ELSEVIER

Food Chemistry 72 (2001) 407–412

Food Chemistry

www.elsevier.com/locate/foodchem

Rapid Communication

Release of galactose and N-acetylglucosamine during the storage of UHT milk

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Received 25 July 2000; accepted 31 October 2000

Abstract

The changes in the concentration of monosaccharides and the influence of dephosphorylation of sugar phosphates thereon were studied in four batches of UHT milk, (two commercial and two experimental batches produced in a pilot plant) during storage at 10–30°C for 120 days. An increase in galactose and N-acetylglucosamine, together with a decrease in N-acetylglucosamine-1P and galactose-1P, were found, which were larger as the temperature of storage increased. The effects of microbial content and thermal treatment on these processes were evaluated. The contribution of dephosphorylation of the phosphorylated sugars to the total increment in N-acetylglucosamine and galactose was examined. While, in most cases, the monosaccharide increases found were consistent with the disappearance of phosphorylated sugars, changes in galactose during storage at 30°C seemed to be due to other processes, probably involving glycosidases, that may have potential detrimental consequences to milk stability.

Keywords: UHT milk; Phosphorylated carbohydrates; NAGA; Galactose

1. Introduction

The quality of UHT-processed milk is influenced by the microbiological characteristics of the raw material, the heat treatment applied and the conditions of storage. Under inappropriate storage conditions, changes in milk can be more important than those caused by heat treatment, leading to an unacceptable product (Corzo, Lopez-Fandiño, Delgado, Ramos & Olano, 1994). The biochemical processes that occur during the shelf-life of UHT milk are related, among other factors, to enzymatic activities from residual or reactivated heat-stable enzymes (lipases, proteinases, phosphatases and glycosidases) either indigenous or of bacterial origin (Andrews & Pallavicini, 1973; Burton, 1988; Fairbairn & Law, 1986; Harwalkar, 1992; Mann, Mawhinney & Marshall, 1984; Renner, 1988).

Carbohydrates present in milk include free monosaccharides, oligosaccharides, amino sugars, sugar phosphates, as well as glycosyl residues bound to both proteins and lipids (Walstra & Jenness, 1984). Free monosaccharides, such as glucose, galactose and *myo*-inositol are found at levels of 0.7–12 mg/100 ml in bovine milk (Renner, 1989) and free N-acetylglucosamine has been found at 1.1–11 mg/100 ml (Hoff, 1963; Ruas-Madiedo, Reyes-Gavilán, Olano & Villamiel, 2000; Walstra & Jenness, 1984). Levels of monosaccharides in milk can change as a consequence of thermal processing (Olano, Calvo, Ramos & Morais, 1989) and/or during storage (Ruas-Madiedo et al., 2000; Troyano, Olano & Martínez-Castro, 1994).

In milk, carbohydrate moieties linked to proteins are present on the surface of the micelles, bound to κ-casein, (Dziuba & Minkiewicz, 1996) and on the outer layer of the milk fat globule membranes (Horisberger, Rosset & Vonlanthen, 1977). These glycoproteins have a stabilizing effect and it has been suggested that removal of carbohydrates could facilitate the collapse of milk fat globules and/or the precipitation of casein micelles (Marin et al., 1984). If glycosidases were active, they could act on these complex carbohydrates, leading to the release of monosaccharides.

In a preliminary study, Recio, Villamiel, Martínez-Castro and Olano (1998) suggested that, together with

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0308-8146/01/$ - see front matter
PII: S0308-8146(00)00310-1

proteolysis, glycolysis could cause degradation of UHT milk during storage at room temperature. These authors observed a substantial increase in the contents of several monosaccharides, such as galactose, N-acetylglucosamine and N-acetylgalactosamine, in commercial UHT milks, particularly in those that showed high proteolytic activities. Thus, it was suggested that the increase in these monosaccharides could be due to the presence of residual glycosidase activities from psychrotrophs, probably as a consequence of a poor microbiological quality of the raw milk and/or to a mild UHT heat treatment.

Since milk contains free phosphorylated sugars, it is also possible that dephosphorylation of these compounds could contribute to an increase in the monosaccharide levels. Phosphatases have been shown to reactivate during the storage of UHT milk (Burton, 1988), and present a broad substrate spectrum [see BRENDA© enzyme database through ExPASy WWW server (Appel, Bairoch & Hochstrasser, 1994) from the Swiss Institute of Bioinformatics], which would include phosphorylated sugars. Therefore, an increase in monosaccharides occurring during storage of UHT milk may not only be related to the degradation of oligosaccharides from glycoproteins. Nevertheless, to the best of our knowledge, no data were found in the literature regarding the contribution of dephosphorylation to the increase in the concentration of monosaccharides during the storage of UHT milk.

The objective of this paper was to examine the levels of monosaccharides, particularly galactose and N-acetyl glucoosamine, during the storage of UHT milk, and to evaluate the contribution of dephosphorylation of their corresponding sugar phosphates thereon. The influence of the initial microbiological quality of milk and the intensity of the heat treatment on those changes were also evaluated.

2. Materials and methods

2.1. Milk samples

2.1.1. Commercial UHT milk

An industrial UHT dairy plant provided two batches of commercial direct UHT milk, one of whole milk (W; 150°C, 2.4 s) and the other one of skim milk (S; 148°C, 2.4 s). Containers (1 l) from both batches were stored at 20 and 30°C. After 0, 15, 30, 60, 90 and 120 days of storage, one container of each batch was opened for analysis.

2.1.2. Experimental UHT milk

Two UHT milk batches, E-I and E-II, were UHT processed in the pilot plant of the Veterinary School at Universitat Autónoma de Barcelona, using two raw milk batches provided from a local dairy farm on 2 consecutive days. With the aim of promoting the growth of psychrotrophs and, thus, the concentration of potentially thermoresistant enzymes, raw milks were held at 4°C for 24 h before UHT processing. Prior to heating, milk was skimmed to a level of 2.0±0.2% fat and homogenised at 20 MPa and 65°C. Heat treatments were carried out at 137°C for 4 s in a pilot UHT plant using a tubular heat-exchanger (Finamat 6500/010 Gea Finnah) at a flow rate of 1000 1/h. Heated milk was packed under aseptic conditions in 200 ml sterile containers (Tetra-Pack®). Both batches were stored at 10, 20 and 30°C. Samples were taken for analysis at 0, 15, 30, 60, 90 and 120 days of storage.

2.1.3. Inoculated sterile milk samples

To test the ability of bacteria to degrade phosphorylated sugars, one sterile and one pasteurized commercial milks were purchased from a local store. The sterile milk was used within its "use by date", and the pasteurised milk was kept at 4°C until the expiration date, to promote bacterial growth. Handling of these samples was done under sterile conditions. One control aliquot of each milk was taken at the time of package opening and analysed immediately. A second control aliquot of each milk, as well as three inoculated samples, 1:100, 1:1000, 1:10000 (pasteurised milk/sterile milk) were incubated at 30°C for 24 h. All samples were prepared and analysed for phosphorylated monosaccharides.

2.2. Microbiological analysis

Total and psychrotrophic bacterial counts were determined on raw milk by standard methods (Marshall, 1993).

2.3. Lactulose determination

Lactulose was determined by gas–liquid chromatography (GLC) of the trimethylsilyl derivatives of the free carbohydrate fraction using a 3 m×1.0 mm internal diameter, stainless steel column (Chrompack, Middelburg, The Netherlands) packed with 2% OV-17 on non-silanized 120/140 Volaspher A-2 (Merck, Damstadt, Germany), following the method described by Olano, Calvo and Reglero (1986). Phenyl-β-D-glucoside (Sigma Chemical Co., St Louis, MO) was used as internal standard.

2.4. Analysis of monosaccharides

Free monosaccharides and *myo*-inositol were analysed by GLC as their trimethylsilyl derivatives using a fused silica column (25 m×0.2 mm) coated with methyl silicone, following Troyano, Olano, Fernández-Diaz, Sanz and Martínez-Castro (1991). Methyl-α-D-galactopyranoside (Sigma) was used as internal standard.

2.5. Analysis of phosphorylated monosaccharides

Milk samples were prepared as follows: samples (1 ml), ready to be analysed, contained 800 μl of milk, 0.05 M EDTA and 20% D_2O, at pH 9.5. As samples showed turbidity due to fat, they were centrifuged at 8000 ***g*** for 30 min at 10°C in a Biofuge 22R, 3743 rotor (Heraeus, Hanau, Germany), the upper layer discarded and the sample introduced into a 5 mm NMR tube.

One-dimensional-^{1}H-decoupled ^{31}P-FT-NMR spectra were obtained at 30°C in a Varian Unity-INOVA-400 spectrometer, at a frequency of 161.892 MHz, using a 90° pulse, spectral width of 6549 Hz, delay time of 2 s, acquisition time of 1.6 s, and 1700 transients. Mathematical treatment of the raw data was done with Varian NMR software. Estimation of the concentration of N-acetylglucosamine-1-phosphate (NAGA-1P) and galactose-1-phosphate (Gal-1P) in the samples under the above spectral conditions was done by multiplying the area under the resonances of interest from milk spectra by the concentration/area ratio, that was previously obtained from the spectrum of milk with known amounts of NAGA-1P and Gal-1P.

All analyses, except for those done by NMR, were performed in duplicate.

3. Results and discussion

3.1. Characteristics of the UHT milk batches before storage

The total bacterial counts in raw milks used for batches W and S were less than 5 log CFU/ml. An estimation of the intensity of heat damage was obtained from the determination of lactulose in milk after processing. A noticeable formation of lactulose was observed in both milk batches after UHT treatment, being higher in batch W (Table 1). The levels of lactulose were within previously reported ranges for direct UHT milks (Corzo, López-Fandiño, Delgado, Ramos & Olano 1994; López-Fandiño, Corzo, Villamiel, Delgado, Olano & Ramos, 1993).

In the case of the experimental batches, E-I and E-II, to enhance the enzymatic activities during storage, raw milk of poor microbiological quality was used. Both E-I and E-II milks, held at 4°C for 24 h, showed high initial bacterial counts: 7.04 and 7.20 log CFU/ml of total bacteria and 4.86 and 5.72 log CFU/ml of psychrotrophs, respectively. The low values of lactulose in batches E-I and E-II (Table 1) were consistent with the relatively mild UHT treatment applied. Thus, although milk processing was done by an indirect UHT method, lactulose contents were similar to those previously reported for direct UHT milks (López-Fandiño et al., 1993).

In general terms, the concentrations of monosaccharides found in all batches immediately after processing (Table 1) were close to those reported in the literature for commercial UHT milks (Recio et al., 1998; Troyano, Villamiel, Olano, Sanz & Martínez-Castro, 1996). The highest levels of galactose were observed in E-I and E-II.

3.2. Changes in the monosaccharide fraction during storage

Hardly any changes were observed in the monosaccharide fraction of the commercial UHT batches (data not shown), other than a considerable increase in the concentration of galactose, found after 30 days of storage at 30°C. This increase was similar in both batches, W and S (Fig. 1).

With regard to E-I and E-II, substantial changes were observed in the concentrations of galactose and N-acetylglucosamine, which increased with time and temperature of storage (Figs. 2 and 3). The changes in galactose levels were more prominent when storage occurred at 30°C than at lower temperatures. In general, at 20 and 30°C, N-acetylglucosamine increased markedly during the initial period of storage and then reached a plateau.

Recio et al. (1998) reported that the contents of galactose, N-acetylglucosamine and N-acetylgalactosamine increased during storage at room temperature of commercial UHT milks, and suggested that the increase in these monosaccharides could be due to the presence of residual glycosidase activities from psychrotrophs. Indeed, a β-D-galactosidase was found in *Pseudomonas fluorescens* 26 which displayed a peak of maximum activity at 30°C (Marin & Marshall, 1983). Galactose is commonly found at the end of oligosaccharide chains linked to proteins, therefore glycosidases could have easy access to it (Kornfeld & Kornfeld, 1976). The presence of a high release of galactose in stored UHT samples, particularly at 30°C, may be related to the presence of UHT-resistant glycosidases.

Table 1
Concentrations (mg/100 ml) of carbohydrates in UHT milk immediately after processing

Carbohydrate	Batches			
	W[a]	S[a]	E-I[b]	E-II[b]
Lactulose	36.9	23.9	15.0	16.4
Galactose	7.2	7.1	9.1	14.5
Glucose	7.0	7.5	2.1	6.5
N-acetylgalactosamine	2.5	2.5	2.8	3.0
N-acetylglucosamine	7.7	7.6	8.4	8.1
myo-inositol	2.3	2.3	1.8	1.7

[a] W and S, whole and skim commercial UHT milks, respectively.
[b] E-I and E-II, batches I and II of experimental UHT milks, respectively.

3.3. Changes in the concentrations of phosphorylated monosaccharides during storage

Almost no changes were detected in the amounts of phosphorylated sugars in batches W and S during storage (data not shown), although a slight decrease in Gal-1P was observed at the end of the storage period at 30°C in batch S.

Together with the increase in N-acetylglucosamine and galactose, an appreciable decrease in NAGA-1P and Gal-1P was observed during the storage of the experimental UHT milk batches (Table 2). In general, dephosphorylation was more active when the temperature of storage increased from 10 to 30°C. NAGA-1P was dephosphorylated at a higher rate than Gal-1P.

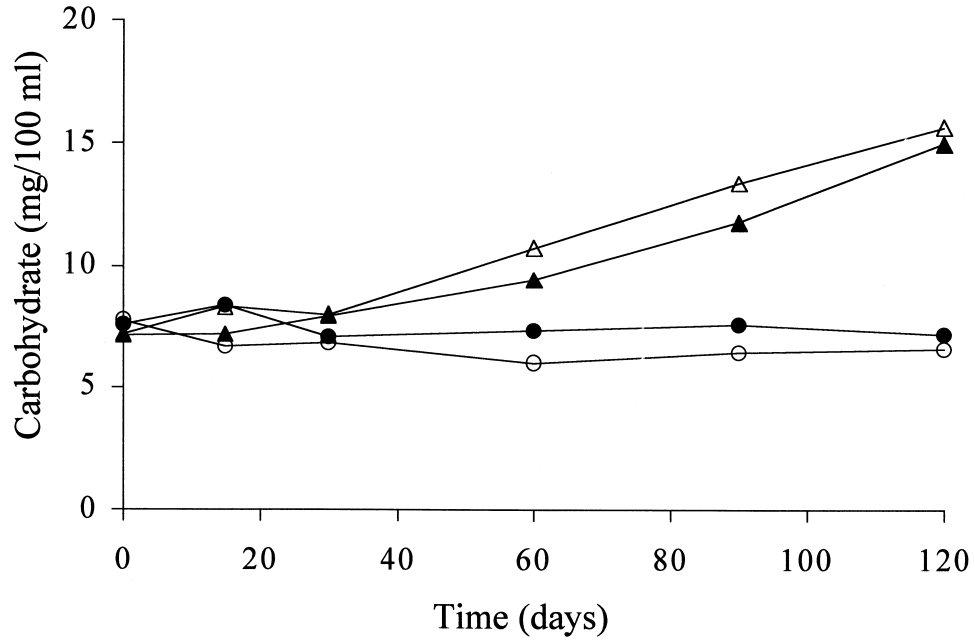

Fig. 1. Changes in the concentration (mg/100 ml) of galactose (△,▲) and N-acetylglucosamine (○,●) during storage of whole (W; △,○) and skim (S; ▲,●) commercial UHT milk at 30°C.

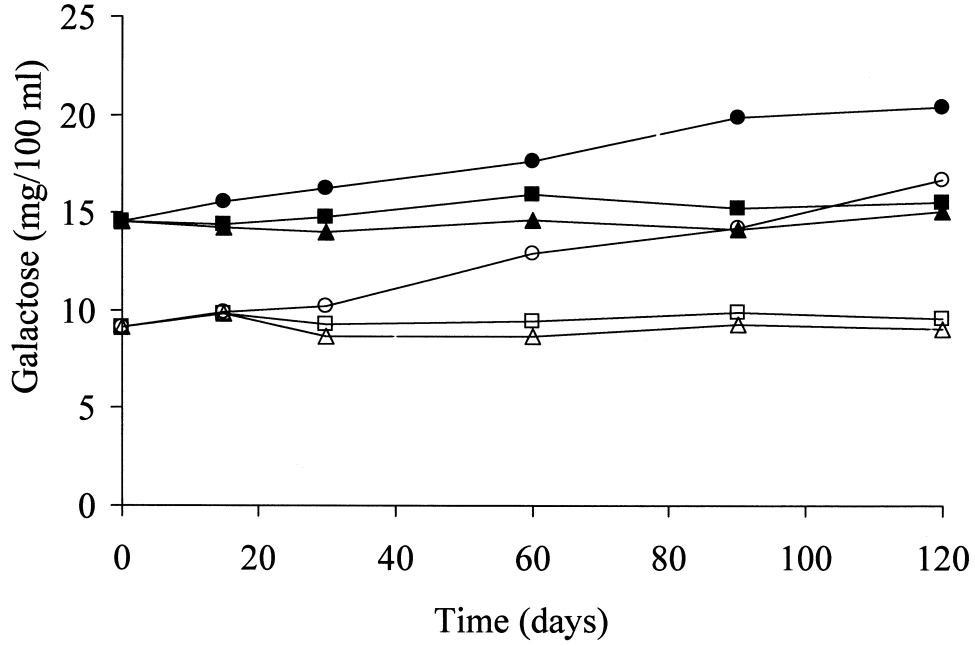

Fig. 2. Changes in the concentration (mg/100 ml) of galactose during storage of experimental UHT milk at 10 (△,▲), 20 (□,■) and 30°C (○,●). Batch E-I: (△,□,○); batch E-II: (▲,■,●).

Dephosphorylation processes can have an enzymatic origin, and some phosphatases were shown to survive UHT processing of milk (Burton, 1988). In addition, phosphatases from psychrotrophic bacteria have been found in pasteurised milk, which interfered with the detection of indigenous phosphatase activity (Knight & Fryer, 1989). We have observed that the incubation of uninoculated sterile milk at 30°C for 24 h did not cause any changes on the amounts of phosphorylated sugars, while the incubation of the same sterile milk, containing an inoculum of pasteurised milk, at a ratio of 1:10 000 and above, led to a complete dephosphorylation of both NAGA-1P and Gal-1P (results not shown). In addition, the fastest rate of dephosphorylation of sugar phosphates during storage occurred in those milk batches with the highest initial psychrotrophic counts and subjected to the mildest heat treatments (E-I and E-II). Therefore, it seems likely that heat-resistant phosphatases of microbial origin would have been responsible for the decrease of both NAGA-1P and Gal-1P.

Table 2
Concentrations (μM) of phosphorylated carbohydrates during the storage of experimental UHT milk batches

Time (days)	Batch E-I						Batch E-II					
	Gal-1P[a]			NAGA-1P[b]			Gal-1P			NAGA-1P		
	Storage temperature (°C)						Storage temperature (°C)					
	10	20	30	10	20	30	10	20	30	10	20	30
0	117	117	117	393	393	393	123	123	123	3367	367	367
15	156	109	81	383	364	262	nd[c]	117	94	nd	159	0
30	nd	125	nd	nd	256	nd	98	86	nd	217	70	nd
60	125	113	46	317	147	0	78	0	nd	94	0	nd
90	103	66	0	262	39	0	67	nd	nd	79	nd	nd
120	109	73	0	215	0	0	0	nd	0	0	nd	0

[a] Galactose-1-phosphate.
[b] N-acetylglucosamine-1-phosphate.
[c] Not determined.

3.4. Pathways for the increase in monosaccharides

In order to distinguish whether the increases in NAGA and galactose were caused by the dephosphorylation of NAGA-1P and galactose-1P, or to other processes, the potential contribution of dephosphorylation was evaluated.

During storage of the experimental batches, the decrease in NAGA-1P very closely followed similar increase in N-acetylglucosamine. This was particularly obvious during storage of E-II at 20°C: NAGA-1P disappeared after 60 days at 20°C or 15 days at 30°C (Table 2), when N-acetylglucosamine reached the maximum levels (Fig. 3). This observation could be explained by a limited availability of substrate, since once NAGA-1P was exhausted, no further increase in N-acetylglucosamine was observed. Moreover, at 20–30°C the maximum increase in N-acetylglucosamine was of about 8.5 mg/100 ml for E-I and E-II milks while, for the same samples, it was estimated that dephosphorylation of available NAGA-1P could have yielded approximately 8 mg/100 ml of N-acetylglucosamine. Therefore, the dephosphorylation of NAGA-1P appears to be the major process responsible for the increase in N-acetylglucosamine at all temperatures assayed.

A considerable decrease in the content of Gal-1P was observed during storage of E-I and E-II at 10–20°C,

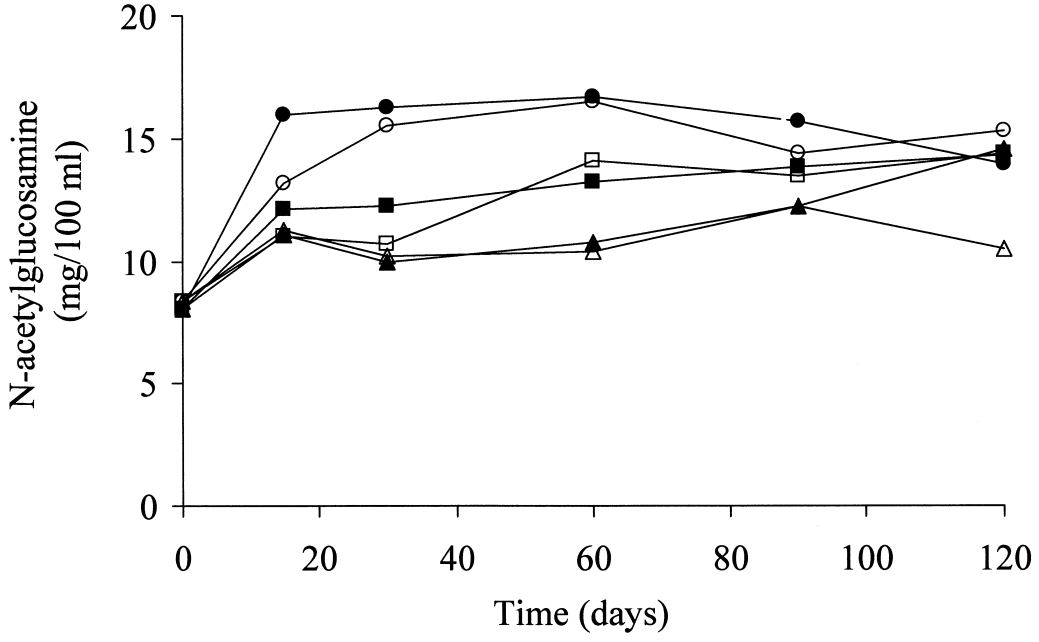

Fig. 3. Changes in the concentration (mg/100 ml) of N-acetylglucosamine during storage of experimental UHT milk at 10 (△,▲), 20 (□,■) and 30°C (○,●). Batch E-I: (△,□,○); batch E-II: (▲,■,●).

which was consistent with the amount of free galactose formed. However, at 30°C, the decrease in Gal-1P level seemed to contribute only partially to the increase in galactose content. At this temperature, while the dephosphorylation of Gal-1P contributed to a maximum of approximately 2 mg/100 ml of galactose, the actual increase in galactose found was 7.7 mg/100 ml. This suggests that, during storage at 30°C, the increase in galactose could be mainly attributed to other processes, such as glycosidase activities (Recio et al., 1998), although dephosphorylation of Gal-1P would contribute to a certain degree.

In conclusion, the increase in monosaccharides during the storage of UHT milk may be due to different processes, which can be more or less active, depending on the temperature of storage. A distinction among the different processes that can take place is important since the substrates are different. At 10 and 20°C the increase in galactose could be explained by dephosphorylation of phosphorylated sugars that, in principle, may not alter the milk characteristics. But the increase in galactose that occurred when milk was stored at 30°C came from other sources which, may contribute to destabilisation of milk. This occurred for all the samples used in this study, both commercial and experimental; thus it may be a process to consider when UHT milk is kept at temperatures of about 30°C, as frequently reached in hot countries during summer.

Acknowledgements

This work was financially supported by the Comisión Interministerial de Ciencia y Tecnología (C.I.C.Y.T.), project PETRI 95-0223-OP. J. Belloque acknowledges Comunidad Autònoma de Madrid for a fellowship. The authors thank Dr. B. Guamis and Dr. J. M. Quevedo from Universitat Autonoma de Barcelona for the pilot plant experiments. We also thank Mr. A. Alaminos for technical assistance and Dr. M. L. Jimeno for valuable advice.

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