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# Oxidative damage to proteins related to metals and antioxidant defenses in breastmilk

*Daño oxidativo a las proteínas relacionado con metales y defensas antioxidantes en la leche materna*

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## Abstract

**Introduction:** Breast milk contains molecules needed for the development of children; the integrity and function of these molecules is affected by the presence of pro-oxidants. Protein carbonyls are mainly produced as a result of the interaction of metals with reactive oxygen species (ROS), which may initiate a chain reaction that promotes molecular oxidation.

**Objective:** This study aimed to determine the association between the concentration of protein carbonyls with the concentration of trace elements (lead [Pb], cadmium [Cd] and selenium [Se]), superoxide radical ($O_2^{\bullet-}$) production, and glutathione (GSH) content, as well with the activity of the main antioxidant enzymes (superoxide dismutase [SOD], catalase [CAT], glutathione peroxidase [GPx], glutathione reductase [GR] and glutathione S-transferase [GST]) in breast milk.

**Methods:** In this study 108 transitional milk samples (7-10 days) were analyzed. Antioxidant enzyme activities, $O_2^{\bullet-}$ production, protein carbonyl and GSH concentrations were analyzed by spectrophotometry. Trace element concentration was quantified by atomic absorption spectrophotometry. Generalized linear modelling was used to assess the relationship between protein carbonyls concentration with oxidative stress indicators and trace elements concentration.

**Results:** Cd and Pb were detected in 21.3 and 55.6% of breast milk samples, respectively. The median concentration of Cd was 0.01 μg $L^{-1}$ (0.01-3.52 μg $L^{-1}$) and Pb concentration was 2.61 μg $L^{-1}$ (0.08-195.20 μg $L^{-1}$). According to the best-fit model, the main factors contributing to protein carbonyl concentrations were the activity of GPx, GR, and concentration of GSH, Se, Pb and Cd.

**Conclusions:** According to the generalized linear model, the activity of GPx and GR, could help explain protein oxidation induced by Pb and Cd in breast milk.

**Key words:**

Antioxidants. Breast milk. Protein carbonyls. Trace elements.

## Resumen

**Introducción:** la leche materna contiene las moléculas necesarias para el desarrollo de los niños; la integridad y función de estas moléculas se afecta por la presencia de prooxidantes. Los carbonilos proteicos se producen principalmente como resultado de la interacción de metales con especies reactivas de oxígeno (ERO), los cuales pueden iniciar una reacción en cadena que promueve la oxidación molecular.

**Objetivo:** este estudio tiene como objetivo determinar la asociación entre la concentración de carbonilos proteicos con la concentración de elementos traza (plomo [Pb], cadmio [Cd] y selenio [Se]), producción de radical superóxido ($O_2^{\bullet-}$), y contenido de glutatión (GSH), así como con la actividad de las principales enzimas (superóxido dismutasa [SOD], catalasa [CAT], glutatión peroxidasa [GPx], glutatión reductasa [GR] y glutatión S-transferasa [GST]) en leche materna.

**Métodos:** en este estudio se analizaron 108 muestras de leche de transición (7-10 días). La actividad de las enzimas antioxidantes, producción de $O_2^{\bullet-}$, concentración de carbonilos proteicos y GSH se analizaron por espectrofotometría. La concentración de elementos traza se cuantificó por espectrometría de absorción atómica. Se utilizó un modelo lineal generalizado para evaluar la relación entre la concentración de carbonilos proteicos con los indicadores de estrés oxidativo y las concentraciones de elementos traza.

**Resultados:** Cd y Pb fueron detectados en 21,3 y 55,6% de las muestras de leche materna, respectivamente. La mediana de la concentración de Cd fue 0,01 μg $l^{-1}$ (0,01-3,52 μg $l^{-1}$) y para la concentración de Pb fue 2,61 μg $l^{-1}$ (0,08-195,20 μg $l^{-1}$). De acuerdo con el modelo de mejor ajuste, los principales factores de afectan la concentración de carbonilos proteicos, son la actividad de GPx y GR, y las concentraciones de GSH, Se, Pb y Cd.

**Conclusiones:** de acuerdo con el modelo lineal generalizado, la actividad de GPx y GR podría ayudar a explicar la oxidación proteica, inducida por Pb y Cd en leche materna.

**Palabras clave:**

Antioxidantes. Leche materna. Carbonilos proteicos. Elementos traza.

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## INTRODUCTION

Breast milk is the food most recommended for children, particularly during the first 6 months of life. Although the composition of milk is variable along the duration of lactation, it contains molecules, such as carbohydrates (70 g $L^{-1}$), lipids (38 g $L^{-1}$) and proteins (9 g $L^{-1}$), needed for the child's development (1,2). Depending on their composition, proteins in breast milk have different functions; some of the most studied proteins are casein (stimulation of the immune system); $\alpha$-lactalbumin (synthesis of lactose); lactoferrin (bacteriostatic effect on intestine); immunoglobulins (Ig) (antibody formation); enzymes (bactericides, hydrolytic, antioxidants) among other proteins (1,3).

Integrity and function of the proteins in breast milk is affected by the presence of pro-oxidants, such as metals and trace elements (4). The protein carbonyls are mainly produced as a result of oxidation of amino acid side chains, 450 sites with high probability of carbonylation have been registered (5). This irreversible process is commonly a result of the interaction of metals with reactive oxygen species (ROS) (5). Production of ROS is carried out naturally as a result of aerobic metabolism, but it may increase in the presence of certain elements, such as lead (Pb) and cadmium (Cd), which have been reported in breast milk (6). These elements, upon contact with ROS, can induce Haber-Weiss and Fenton reactions generating new radicals and initiating a chain reaction that promotes molecular oxidation (4,5). However, antioxidant molecules in breast milk counteract oxidative damage to proteins and other functional molecules. The main antioxidant enzymes reported in breast milk are superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione S-transferase (GST). Glutathione (GSH) is another antioxidant present in breast milk, which has been extensively studied because of its interaction with glutathione-dependent antioxidant enzymes (7). In addition, breast milk contains selenium (Se), a microelement needed for Se-dependent antioxidant enzymes including GPx and GR (8).

Mathematical models are currently used in biology to describe processes, by including various factors, in order to predict or explain in a simple manner a biological phenomenon. Multivariate statistics allows evaluating multiple independent variables at the same time, simplifying the analysis (9). To find the contribution of the predictor variables with respect to the response variable, when the assumptions of normality are not met, it is recommended to use generalized linear models (GLM) (10). In previous studies, GLM is used to describe the relationship between trace elements and antioxidant defenses in various tissues (11-13). This study aimed to determine the association between the concentration of protein carbonyls with the concentration of trace elements (Pb, Cd and Se), $O_2^{\bullet-}$ production and GSH content, as well as with the activity of the main antioxidant enzymes (SOD, CAT, GPx, GR and GST) in breast milk of women of Baja California Sur.

## METHODS

### STUDY GROUP

Breast milk samples were collected from 108 lactating women in La Paz, Baja California Sur, México, who were between the first and second week postpartum therefore it was transitional milk (7-10 days postpartum). Breast milk samples were collected at home by a volunteer nurse. Samples were collected in plastic tubes of about 35 mL, with an extractor, and then these were refrigerated and were transported to the laboratory to be stored at -80 °C until analysis. Informed consent was obtained and a specialized questionnaire was completed. Both the informed consent and questionnaire were approved by Capítulo Baja California Sur de la Academia Nacional Mexicana de Bioética A. C. The sampling included healthy women who were in their second week of lactation. The age of mothers was 15 to 44 (26.3 ± 6.07) years old. Samples (approximately 35 mL) were collected with the help of a nurse and were immediately stored at -80 °C.

### SAMPLE ANALYSES

#### Antioxidant enzyme activity

##### *Superoxide dismutase (SOD, EC 1.15.1.1) activity*

The activity of superoxide dismutase (SOD) was quantified using the method of Suzuki (2000) (14), based on the inhibition of the reduction of nitroblue tetrazolium (NBT). One unit (U) of SOD activity is defined as the amount of enzyme required to inhibit the maximum reaction by 50%. Results are shown in U $mg^{-1}$ protein.

##### *Catalase (CAT, EC 1.11.1.6) activity*

The activity of catalase (CAT) was determined following the disappearance of $H_2O_2$ at 240 nm (15). One unit of CAT activity is defined as the quantity of enzyme required to reduce 1 µM $H_2O_2$ per minute. Results were expressed in U $mg^{-1}$ protein.

##### *Glutathione peroxidase (GPx, EC 1.11.1.9) activity*

Glutathione peroxidase (GPx) activity was evaluated spectrophotometrically, the decrease in the concentration of reduced nicotinamide adenine dinucleotide phosphate (NADPH) was measured in a coupled assay with glutathione reductase (GR) (16,17). One unit of GPx activity is defined as the amount of enzyme that oxidizes 1 µM NADPH per minute. Results are shown in U $mg^{-1}$ protein.

### *Glutathione S-transferase (GST, EC 2.5.1.18) activity*

Glutathione S-transferase (GST) activity was determined by following the formation of dinitrobenzene glutathione thioether complex formed by the conjugation of GSH with 1-chloro 2,4- dinitrobenzene (CDNB) (18). One unit of GST activity is defined as the amount of enzyme that catalyzes the conjugation of 1 μM CDNB. Results are shown in U $mg^{-1}$ protein.

### *Glutathione reductase (EC 1.8.1.7) activity*

The activity of glutathione reductase (GR) was measured as the reduction in the absorbance of NADPH (17,19). One unit of GR activity is defined as the amount of enzyme that oxidizes 1 μM NADPH per minute. Results are shown in U $mg^{-1}$ protein.

## TOTAL PROTEIN CONTENT

Protein content in milk samples was measured for the purpose of standardizing the data. The method of Bradford (20) was applied, by using a Bio-Rad© kit (Laboratories Hercules, CA) adapted to microplate. All results are expressed in milligrams per milliliter (mg $mL^{-1}$).

## GLUTATHIONE CONCENTRATION

To determine the GSH content, the method of Griffith (21), as modified by Hermes-Lima and Storey (22) was used. This method follows the detection of the product formed by the reaction of GSH with 5'5-dithiobis 2-nitrobenzoic acid (DTNB). The results were expressed in nmol of glutathione equivalents (GSH-Eq) per milligram of protein.

## SUPEROXIDE RADICAL PRODUCTION

Superoxide radical ($O_2^{\bullet-}$) production was quantified following the reduction of ferricytochrome c to ferrocytochrome c (23). Data were expressed in nmol of $O_2^{\bullet-}$ per milligram of protein per minute.

## PROTEIN CARBONYL CONCENTRATION

Oxidative damage to proteins was determined as the concentration of protein carbonyls. The complex formed between protein carbonyl derivatives and 2, 4-dinitrophenyl hydrazine (DNPH) was measured (24). The protein carbonyl concentration is shown in μmol of protein carbonyls per milligram of proteins.

## TRACE ELEMENTS

Trace element concentration is quantified by atomic absorption spectrophotometer (25). Approximately 10 mL (≈ 10 g) of whole milk were digested with 70% nitric acid ($HNO_3$) and 30% $H_2O_2$ in a microwave (Mars 5x, CEM, Matthew, North Carolina, USA) and then filtered. The concentrations of Se, were measured using a hydride system coupled to an atomic absorption spectrophotometer (XplorAA, GBC, Australia); Cd and Pb were quantified by atomic absorption spectrophotometer (HG 3000, GBC, Australia) using an air acetylene flame. The analysis was performed in duplicate. Detection limits were 0.5 μ $L^{-1}$ for Se, 0.011 mg $L^{-1}$ Cd and 0.15 mg $L^{-1}$ Pb. A recovery rate ≥ 90% was obtained.

## STATISTICAL ANALYSES

The descriptive statistics, median, range, 25th and 75th percentiles were evaluated. In the case of data of trace elements that fell below the detection limit, the half-value of the specific detection limit for each variable was used to fulfill base data (26). Data distribution was assessed by Kolmogorov-Smirnov normality tests. The relationship between variables was explored using Spearman correlations.

A generalized linear regression model analysis (GLM) was chosen given lack of normality of data ($p < 0.05$) (10). Considering that the variables were continuous and positive, a gamma distribution error was used due to its flexibility, from a curve with bias to the right (when, in relation to $\mu^2$, the dispersion parameter *v*, is smaller) to a symmetrical Gaussian curve (for larger values of $\nu$) (27). A GLM was performed in order to explain the relationship between protein carbonyl concentration (response variable) with the explanatory variables, activity of antioxidant enzymes (GPx, GR, SOD, CAT), $O_2^{\bullet-}$ production rate, and concentration of GSH, Se, Cd and Pb. A *log* canonical link function was used in order to transform the response variable, allowing a linear relationship between the dependent variable and independent variables (28,29). The GLM was performed starting from a maximal model including the variables mentioned above ($k = 9$) ($\beta = 30.522$, standard error = 6.7386, $p < 0.01$, scale 439.947, residual deviance = 47514.298). In order to obtain the minimal adequate model a backwards procedure was employed; the alternative models were evaluated by using the deviance as criterion of goodness of fit and considering the contribution of each variable to the model, the residuals as validation method were used, although these were not showed in this paper. Statistical analyzes were performed using SPSS (version 20), and statistical significance was considered at $\alpha = 0.05$.

## RESULTS

Antioxidant enzyme (GPx, GR, SOD and CAT) activities, $O_2^{\bullet-}$ production, and protein carbonyl, GSH, Se, Pb and Cd concentrations are shown in table I. Among the trace elements data, Cd concentration was below the detection limit in 78.7% of the samples and Pb concentration in 44.4% of the samples.

The Spearman correlation matrix suggests correlations between trace element concentrations and oxidative stress indicators (Table II).

**Table I.** Antioxidant enzymes activity, $O_2^{\bullet-}$ rate of production and protein carbonyl, selenium, lead and cadmium concentration in breast milk of women lactating (n = 108) of Baja California Sur, México

| Variable | Median | Range | P25 | P75 | < DL |
|---|---|---|---|---|---|
| PC | 21.690 | 0.56-99.24 | 6.43 | 47.52 | |
| GPx | 0.06 | 0.00-10.20 | 0.02 | 0.21 | |
| GR | 0.02 | 0.00-7.32 | 0.01 | 0.05 | |
| SOD | 198.17 | 1.65-2117.96 | 39.98 | 775.45 | |
| CAT | 0.25 | 0.00-4.58 | 0.10 | 0.49 | |
| $O_2^{\bullet-}$ | 0.01 | 0.00-0.16 | 0.00 | 0.02 | |
| GSH | 21.95 | 1.42-253.43 | 12.18 | 38.35 | |
| Se | 19.79 | 6.32-56.13 | 15.34 | 27.81 | |
| Pb | 2.61 | 0.08–195.20 | 0.08 | 12.87 | 44.4% |
| Cd | 0.01 | 0.01-3.52 | 0.01 | 0.01 | 78.7% |

*P25-P75: 25th and 75th percentiles; PC: protein carbonyl concentration, µmol mg-1 protein; GPx: glutathione peroxidase activity, U mg-1 protein; GR: glutathione reductase activity, U mg-1 protein; SOD: superoxide dismutase activity, U mg-1 protein; CAT: catalase activity, U mg-1 protein; $O_2^{\bullet-}$: rate of production of superoxide radical, nm min-1 mg-1 protein; GSH: glutathione concentration, nmol mg-1 protein; Se: selenium concentration, µg L-1; Pb: lead concentration, µg L-1; Cd: cadmium concentration, µg L-1.*

**Table II.** Spearman correlation coefficients between indicators of oxidative stress and trace element concentrations in breast milk

| | Se | Cd | Pb |
|---|---|---|---|
| PC | 0.28 | 0.02 | -0.27 |
| GPx | -0.24 | 0.22 | |
| SOD | -0.2 | | |
| Se | | | -0.24 |
| Cd | | | 0.25 |
| Pb | -0.24 | 0.25 | |

*TBARS: thiobarbituric acid reactive substances; CP: protein carbonyl; GPX: glutathione peroxidase; SOD: superoxide dismutase; GR: glutathione reductase; GST: glutathione-S- transferase; CAT: catalase; GSH: glutathione; SE: selenium; CD: cadmium; PB: lead. Statistically significant results (p < 0.05).*

Concentration of Se was significantly and positively correlated with protein carbonyl levels ($r = 0.33$, $p < 0.05$) and negatively correlated with SOD activity ($r = -0.21$, $p < 0.05$). The concentration of Cd was positively related to the activity of GPx and to Pb levels ($r = 0.22$ and $r = 0.24$, $p < 0.05$, respectively). Pb concentration was negatively correlated with protein carbonyl content ($r = -0.27$, $p < 0.05$) and Se concentration ($r = -0.24$, $p < 0.05$) correlated with the activity of GPx, and was positively correlated with Cd concentration ($r = 0.25$, $p < 0.05$) (Table II).

Minimal adequate model included activity of the antioxidant enzymes GPx and GR, concentration of GSH and trace elements Se, Pb and Cd ($\beta = 19.570$, Est error = 5.2839, $p < 0.01$, scale 446.731, residual deviance = 51486.957) (Table III). Fitted model explained 38% of the variance of the response variable. The equation of the minimal model fitted (Eq. 1) predicted a median value of protein carbonyl concentration of 21.689 µmol mg$^{-1}$ in comparison with the observed median (29.849 µmol mg$^{-1}$), therefore the model fits the data in a 37.6%.

$$CP = e^{19.570-3.995(GPx)-4.715(GR)-0.136(GSH)+0.759(Se)+0.179(Pb)-10.913(Cd)} \quad (1)$$

## DISCUSSION

In the present study, the median concentration of Cd was 0.01µg L$^{-1}$, which is below the limit allowed by the World Health Organization (WHO, 1 µg L$^{-1}$) (30). The values reported by Gundacker et al. (2006) were 0.086 µg L$^{-1}$ of Cd; decreased concentration of Cd has been associated with the intake of supplements, which contain vitamins and minerals that are likely to improve the activity of antioxidant enzymes or are antioxidants themselves (6). Cd itself is not able to generate ROS, but it affects ROS production, and the cellular redox state; therefore, causing a state of oxidative stress (31). In addition, Cd has the ability to replace the iron (Fe) in protein structures (31). Cd is responsible for increasing the concentration of free Fe which, via the Fenton reaction, can produce ROS such as $^{\bullet}OH$. Moreover, Cd binds to the thiol groups in proteins, including GSH (31). The binding of Cd to sulfhydryl groups can cause the decrease of functional GSH. Prolonged exposure to Cd can increase ROS production, affecting antioxidant defenses because it can also affect the structure and function of GSH-dependent antioxidant enzymes (31,32). In the present study, a positive correlation between the concentration of Cd and GPx activity ($r = 0.22$) was found. It would be expected that GPx activity diminished with increased Cd and the corresponding decrease in

**Table III.** Coefficients of the minimal model fitted in a generalized linear model analysis (GLM) (k = 6) with gamma error distribution for protein carbonyl (PC) concentration (μmol $mg^{-1}$ protein) in breast milk of women of Baja California Sur, México (n = 108)

| Response variable | Parameters | β | Standard error | 95% Wald confidence interval | | Hypothesis test | | |
|---|---|---|---|---|---|---|---|---|
| | | | | Lower | Superior | Wald Chi-square | df | p |
| PC | (Intercept) | 19.570 | 5.2839 | 9.214 | 29.927 | 13.717 | 1 | < 0.01 |
| | GPX | -3.995 | 1.5900 | -7.111 | -0.878 | 6.312 | 1 | 0.01 |
| | GR | -4.715 | 1.8115 | -8.266 | -1.165 | 6.776 | 1 | < 0.01 |
| | GSH | -0.136 | 0.0553 | -0.245 | -0.028 | 6.089 | 1 | 0.01 |
| | Se | 0.759 | 0.2231 | 0.322 | 1.196 | 11.577 | 1 | < 0.01 |
| | Pb | 0.179 | 0.0439 | 0.093 | 0.265 | 16.578 | 1 | < 0.01 |
| | Cd | -10.913 | 2.8405 | -16.480 | -5.345 | 14.759 | 1 | < 0.01 |
| | Scale | 476.731 | 64.8749 | 365.123 | 622.454 | | | |

*df: Degrees of freedom; PC: protein carbonyl concentration, μmol $mg^{-1}$ protein; GPx: glutathione peroxidase activity, U $mg^{-1}$ protein; GR: glutathione reductase activity, U $mg^{-1}$ protein; GSH: glutathione concentration, nmol $mg^{-1}$ protein; Se: selenium, μg $L^{-1}$; Pb: lead, μg $L^{-1}$; Cd: cadmium, μg $L^{-1}$.*

GSH concentration. This is in contrast with what was reported by Cuypers et al. (2010) (31).

The median concentration of Pb reported in this study was 2.61 μg $L^{-1}$, below the limit established by WHO (33). Furthermore, the presence of elevated concentrations of Pb affects the activity of antioxidant enzymes such as GR, GPx, CAT, among others (34). Lead has an affinity for thiol groups, including that of GSH, and affects the molecule's function, and consequently can affect the activity of antioxidant enzymes that use a thiol group as hydrogen donor (GPx and GST) (34). Lead also forms a complex with Se, consequently the concentration of free Se is reduced. Se is a cofactor of the selenoprotein GPx; therefore, its activity is indirectly affected by Pb. CAT is another antioxidant enzyme the activity of which can be affected by Pb, because the latter inhibits the synthesis of heme, which is a basic component in the structure of this antioxidant enzyme (34).

In this study, the median concentration of Se was 19.8 μg $L^{-1}$, within the range reported previously. Studies performed in women of Ontario, Canada, had 17.7 μg Se $L^{-1}$ (range 13-25 μg $L^{-1}$) (35). In Brazil 36, the average Se concentration was 14.06 μg $L^{-1}$ (10.0 - 24.7 μg $L^{-1}$), no significant correlation was found with body mass index (BMI) ($p = 0.74$), or Se supplementation ($p = 0.60$). However, in people with high fish intake, a higher Se concentration in breast milk was observed (36). Marine food has an important contribution to the Se concentration in the body (37). Se concentration is significantly correlated with protein carbonyl content and SOD and GPx activities (Table II). Most studies suggest that in the presence of Se, the activity of the enzyme GPx is enhanced; therefore, Se may contribute to counteract molecular oxidation (12).

In earlier studies, the main enzyme involved in preventing oxidative damage to proteins was GPx. The activity of GPx in smoking mothers was reported at 105.1 U $L^{-1}$ (approximately 0.012 U $mg^{-1}$ protein, assuming 9 g $L^{-1}$ of breast milk), while in non-smoker women GPx activity was 90.8 U $L^{-1}$ [1] (approximately 0.010 U $mg^{-1}$); the difference was attributed to the Cd present in the cigarette (38). Moreover, it has been reported an increase in the activity of GPx (79%) related to low concentrations of Pb ($p < 0.001$) in human erythrocytes, the opposite occurs at high concentrations of Pb, thus there is a relationship between this element and activity of GPx (39). If the concentration of Pb and Cd affects the activity of GPx, this can interfere with its antioxidant action, increasing the risk of protein oxidation by ROS. Protein carbonyl content can be an indicator of oxidative damage.

According to the GLM, GPx and GR activities, as well as the concentrations of Se, Cd and Pb appear to be directly related to the concentration of protein carbonyls in breast milk. Previously, protein carbonyl concentration has been linked to oxidative stress indicators, such as antioxidant enzymes and prooxidant molecules (40). Apparently, GPx and GR enzymes are the main responsible of preventing oxidation induced by Pb and Cd (7). Selenium concentration is related to GPx activity and is a cofactor of this enzyme ($r = -0.24$, $p < 0.05$) (8). Cd and Pb provides information to the model, however, the results about Cd should be taken with caution due to the low proportion of data detected in breast milk (21% undetected data) (26). The obtained model helps to explain the relationship between the factors involved in the oxidation of proteins, which determines the concentration of protein carbonyls in breast milk.

## CONCLUSIONS

The concentrations of Cd and Pb found in breast milk in this study, are below the limit allowed by WHO. The oxidative dam-

age to proteins can be induced in part by Cd, potentially due to a decrease in GPX activity. On the other hand, lead affects the activity of GPx and GR, as well as the concentration of free Se. Se concentration reported in breast milk in this study is within the range of previous studies, and it is correlated with protein carbonyl content and GPx activity. According to the GLMs, GPx and GR activities and Se (a cofactor of GPx) might help to explain the protein oxidation induced by Pb and Cd in breast milk of women from Baja California Peninsula.

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