids. For example, due to large within-person variation, repeated measurements from the same mother over time are required to accurately determine breast milk β-cryptoxanthin and lycopene for that individual. In contrast, due to large between-person variation, larger sample sizes are needed to accurately measure changes in α- and β-carotene in breastmilk for a population. For example, to document significant differences in small changes, e.g., 25%, in breastmilk β-carotene concentration between two groups of mothers, approximately 111 women per group would be needed (two-sided t test with $\alpha = 0.05$ and a power of 80%). In contrast, only 40 women per group would be needed to detect the same changes in β-cryptoxanthin concentrations.

Problems with approximating a nutrient by a single sampling are well documented.[23,24] A number of maternal factors influence variations in concentrations of nutrients. The factors governing secretion of carotenoids into milk are not understood, but appear to be related to stage of lactation, maternal dietary patterns, and feeding patterns. In one study, milks of German mothers had the highest fat content in the afternoon while those of English mothers had highest fat contents in the evening.[25] In contrast, of the six mothers we surveyed, five had the highest fat and carotenoid contents in morning milk *(Figures 2–4).* In 20 Houston mothers, total energy concentrations (kcal/L milk) increased during the day and were highest in the evening.[26] Changes in nutrient concentrations in breastmilk may also be related to hormonal stimulation.[27] In particular, prolactin release is related to sleep-awake cycles and may influence feeding patterns of the mother-infant pair.[28]

In addition to variations discussed above in carotenoids, fat, and energy, variations have been reported in folacin,[29] vitamin B_6,[30] cholesterol,[26] and protein[31] over the 24-hr period. For these reasons, it is generally agreed that where possible, 24-hr pools should be analyzed. However, for many studies, this will be impractical. Thus we have developed abbreviated sampling protocols to approximate total daily output of breastmilk carotenoids. The data in *Figure 5* show that two midafternoon samples will approximate the average 24-hr carotenoid concentration in breastmilk samples of our population. A single midafternoon sample provided an only slightly less better approximation in this study. However, due to the technical difficulties in uniformly dispersing, hydrolyzing, and analyzing milk samples, we recommend that when possible, two midafternoon samples be used. Our data are consistent with previous recommendations[32] that two midafternoon samples be used to approximate the average 24-hr lipid concentrations in breastmilk.

Carotenoids are associated with lipid in milk *(Figure 6)* and therefore enriched in hind milk. For this reason, a complete breast emptying is preferred when quantitation of total breast milk carotenoids is desired. However, when quantitating carotenoids in milk samples from partial breast emptyings, carotenoid concentrations are most appropriately expressed relative to lipid. In addition, as reported earlier for energy concentrations,[26] lipid and carotenoid concentrations are inversely related to volume. Therefore, the volume of milk that the infant will consume as well as the concentrations of nutrients in the milk should be considered when assessing adequacy of breastmilk to supply infant needs.

In summary, the major breastmilk carotenoids, β-cryptoxanthin, lycopene, α-carotene, and β-carotene have been analyzed and quantitated in mature breastmilk from 18 normal mothers. Concentrations of breastmilk carotenoids are about one-tenth those of serum and are strongly related to lipid. There is significant variation in carotenoids both within and between individuals. The largest variability between individuals (~20 fold) was seen with α-carotene and β-carotene in this population, while lycopene and β-cryptoxanthin varied the most within individuals. Carotenoid concentrations varied significantly over a 24-hr period in an individual and the concentration pattern of individual mothers varied from day to day. A reliable estimate of 24-hr concentrations is provided by two midafternoon samples.

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