The effect of trace minerals on the stability of retinol acetate, cholecalciferol and selenomethionine stability within premixes

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Abstract

This study compared the effect of an organic proteinate mineral source and an inorganic sulphate mineral source in relation to their effect on the stability of retinol acetate and cholecalciferol within simulated premixes, while comparing the stability of two different selenomethionine (SeMet) sources (selenium enriched yeast (SeYeast) and the chemically synthesised L-SeMet) in the presence of inorganic sulphate mineral sources within simulated premixes. Four vitamin-trace mineral premixes, two containing organic trace mineral sources in the form of proteinates and two containing inorganic trace mineral sources in the form of sulphates, were formulated so that, when added to a complete broiler feed at the appropriate inclusion rates, they contained the same amount of retinol acetate and cholecalciferol and varying levels of trace minerals (National Research Council recommended level, commonly used industry level or a reduced inclusion level). The two SeMet-trace mineral premixes were formulated to contain commonly used industry levels of vitamins and trace minerals. The two SeMet-trace mineral premixes differed in the source of SeMet. One premix contained chemically synthesised L-SeMet while the other contained SeYeast. The vitamin content of the four vitamin-trace mineral premixes was analysed after 14 and 84 days in storage by ultra-high performance liquid chromatography and the amount present within each of the premixes was compared to the quantity determined prior to storage. In general, the premixes formulated with the sulphate trace mineral source were found to have higher losses of retinol acetate and cholecalciferol than those formulated with the proteinate trace mineral source. The inclusion of the proteinate minerals at both National Research Council and reduced inclusion levels significantly ($P \leq 0.05$) increased the stability of both the vitamins when compared to the inorganic sulphate mineral sources included at commonly used industry levels. The SeMet content of the two SeMet-trace mineral premixes was analysed after 49 days in storage by high performance liquid chromatography – inductively coupled plasma mass spectrometry and the amount of SeMet present within each of the samples was compared to the quantity determined prior to storage. SeMet present within the SeYeast was found to be significantly more stable ($P \leq 0.05$) than the chemically synthesised L-SeMet.

Keywords: trace minerals, retinol acetate, cholecalciferol, selenomethionine, stability

1. Introduction

Trace minerals such as copper (Cu), iron (Fe), manganese (Mn), zinc (Zn) and selenium (Se) are essential cofactors for hundreds of cellular enzymes and transcription factors in both poultry and livestock. As such, these are involved in a wide variety of biochemical processes including immune development and response, blood pigment formation, tissue

development, bone development and protection against oxidative stress (Davis and Mertz, 1987; Hambridge *et al.*, 1986; Hurley and Keen, 1987; Morris, 1987; Richards *et al.*, 2010; Sirri *et al.*, 2016; Surai *et al.*, 2018; Yatoo *et al.*, 2013). These trace minerals are usually provided in the diet through the addition of specifically formulated premixes. The majority of the premix trace mineral components are either inorganic trace mineral (ITM) salts, such as

carbonates, sulphates or oxides or, mineral salts bound to organic ligands such as proteins (Sirri *et al.*, 2016). These organic minerals are referred to as organic trace minerals (OTM). The advantages of OTM over their inorganic counterparts in relation to dietary and environmental antagonisms, adsorption and bioavailability have been well documented (Akter *et al.*, 2015; Bao and Choct, 2009; Coelho, 2002; Männer *et al.*, 2006; Miles and Henry, 2000; Nocek *et al.*, 2006; Shurson *et al.*, 2011; Spears, 1996; Yan and Waldroup, 2006).

Selenium differs from the other trace minerals, in relation to both its inorganic and organic sources. It is included in premixes as either inorganic sodium selenite or selenate, or as organic Se, which is mainly in the form of selenomethionine (SeMet; Surai *et al.*, 2018). Numerous organic sources of SeMet have been approved for use within the poultry industry and they include natural and chemically synthesised SeMet, Se enriched yeast, Zn-SeMet and OH-SeMet (Surai *et al.*, 2018). Similar to the other trace minerals, the advantages of using organic Se sources over inorganic sources have been well documented (Brozmanová *et al.*, 2010; Fisinin *et al.*, 2008; Gosetti *et al.*, 2007; Ip, 1986; Rahmanto and Davies, 2011; Surai and Fisinin, 2014; Zhao *et al.*, 2017).

Many of the studies in relation to OTM have focused on comparing the effect of just one specific source of OTM to an ITM in relation to the stability of numerous vitamins and other premix components. In general, OTM are grouped together under this blanket term, even though there are multiple sources whose structure and characteristics vary. The differences in structure and characteristics between OTMs can be attributed to the type of bonding groups used and the production process (Murphy, 2018). The type of ligand and the production process has a direct relationship on the bond strength between the ligand and the mineral salts. The bond strength may affect the stability of the mineral during the digestive process and with other premix components, such as vitamins, whilst in storage.

Trace minerals are usually formulated into premixes alongside other essential components, such as vitamins. Vitamins are biologically active biochemicals and are quite sensitive to their physical and chemical environment. Individual vitamins have varying degrees of sensitivity to numerous environmental degradation factors, including humidity, light, heat and pH (Shurson *et al.*, 2011). In the presence of trace minerals, vitamins can undergo significant degradation, due to oxidation, under normal storage conditions (Coelho, 1991; Shurson *et al.*, 2011). The presence of pro-oxidative trace minerals within premixes can lead to Fenton-type oxidising reactions and the subsequent degradation and destruction of compounds, such as retinol and cholecalciferol. Retinol acetate, the most commonly used source of vitamin A in animal

premixes, contains five double bonds within its structure, which are highly susceptible to oxidation (Coelho, 2002). Cholecalciferol contains a free phenolic hydroxyl group that can be easily oxidised (Coelho, 2002). High levels of vitamin degradation in storage can attribute to the suboptimal performance of numerous biochemical processes which can eventually lead to livestock developing deficiency diseases.

One aim of this study was to evaluate and compare the differences between an organic proteinate trace mineral and an inorganic sulphate source in relation to their effect on the stability of retinol acetate and cholecalciferol within a simulated premix. The second aim was to evaluate and compare the effect of SeMet source on the stability of SeMet in the presence of ITM within a simulated premix.

2. Materials and methods

Acetic acid, cholecalciferol (European pharmacopoeia reference standard), dimethyl sulphoxide, hydrochloric acid, methanol, nitric acid, protease XIV, retinol acetate (European pharmacopoeia reference standard), SeMet (standard powder >98% by TLC), tetrahydrofuran, trifluoroacetic and Tris were obtained from Sigma-Aldrich, Arklow, Ireland and were of ACS grade or higher, where appropriate. Four vitamin-trace mineral premixes, two containing OTM sources in the form of proteinates and two containing inorganic sulphates, were formulated using feed grade materials (Table S1, S2 and S3) to mimic commercial premixes. The premixes were formulated so that, when added to complete broiler feed at the appropriate inclusion rates (Table S2 and S3), they would all contain the same amount of retinol acetate and cholecalciferol (Table S4). The premixes differed, however, on trace mineral level (Table S4). The premixes were formulated so that the two OTM premixes would contain either NRC (National Research Council, 1994) or a reduced level (TRT) (Qiu *et al.*, 2020) of the trace mineral requirements for broilers when added to the complete feed at the appropriate inclusion rates (Table S2). The ITM premixes were formulated to contain NRC (National Research Council, 1994) and commonly used industry mineral levels when added to the complete feed at the appropriate inclusion rates (Table S3).

Two SeMet trace mineral premixes were formulated (Table S5) using feed grade materials to mimic commercial premixes. Both premixes contained ITM in the form of sulphates but differed in relation to SeMet source. One the premixes contained a selenium enriched yeast (SeYeast) while the other contained a chemically synthesised L-SeMet. These two premixes were formulated so that they both would contain commonly used industry levels of vitamins and trace minerals (Table S6).

Vitamin analysis

Upon receipt at the facility, the four premixes were thoroughly mixed. Each premix was weighed out six times (OTM TRT, 4.4 g; OTM NRC, 8 g; ITM NRC, 4.8 g; ITM Industry levels, 4.4 g) into 15 ml sterlin tubes (Sarstedt, Wexford, Ireland) to allow for both retinol acetate and cholecalciferol to be analysed separately and in triplicate. The premixes were weighed out so that each tube could contain equal amounts of retinol acetate and cholecalciferol, and this was accomplished by using the inclusion rate of each of the premixes as a guide. The remainder of each premixes was then placed in storage in a dark room with uncontrolled humidity and temperature to mimic the conditions that would be expected within commercial settings. The premixes were mixed every day and only removed to be analysed again after 14 and 84 days in storage.

Sample extraction of both retinol acetate and cholecalciferol from each premix was performed by the addition of dimethyl sulfoxide (DMSO; 20 ml). The premixes were then placed on a IKA KS 260 Basic plate shaker (Ika England Ltd, Oxford, England) for 30 min at 500 rpm and centrifuged in an Eppendorf Centrifuge 5810 (Eppendorf, Stevenage, UK) for 5 min at 3,220×g. The cholecalciferol samples were filtered using 3 ml BD leur lock syringes (Becton Dickson, Franklin Lakes, NJ, USA) and Chromafil RC-45/25 filters (Macherey-Nagel, Düren, Germany) into 2 ml amber glass vials (Agilent Technologies, Cork, Ireland) and capped. The retinol samples were filtered and then diluted down by adding 250 µl to 1.75 ml of DMSO and were further filtered into the amber glass vials and capped.

The quantitative analysis of retinol acetate and cholecalciferol was performed using an adapted version of the reverse phase HPLC method for simultaneous determination of fat-soluble vitamins developed by Joseph (2012). The ultra-high performance liquid chromatography analysis of the samples was performed using an Agilent 1290 Infinity LC System (Agilent Technologies, Cork, Ireland), consisting of a binary pump with an integrated vacuum degasser, autosampler and thermostatted column compartment containing a Zorbax RRHD Eclipse Plus C18, 2.1×100 mm, 1.8 µm column and a Diode array detector (Agilent Technologies). The chromatographic parameters were adapted from Joseph (2012) with a few alterations (Table S7).

Selenomethionine analysis

On receipt, the two premixes were thoroughly mixed, and each was weighed out in triplicate. The remainder of the premix was then stored in an environment similar to that of the vitamin-trace mineral premixes mentioned above. Similarly, the premixes were mixed every day and were only removed to be analysed again after 49 days in storage.

SeMet analysis was carried out by HPLC-inductively coupled plasma mass spectrometry (HPLC-ICP-MS) on an Agilent Zorbax RX-C8 4.6×250 mm (5 µm) column using an Agilent Technologies 1260 infinity series HPLC system connected to an Agilent Technologies 7700× series ICP-MS (Agilent Technologies). The analysis was carried out as described by Ward *et al.* (2013) Approximately 0.04 g of the sample was mixed with protease enzyme solution (80 mg protease XIV in 0.5 ml of Tris buffer pH 7.5) and vortexed for 2 min. Samples were ultrasonicated for 25 seconds at 80% amplitude in an Ultrawave ultrasonic bath (Ultrawave, Cardiff, UK). These samples were extracted for 15 minutes at a power output of 30 W with a CEM Discover microwave equipped with an Explorer SP-D Plus 24/48 autosampler (CEM Corporation, Charlotte, NC, USA). Extracts were centrifuged in a Hettich Universal 320R centrifuge (Hettich GmbH, Tuttlingen, Germany) at 19,060×g for 3 min. The supernatant was transferred to a 15 ml centrifuge tube. The volume was made up to 15 ml using deionised water and mixed well before removing an aliquot (2 ml) for filtration (0.2 µm) and dilution with deionised water before analysis (injection volume 45 µl). The isocratic chromatography for this analysis had a mobile phase of 0.1% trifluoroacetic acid with 2% MeOH in deionised water at a flow rate of 1 ml/min and the column temperature was set at 25 °C.

Statistical analysis

To ensure the reproducibility of the results, all the experiments were repeated in triplicate and a mean value with standard deviation was considered. The samples were evaluated for significant differences with Minitab 19.2020.1. The samples were subjected to a one-way analysis of variance (ANOVA) test with a *post hoc* Tukey HSD test. Significance was attained at $P < 0.05$. The data was presented as the mean of the triplicate experiments.

3. Results

Retinol acetate

Figure 1 illustrates the effects of the various trace mineral sources and inclusion rates on the stability of retinol acetate within the simulated premixes. After two weeks in storage, the two premixes containing the OTM had no negative impact on the stability of retinol acetate. Their inorganic counterparts, ITM NRC and ITM Ind, elicited a loss of $8.17 \pm 6.31\%$ (6,146 IU/g DM to 5,644 IU/g DM) and $21.72 \pm 1.16\%$ (7,500 IU/g DM to 5,871 IU/g DM) respectively, which were both significantly higher losses ($P \leq 0.05$) when compared to both OTM samples. After a further ten weeks in storage, the OTM premixes elicited a slight loss in retinol acetate (OTM TRT; $4.04 \pm 2.33\%$: 7,431 IU/g DM to 7,130 IU/g DM, OTM NRC; $3.88 \pm 2.49\%$: 3,596 IU/g DM to 3,318 IU/g DM) when compared to the initial pre-storage results. The ITM NRC premix elicited higher

Figure 1. Retinol acetate loss in the presence of various sources and inclusion rates of copper (Cu), iron (Fe), manganese (Mn) and zinc (Zn). Data are a means of triplicate experiments. Average values lacking a common superscript differ in statistical significance according to one-way ANOVA and separation by Tukey's test ($P \leq 0.05$). OTM TRT = organic trace minerals reduced inclusion rate; OTM NRC = organic trace minerals NRC inclusion rate; ITM NRC = inorganic trace minerals NRC inclusion rate; ITM Ind = inorganic trace minerals industry inclusion rates.

retinol acetate loss ($11.47 \pm 4.75\%$: 6,146 IU/g DM to 5,442 IU/g DM) than both the OTM premixes, while ITM Ind had significantly higher ($28.7 \pm 2.08\%$: 7,500 IU/g DM to 5,347 IU/g DM; $P \leq 0.05$) retinol acetate loss than both the OTM premixes.

Cholecalciferol

Figure 2 illustrates the effects of the various trace mineral sources and inclusion rates on the stability of cholecalciferol within the simulated premixes. After two weeks in storage, three of the four premixes, OTM NRC, ITM NRC and OTM Ind, elicited a loss of cholecalciferol. OTM NRC lost $7.67 \pm 4.35\%$ (983 IU/g DM to 907 IU/g DM) when compared to the pre-storage quantity of cholecalciferol. This loss was significantly higher ($P \leq 0.05$) than OTM TRT but significantly lower ($P \leq 0.05$) than ITM NRC and ITM Ind, which lost $51.46 \pm 4.2\%$ (1,443 IU/g DM to 700 IU/g DM) and $51.9 \pm 5.1\%$ (1,811 IU/g DM to 871 IU/g DM) respectively. After twelve weeks in storage, it all four premixes had decreased levels of cholecalciferol when compared to the initial quantities measured before storage. However, the OTM TRT ($7.48 \pm 3.95\%$: 1,735 IU/g DM to 2,045 IU/g DM) and OTM NRC ($20.38 \pm 4.7\%$: 983 IU/g DM to 783 IU/g DM) elicited significantly fewer ($P \leq 0.05$) losses of cholecalciferol when compared to ITM NRC ($48.93 \pm 2.76\%$:

1,443 IU/g DM to 700 IU/g DM to 737 IU/g DM) and ITM Ind ($48.38 \pm 2.39\%$: 1,811 IU/g DM to 935 IU/g DM).

Selenomethionine analysis

Figure 3 illustrates the effect of a sulphate trace mineral source on the stability of two different organic SeMet sources within simulated premixes. After a seven-week storage period it was noted that the level of SeMet present within SeYeast had only very slightly decreased ($0.29 \pm 1.83\%$) while the chemically synthesised L-SeMet exhibited a significant loss ($P < 0.05$) of $11.7 \pm 2.51\%$ when compared to the initial SeMet content measured pre-storage. The SeMet present as chemically synthesised L-SeMet elicited significantly higher ($P < 0.05$) SeMet loss when compared to the SeYeast source.

4. Discussion

Retinol acetate and cholecalciferol stability were significantly ($P \leq 0.05$) increased within vitamin-trace mineral premixes through the inclusion of OTM sources instead of ITM sulphate sources. The data indicated that, in general, increased levels of trace minerals will increase retinol acetate and cholecalciferol degradation in line with the duration of time in storage. The stability of retinol acetate within simulated animal premixes has been well

Figure 2. Cholecalciferol loss in the presence of various sources and inclusion rates of copper (Cu), iron (Fe), manganese (Mn) and zinc (Zn). Data are a means of triplicate experiments. Average values lacking a common superscript differ in statistical significance according to one-way ANOVA and separation by Tukey's test ($P \leq 0.05$). OTM TRT = organic trace minerals reduced inclusion rate; OTM NRC = organic trace minerals NRC inclusion rate; ITM NRC = inorganic trace minerals NRC inclusion rate; ITM Ind = inorganic trace minerals industry inclusion rates.

Figure 3. Selenomethionine (SeMet) loss between two SeMet sources in the presence of NRC recommended inclusion rates of vitamins and trace minerals after a seven-week storage period. Data are a means of triplicate experiments. Average values lacking a common superscript differ in statistical significance according to one-way ANOVA and separation by Tukey's test ($P \leq 0.05$). SeYeast = selenium enriched yeast; Chem SeMet = chemically synthesised L-selenomethionine.

documented over the years. Numerous studies have examined and compared the effect of number of variables (Coelho, 2002; Shurson *et al.*, 2011; Tavčar-Kalcher and Vengušt, 2007; Yang *et al.*, 2019; Zhuge and Klopfenstein, 1986). Coelho (2002) examined numerous variables in relation to vitamin stability within premixes, including the comparison of OTM to ITM sources, and noted that OTM elicited a retinol acetate loss of 15% after three months in storage compared 35% with sulphate sources. Shurson *et al.* (2011), in a similar experiment, compared a specific metal amino acid complex and sulphate sources in relation to their effect on the stability of various vitamins within a simulated premix. Their findings indicated that ITM significantly ($P \leq 0.05$) increased retinol acetate loss when compared to the OTM, with the inorganic sulphate source eliciting a loss of 26.91% over three months compared to 9.2% for OTM.

The results generated from this study correlated with those reported by Coelho (2002) and Shurson *et al.* (2011), as the data suggested that OTM elicited lower retinol acetate losses than ITM sources. Retinol acetate contains five double bonds within its structure and so is highly susceptible to degradation by oxidation (Coelho, 2002). This can be seen from the results, as after two weeks in the presence of ITM, the amount of stable retinol acetate present within ITM NRC and ITM Ind dropped by 8.1%

and 21.7%, respectively. After a further ten weeks, the retinol acetate losses in both inorganic premixes increased to 11.5 and 28.7% respectively. While the retinol acetate loss within ITM premixes after 12 weeks was higher than after two weeks, the rate at which the levels decreased had slowed. This indicated that retinol acetate was oxidised at a rapid rate initially and degradation slowed over time. In contrast, the retinol acetate loss in the presence of OTM was significantly less ($P \leq 0.05$) after two weeks and, after twelve weeks in storage OTM NRC still retained more retinol acetate than its inorganic NRC counterpart. It should be noted, however, that under normal circumstances OTM would not be included at NRC levels, and this treatment represented an extreme case which was included to provide a direct comparison relative to the ITM premixes. These results indicated that the binding of organic ligands to trace minerals can limit their interaction with the environment, and subsequently reduce the formation of reactive oxygen species (ROS). By limiting the formation of ROS, proteinates can significantly ($P \leq 0.05$) reduce degradation rate of retinol acetate within a vitamin-trace mineral premix, conferring increased stability of retinol acetate when compared to premixes containing ITM.

The stability of cholecalciferol within simulated animal premixes has been documented over the years, but has received less focus than retinol acetate. Studies have examined and compared the effect of a number of variables on the stability of cholecalciferol within premixes (Shurson *et al.*, 2011; Yang *et al.*, 2019, 2021). The study performed by Shurson *et al.* (2011) was the same that for retinol acetate. They noted that the OTM incurred a cholecalciferol loss of 9.1% after three months in storage, compared to 13.44% in the premixes formulated with ITM sulphate sources. These results partially correlated with the results obtained in the current experiment, and differed only due to the fact that, while Shurson noted there was a difference in relation to cholecalciferol loss between both mineral sources, this difference was found to be insignificant. The results obtained from the current experiment noted that, at both time points, both OTM samples elicited significantly ($P \leq 0.05$) lower cholecalciferol loss than their inorganic counterparts. Cholecalciferol contains a free phenolic hydroxyl group that can be readily and rapidly oxidised (Coelho, 2002). This was observed in the results, and, after only two weeks in storage, both ITM NRC and ITM Ind were found to have lost approximately 52% each. After a further 10 weeks, there was no further cholecalciferol loss. Similar to retinol acetate, this indicated that cholecalciferol was rapidly oxidised initially and that which remained was relatively stable for the remainder of the time in storage.

The results from the current experiment differed from others regarding cholecalciferol stability in that the level of losses seen within the premixes containing the ITM sulphate sources was substantially higher than reported in

other experiments. The range of cholecalciferol loss noted after three months from a number of other experiments ranged from 5-20% (Coelho, 2002; Shurson *et al.*, 2011; Yang *et al.*, 2019, 2021). While this was unexpected, there are numerous reasons why an increased loss in cholecalciferol were noted, which include environmental factors, vitamin manufacturing process, redox reactions, friction and premix formulation (Coelho, 2002). An alteration in any one of these variables could easily lead to a substantial increase in cholecalciferol losses and poorer stability. One possible explanation was that the daily mixing of the premixes caused an increase in the usual friction seen within premixes. This can lead to the erosion of the vitamin coating, which would expose the nutrient to variables which would increase cholecalciferol degradation.

It was clearly seen that organic proteinate sources elicited a significantly ($P \leq 0.05$) lower level of cholecalciferol loss than their ITM counterparts at both time points. These results indicated that the binding of a proteinate to trace minerals can limit interactions within the premixes and reduce the formation of ROS. By limiting the formation of ROS, proteinate OTM can significantly ($P \leq 0.05$) lower the rate by which cholecalciferol is degraded within a vitamin-trace mineral premix, increasing stability compared to ITM premixes containing sulphate sources.

The data on the effect of source on the stability of SeMet in the presence of ITM demonstrated that chemically synthesised L-SeMet was significantly ($P \leq 0.05$) less stable than SeYeast when formulated into a premix containing ITM sources. A report by EFSA (2013) stated that synthesised L-SeMet is unstable in premixtures containing compounds of trace elements, with a SeMet loss of 45, 46 and 63% seen after three, six and nine months, respectively. The instability of chemically synthesised L-SeMet within a premix may be due to the fact its purified form can be easily oxidised (Surai *et al.*, 2018). The presence of pro-oxidative minerals increases the formation of ROS, which subsequently oxidise SeMet. While the current experiment correlated with the EFSA report (2013) in relation to the instability of chemically synthesised L-SeMet in the presence of trace minerals, it showed that SeYeast source was significantly more stable ($P \leq 0.05$).

The importance of SeMet within the diet of poultry has been well documented especially during times of stress (Schrauzer, 2006; Surai *et al.*, 2018). The inclusion of SeYeast in vitamin-trace mineral premixes over chemically synthesised L-SeMet allows for a more stable source of SeMet and enhances the building of Se tissue reserves. These can be used by the poultry in times of stress to limit issues in productive and reproductive performances due to suboptimal levels of dietary Se.

5. Conclusions

Overall, the inclusion of OTM proteinate sources into vitamin-trace mineral premixes significantly ($P \leq 0.05$) increased the stability of fat-soluble vitamins during storage when compared to their ITM sulphate counterparts. In addition, it was determined that the SeMet present within SeYeast was significantly ($P \leq 0.05$) more stable than chemically synthesised L-SeMet when formulated into a vitamin-trace mineral premix containing ITM sulphate sources.

Supplementary material

Supplementary material can be found online at <https://doi.org/10.3920/JAAN2021.0002>.

Table S1. The formulation of the vitamin premix.

Table S2. Formulation of the poultry vitamin premixes containing organic trace minerals.

Table S3. Formulation of the poultry vitamin premixes containing inorganic trace minerals.

Table S4. Nutrient concentration per kg/DM of complete broiler feed after addition of the vitamin premixes.

Table S5. Formulation of the poultry selenium-inorganic trace mineral premixes.

Table S6. Nutrient concentration per kg/DM of complete broiler feed after addition of the selenium premixes.

Table S7. Chromatographic parameters used for the Agilent 1290 Infinity LC system.

Conflict of interest

The authors are currently or were all employed by Alltech Ireland at the time of this experiment. Alltech also manufactured and sell the organic trace mineral sources tested within this experiment.

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