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Thermal Kinetics Denaturation of Buffalo Milk Immunoglobulins

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Abstract: The denaturation rate of immunoglobulins (Igs) in buffalo milk was determined in the temperature range of 63-88°C for holding time 5-15 min by Single Radial Immunodiffusion (SRID) technique. Data revealed that the IgG and IgM were incompletely denatured upon heating up to 88°C for 15 min; whereas IgA was completely denatured at any of these temperatures, which means that the IgA was the most heat sensitive of Igs and these heat treatments had a destructive effect on the IgA content of milk samples. The kinetic and thermodynamic parameters for heat-induced denaturation of buffalo milk Igs were determined, which indicate that the quantity of Igs in dairy products is dependenton thermal treatment. The reaction order obtained for the thermal denaturation of IgG and IgM was second order. The velocity constant rate (k) and thermal destruction coefficient (Z) values of IgG and IgM were gradually increased, while both of the decimal reduction time (D_u) and temperature coefficient (Q_{10}) values were decreased with increasing temperature. The rate of denaturation (k) in buffalo milk Igs was in low values at low temperature 63°C when compared to this at high temperature 88°C. In contrast, D_u and Q_{10} values were higher at 63°C when compared to those at 88°C. Values of k, E_a and Q_{10} were higher in the case of IgG than those in IgM. These results should be taken into account in the design of heat treatments of milk in order to preserve the biological functions of Igs when added to formula milk or other hyperimmune products.

Key words: Buffalo milk, immunoglobulins, thermal stability and kinetic parameters

INTRODUCTION

When milk is heated, the whey proteins denature. The minor whey proteins such as lactoferrin, bovine serum albumin and the immunoglobulins begin to denature at temperatures as low as 65°C and there for significant denaturation is observed under pasteurization conditions (about 72°C 15 sec). The major whey proteins, β-Lg and α-La are more heat stable and significant denaturation occurs only at temperatures above about 70-75°C (Fox, 1995).

The kinetic and thermodynamic parameters are consistent with the denaturation or unfolding reactions being rate determining in thelow temperature ranges, whereas these parameters are consistent with the aggregation reaction being rate determining in the higher temperature ranges (Anema and McKenna, 1996; Oldfield *et al*., 1998). Also, it is known that whey protein denaturation is observed at sufficiently high pressures and like heating, denaturation levels increase with increasing pressure or duration of treatment once a threshold pressure is exceeded. Unlike the heat treatment of milk, where the minor whey proteins (lactoferrin, bovine serum albumin and the Igs) are most labile (Huppertz *et al*., 2004; Hinrichs and Rademacher, 2005; Anema *et al*., 2005).

Pasteurization of market milk is an important attainment for hygienic safety of milk. According to the FAO/WHO definition (Fox, 1989), it is a process applied to a product with the aim of avoiding

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public health hazards associated with milk by heat treatment, which is consistent with minimal chemical, physical and organoleptic changes in the product. The minimal temperature-time relations that fulfill these requirements are at 63°C 30 min or 72°C 15 sec. A more severe heat treatment is incompatible with the FAW/WHO definition and gives an unnecessary over processing. Therefore the development of suitable, sensitive methods is important for the purpose of control. Knowledge of the genuine content of Igs, their fluctuations as well as their denaturation kinetics is necessary for a quantitative evaluation.

There were five classes of Igs; IgG (IgG_1 and IgG_2), IgM, IgA, IgD and IgE with similar basic Y-shaped monomer structure consisting of four subunits, two light chains [Molecule Weight (MW) = 22-27 Kilo Dalton (KD)] and two heavy chains (MW = 54-76 KD), joined by disulfide bridges (Larson, 1992). The ones that are present in significant concentration in bovine colostrum are IgG_1, IgG_2, IgM and IgA (Walstra and Jenness, 1984). IgG_1 and IgG_2 are monomers with about the same molecular weight (146-163 KD). IgA, with a molecular weight of 385-430 KD, consists of two monomers joined with the so-called J-chain and the secretory component, whereas IgM is pentamer with a molecular weight of 1030 KD.

Although studies investigating the thermal destruction of human milk Igs have been reported (Morgan *et al*., 1986), there is a little information available for bovine and buffalo milk Igs and no systematic study on the kinetics of thermal destruction of buffalo Igs has been reported.

Therefore, the objectives of this study was to determine the heat denaturation of the buffalo milk Igs by measuring the loss of Igs using SRID technique. Also, to calculate kinetic parameters for the denaturation process which enabled prediction of the behavior of Igs in the range of temperatures for common pasteurization treatments as well as to select the best treatments in order to preserve structures.

MATERIALS AND METHODS

Milk Samples

Raw buffalo milk were collected from the dairy herd of Faculty of Agriculture, Cairo University, Giza, Egypt, during the period from October 2005 until July 2006. Immediately after complete milking of individual animal, the milk was thoroughly mixed and about 50 mL was taken in a clean stopper sample bottle in an ice box and transferred to the dairy laboratory, National Research Centre. Milk samples were analysed for Total Solids (TS) by the drying oven method at 105°C for 3 h as described by AOAC (1990). The Total Protein (TP) of milk was determined by kejldahl method (Ling, 1963). The average of three replicates was taken for each sample.

Samples Preparation

Milk samples were defatted by using the laboratory centrifuge at 4000 rpm for 30 min. Acid whey was prepared by isoelectric coagulation of casein at pH 4.6 using 1N HCl solution and centrifuging at 10000 rpm for 15 min.

Heat Treatments

About 15 mL buffalo raw milk in test tubes were covered with Para film, then heated in water bath at 63-88°C for 5-15 min. At specified time, individual test tubes were removed from the water bath and immediately cooled in an ice water bath. Each thermal treatment was conducted in triplicate.

Precipitation of Immunoglobulins with Ammonium Sulfate

Saturated Ammonium Sulfate (SAS) was prepared by dissolving excess in distilled water until some crystals of ammonium sulfate remained undissolved. Working solution of 80% ammonium sulfate

solutions was freshly prepared by diluting SAS with necessary amount of water. Equal volumes of the WPC and 40% SAS solution were mixed; the formed precipitate was removed by centrifuging, dissolved in the minimum quantity of distilled water and dialysed until the complete removal of the ammonium salt (Nawar, 1999).

Determination of Immunoglobulins Concentration

This determination was obtained using the Single Radial Immunodiffusion (SRID) as described by Mancini *et al*. (1965). Ready made plates for determination of immunoglobulin fractions (IgG, IgM and IgA) in milk samples was supplied from Bethyl Argentina.

Kinetic Calculations

The kinetic calculations of Igs were calculated as described by Anap *et al*. (1987) and Mansour, (1999).

Order of Reaction

To determine the order of the reaction responsible for the losses of Igs a graphical method was used. The concentrations of Igs during holding times were plotted according to the following equations:

Reaction of zero order:	C	=	$- kt + C_0$
Reaction of the 1st order:	$\log C$	=	$kt / 2.3 + \log C_0$
Reaction of 2nd order:	$1/C$	=	$kt + 1 / C_0$
Reaction of 3rd order:	$1/C^2$	=	$2 kt + 1 / C_0^2$

Where: C = Concentration at initial time (C_0)/Concentration at defined time (C_t)

When the form of these equations is compared with that of a straight line ($y = ax + b$) becomes clear that a plot of one of these functions versus time must be a straight line if the law of the corresponding reaction kinetics is fulfilled. The temperature/time dependent results of the measurements were therefore plotted in 4 figures with C, log C, 1/C and $1/C^2$ as ordinates and the same time intervals in each case as the abscissa.

Velocity Constants (k)

It is defined as the rate of denaturation influenced by temperature/time. It was calculated from the slopes of the obtained straight lines.

Decimal Reduction Time (D_v)

Time required to reduce the Igs concentration to 1/10 of its original value at constant temperature, was calculated for the Igs denaturation during the heat treatment between 63-88°C for 5-15 min, using the semi-logarithmic plots of Igs concentration versus holding time by the relationship: D-value = 2.303/k

Activation Energy (E_a)

The temperature dependence of reaction velocity constant has been depicted in Arrhenius plot using the k-values and temperatures according to the Arrhenius relationship with some modifications. The resulted equation can be formulated as follows:

$$\log k_2 - \log k_1 = - E_a / 2.3\ R\ (1/T_2 - 1/T_1)$$

Where: k = Velocity constant, (s^{-1})
R = Universal gas constant (8.314 KJ/mol)
T = Absolute temperature.

Thermal Destruction Coefficient (Z)

Temperature change required to alter rate of destruction, was calculated by negative reciprocal slope of the semi-logarithmic plots of D-values versus heating temperature using the relationship:

$$Z\text{-value} = 2.3\ R_t\ T_1\ T_2 / E_a$$

Temperature Coefficient (Q_{10}-value)

Denote how much faster a reaction takes place when the temperature is raised by 10°C, can be expressed as the factor by which D-value decreases for 10°C rise in temperature. In the same trend, Q_{10}-value can also be expressed by means of the Z-value according to the following relationship: $Q_{10} = 10^{10/Z}$

RESULTS AND DISCUSSION

The gross Composition of Heated Buffalo Milk

Data presented in Table 1 illustrate the Total Solids (TS) and the Total Protein (TP) of heat treatments buffalo milk samples at 63-88°C for 5-15 min.

The TS content of all treatments was lower than that of control sample. On the other hand, the TS content gradually decreased with increasing the temperature and the holding time. These results may be attributed to the heat effect in denaturating and precipitating some of milk proteins. These results were in accordance with that obtained by El-Loly (1996), who reported that the TS contents were 12.02 and 10.21% at 63°C/30 min and 100°C/10 min, respectively.

Concerning to the Total Protein (TP), the trend of heat-treated milk samples was similar to that of TS. Similar results were reported by EI-Loly (1996), who found that the TP content was 2.24 and 2.19% at 63°C/30 min and 100°C /10 min, respectively.

Also, the obtained data were proved by Singh and Waungana (2001), Macej *et al*. (2002, 2004) they reported that the heat treatment of milk during commercial processing operations results in a number of physicochemicalchanges in the milk constituents. Significant changes occurring upon heating milk above 60°C include the denaturation of whey proteins, the interactions between the denatured whey proteins and the casein micelles.

Table 1: Mean values of total solids (TS) and total protein (TP) of buffalo milk as affected by different heat treatments

Temperature (°C)	Time (min)	TS (%)	Loss (%)	TP (%)	Loss (%)
Control	-	14.28	-	3.35	-
63	5	14.12	1.19	3.13	6.57
	10	14.01	1.89	2.97	11.34
	15	13.91	2.59	2.92	12.84
68	5	14.02	1.82	3.01	10.15
	10	13.95	2.31	2.86	14.63
	15	13.85	3.01	2.77	17.31
73	5	13.86	2.94	2.91	13.13
	10	13.74	3.78	2.80	16.41
	15	13.43	5.95	2.72	18.81
78	5	13.03	8.75	2.79	16.72
	10	12.77	10.57	2.60	22.39
	15	12.48	12.61	2.53	24.48
83	5	11.92	16.53	2.56	23.58
	10	11.71	18.00	2.35	29.85
	15	11.49	19.54	2.23	33.43
88	5	11.25	21.22	2.31	31.04
	10	11.17	21.78	2.22	33.73
	15	11.02	22.83	2.18	34.93

Denaturation of Buffalo Milk Immunoglobulins

The Igs stability to heat treatment has been studied using different experimental techniques, such as chromatographic methods (Resmini *et al*., 1989; Lucisano *et al*., 1994; Law, 1995), differential scanning calorimetry (Lindstrom *et al*., 1994) and immunochemical methods(Fukumoto *et al*., 1994; Li-Chan *et al*., 1995). These techniques allowed calculation of the kinetic and thermodynamic parameters for thermaldenaturation of these proteins. The immunochemical methods that are usually employed to study the effect of heat treatment on milk Igshave been based on the reaction between the Igs in milk and the antibodies against them (Fukumoto *et al*., 1994; Li-Chan *et al*., 1995).

We studied the effects of different heat treatments on the structure changes that occur in buffalo milk Igs by measuring the loss of Igs using SRID technique.

Although immunoglobulins make up only 1-2% of the total milk protein, they are very important in the processing of milk into dairy products. Their sensitivity to heat means that can be readily modified by thermal treatments that are part of normal dairy processes. Therefore, it is very interesting to study the effect of thermal treatments on their contents of immunoglobulins.

No precipitation was observed during or after any of heat treatments under study. It may be suggested that the reduction in Igs concentration determined by SRID was due to denaturation or unfolding of the Igs molecules as a result of heat treatments, leading to loss of antigenicity. Although aggregation reactions subsequent to denaturation may have occurred, detection of precipitation would be difficult given the low levels of Igs.

Table 2, showed the degree of denaturation of Igs that increased with temperature of treatment. It could be noticed that the heating caused some losses in Igs. A comparison of immunoglobulin fractions IgG, IgM and IgA content in raw milk and corresponding heated milk indicated that IgG has higher values than IgM and IgA in the control samples. The loss of these fractions after any heat treatment was higher in IgA than IgM and IgG. These results may be attributed to that IgA is the most heat sensitive Igs. Also, IgA non of it was retained in the heat-treated samples, which may be attributed to that IgA the less heat stable Igs and to its low concentration in raw milk. It could be concluded that the Igs were the least heat stable of the whey proteins in milk. This finding was similar to the reported results by Resmini *et al*. (1989) they proved that the Igs were the most heat sensitive components of the whey protein fractions above at 70°C. Also, whey proteins are heat-labile proteins (Fox and McSweeney, 1998). Also, De Wit *et al*. (1983) and De Wit and Klarenbeek (1984) reported the same previous observations, which may be attributed to the high content of disulfide bridges. They also suggested that unfolding of Igs was irreversible and IgA was the most sensitive of the Igs. On the

Table 2: Effect of different heat treatments on the stability of buffalo milk Igs

		Immunoglobulins (mg mL^{-1})					
Temperature (°C)	Time (min)	IgG	Loss (%)	IgM	Loss (%)	IgA	Loss (%)
Control	-	8.71	-	1.91	-	0.04	-
63	5	7.21	17.22	1.23	35.60	0	100
	10	7.09	18.60	1.21	36.65	0	100
	15	6.96	20.09	1.18	38.22	0	100
68	5	6.99	19.75	1.17	38.74	0	100
	10	6.92	20.55	1.15	39.79	0	100
	15	6.81	21.81	1.12	41.36	0	100
73	5	6.88	21.01	1.13	40.84	0	100
	10	6.81	21.81	1.11	41.88	0	100
	15	6.72	22.85	1.08	43.46	0	100
78	5	6.50	25.37	1.08	43.46	0	100
	10	6.42	26.29	1.03	46.07	0	100
	15	6.35	27.10	0.97	49.21	0	100
83	5	5.16	40.76	0.77	59.69	0	100
	10	5.08	41.68	0.73	61.78	0	100
	15	4.92	43.51	0.68	64.40	0	100
88	5	4.21	51.66	0.56	70.68	0	100
	10	4.15	52.35	0.45	76.44	0	100
	15	4.02	53.85	0.40	79.06	0	100

other hand, Lindstrom *et al.* (1994) and Ustunol and Sypien (1997) had demonstrated the denaturation of bovine milk Igs at 80°C and unfolding of IgG and IgM at near that temperature. Whereas, De Wit *et al.* (1983) reported it was at 79°C. While, the IgG fraction was reported to denaturate at 72°C (De Wit and Klarenbeek, 1984).

According to Snezana *et al.* (2007) the investigation of the heat treatments induces the interactions between the major milk proteins and also the formation of co-aggregates. Co-aggregates formed during heat treatments are mostly the result of the disulfide interaction among κ-casein, β-lactoglobulin and α-lactalbumin. Besides, other types of interactions alsoare involved in their formation.

On the other hand, data revealed that commercial pasteurization process results incomplete denaturation of IgG and IgM. But, it had a destructive effect on IgA content of milk samples. The obtained data were in accordance with that reported by Li-Chan *et al.* (1995) and EI-Loly (1996). These results were in accordance with reported by Ustunol and Sypien (1997), who mentioned that the treatment at 80°C for 25 min completely destructive IgA. It is clear from these results that the IgG was the major Igs in milk. Butler (1969), EI-Loly (1996), Fernandez *et al.* (2006) and McMartin *et al.* (2006), they reported the same observation of suffered greater losses of IgG.

The results were contrary to those of Kanno *et al.* (1976), who mentioned that the heating at 70°C for 10 min completely destroyed the agglutinating activity of IgM. We confirmed results of Li-Chan *et al.* (1995) and El-Loly (1996) that commercial processing such as High Temperature Short Time Heating (HTST) pasteurization may not destroy Igs activity of milk. The slight differences were probably due to differences in experimental procedures and heating rates.

Thermal and processing stability of bovine milk Igs have been of interest to preserve the immunoprophylactic or potential of Igs in cow milk. The Igs particularly IgG have been suggested for immunological supplementation of infant formula and other foods (Facon *et al.*, 1993). Li-Chan *et al.*, (1995), they reported that the thermal stability of bovine serum Igs occurred in model systems and in commercially processed milk products. These findings indicatethat the quantity of Igs in dairy products is dependenton thermal treatment.

Data revealed that the IgG and IgM were incompletely denaturation upon heating to 88°C for 15 min, whereas IgA was completely denaturation at any these temperatures, which means that the IgA was the most heat sensitive of Igs and these heat treatments had a destructive effect on the IgA content of milk samples. These results are in accordance with Ustimol and Sypien (1997).

Kinetic Calculations

The concentration of denatured Igs at each measurement time was subjected to kinetic analysis. From the graphics in Fig. 1 and 2 it is observed that order of reaction for the thermal denaturation of

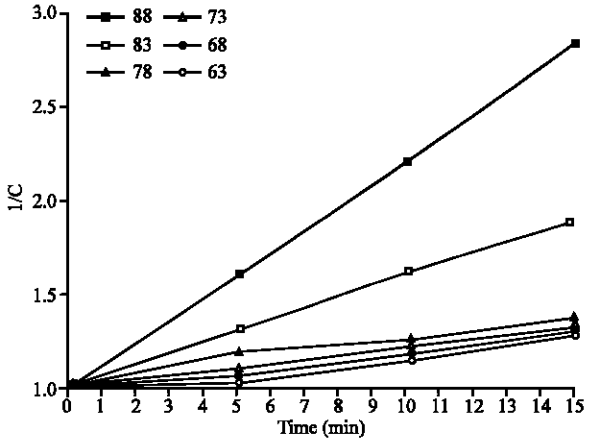

Fig. 1: Plot according to 2nd order reaction of IgG loss

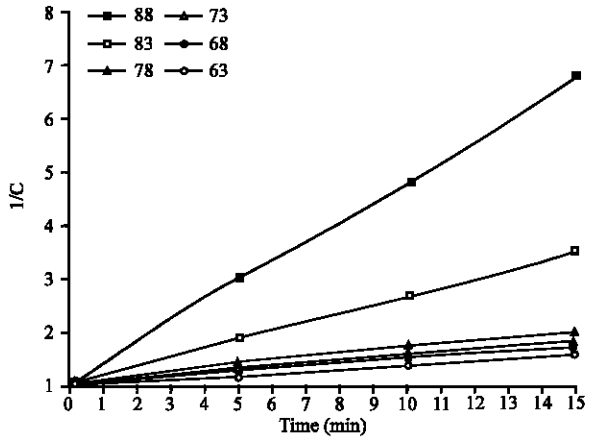

Fig. 2: Plot according to 2nd order reaction of IgM loss

Table 3: The velocity constants (k) and decimal reduction time (Dυ) of immunoglobulins loss reaction at the different treatments of buffalo milk

	K (s^{-1})		D-value (s)	
Temperature (°C)	IgG	IgM	IgG	IgM
63	0.074	0.063	31.12	36.56
68	0.095	0.065	24.24	35.43
73	0.098	0.079	23.50	29.15
78	0.122	0.093	18.88	24.76
83	0.285	0.219	8.08	10.52
88	0.608	0.507	3.79	4.54
Mean	0.214	0.171	18.27	29.59

both IgG and IgM obey the second order reaction during the temperature range of 63-88°C. This finding is in full agreement with that ascribed by Resmini *et al*. (1989) for IgG. But Dominguez *et al*. (1997) found that the denaturation of IgG was best described assuming an apparent reaction order of 1.5.

The velocity constants (k) can be calculated from the slopes of the obtained straight lines. The calculated values of k in s^{-1} of this reaction throughout heat treatments of milk samples at 63-88°C are presented in Table 3. It can be observed that the (k) have IgG and IgM denaturation gradually increased with increasing of heat treatments. Mainer *et al*. (1997) observed the same finding.

The higher values of the rate velocity (k) were observed for IgG than IgM suggest that the denaturation occurs more quickly for IgM than IgG molecules. Generally, the k values for the denaturation in IgG and IgM ranged from 0.074 to 0.608 (with mean value of 0.214) and from 0.063 to 0.507 (with mean value of 0.171), respectively.

The decimal reduction times (D-values) of all studied milk samples were calculated for IgG and IgM during heat treatment between 63 and 88°C, using the plot of Igs concentration holding time.

The slope of each temperature line (K/2.303) was calculated. The reciprocal of these values could be expressed as D-value of each sample. From these data, it is found that the D-values for the denaturation of IgG and IgM were decreased with temperature increasing in all samples. Also, it is clear that the D-values were higher in IgM than those in IgG Table 3. Generally, the D-values of IgG and IgM ranged from 3.79 to 31.12 sec (with mean value of 18.27 sec) and from 4.54 to 36.56 sec (with mean value of 29.59 sec), respectively.

Table 4 gives the mean values of the calculated activation energy (E_a) in the temperature range between 63 and 88°C. Data declared that the highest activation energy required to complete the loss reaction of Igs was in IgG throughout heat treatments of milk samples at 63-88°C. These values were

Table 4: The energy of activation (E_a) of immunoglobulins loss reaction at the different treatments of buffalo milk

Immunoglobulins	E_a (KJ mol^{-1}. K)
IgG	87.455
IgM	86.595

Table 5: Thermal destruction coefficient (Z) and temperature coefficient (Q_{10}) values of immunoglobulins loss reaction at the different treatments of buffalo milk

	Z-value (°C)		Q_{10}-value	
Temperature range (°C)	IgG	IgM	IgG	IgM
63-68	25.05	25.30	2.51	2.48
68-73	25.80	26.05	2.44	2.42
73-78	26.55	26.82	2.38	2.36
78-83	27.32	27.59	2.32	2.30
83-88	28.10	28.38	2.27	2.25
Mean	26.56	26.83	2.38	2.36

87.455 and 86.595 KJ mol^{-1}. K for IgG and IgM, respectively. These results were similar with those reported by Chen *et al*. (2000). It may be indicate that the IgG and IgM were more stable than IgA because a larger amount of energy is needed in order to start denaturation.

IgM showed larger of Z-values than IgA, this finding was opposite with those reported by Chen *et al*. (2000). These results suggested slightly greater stability of IgG than IgM. On the other hand, the values gradually increased with increasing heat treatments. The higher Z and lower E_a values of IgM suggest that the less temperature dependence of the rate of IgG destruction than the rate of IgM (Table 5).

The calculated data of Q_{10} values of denaturated Igs in buffalo milk during the heat treatments are tabulated in Table 5. The results were varied from 2.27 to 2.51 (with mean value 2.38) for IgG and from 2.25 to 2.48 (with mean value 2.36) for IgM. On the other hand, the values decreased with increasing the range of heat treatments.

This could be correlated with the variations between the loss rates in different Igs during the temperature range (63-88°C).

From the previous data, it concluded that the k, E_a and Q_{10} values were higher for IgG than those for IgM at any heating temperature, while the D and Z-values were higher for IgM than those for IgM at the one.

CONCLUSIONS

Generally, data revealed that should be taken into account in the design of heat treatments of milk in order to preserve the biological functions of Igs when added to formula milk or other hyperimmune products. The degree of Igs denaturation increase with increasing treatment temperature. The Igs were the most heat sensitive of whey protein components, which may be attributed to the high content of disulfide bridges or that unfolding of Igs was irreversible. While, IgA was the most sensitive of the Igs.

Kinetic reactions for Igs were highly affected by heating. The reaction order obtained for the thermal denaturation of IgG and IgM were second order. The k and Z-values were increased, while D and Q_{10}-values were decreased with increasing heat treatments of buffalo milk Igs. Values of k, E_a and Q_{10} were higher in the case of IgG than those in IgM at any temperatures, but for the, D and Z-values were in opposite trend. These parameters are considered as important indicator for quantitative describing the mechanism of denaturation occurs during heat treatments.

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