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# 25-Hydroxyvitamin $D_3$ affects vitamin D status similar to vitamin $D_3$ in pigs – but the meat produced has a lower content of vitamin D

Jette Jakobsen[1]*, Hanne Maribo[2], Anette Bysted[1], Helle M. Sommer[1] and Ole Hels[1]

[1]*National Food Institute, Technical University of Denmark, Mørkhøj Bygade 19, DK-2860 Søborg, Denmark*
[2]*Danish Meat Association, Axeltorv 3, DK-1609 Copenhagen V, Denmark*

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In food databases, the specific contents of vitamin $D_3$ and 25-hydroxyvitamin $D_3$ in food have been implemented in the last 10 years. No consensus has yet been established on the relative activity between the components. Therefore, the objective of the present study was to assess the relative activity of 25-hydroxyvitamin $D_3$ compared to vitamin $D_3$. The design was a parallel study in pigs ($n$ 24), which from an age of 12 weeks until slaughter 11 weeks later were fed approximately 55 μg vitamin D/d, as vitamin $D_3$, in a mixture of vitamin $D_3$ and 25-hydroxyvitamin $D_3$, or 25-hydroxyvitamin $D_3$. The end-points measured were plasma 25-hydroxyvitamin $D_3$, and in the liver and loin the content of vitamin $D_3$ and 25-hydroxyvitamin $D_3$. Vitamin $D_3$ and 25-hydroxyvitamin $D_3$ in the feed did not affect 25-hydroxyvitamin $D_3$ in the plasma, liver or loin differently, while a significant effect was shown on vitamin $D_3$ in the liver and loin ($P<0{\cdot}001$). 25-Hydroxyvitamin $D_3$ in the plasma, liver and loin significantly correlates with the sum of vitamin $D_3$ and 25-hydroxyvitamin $D_3$ in the feed ($P<0{\cdot}05$). Therefore, 25-hydroxyvitamin $D_3$ should be regarded as having the same activity as vitamin $D_3$ in food databases. Sole use of 25-hydroxyvitamin $D_3$ as a vitamin D source in pig feed will produce liver and meat with a negligible content of vitamin $D_3$, while an increased content of vitamin $D_3$ in the feed will produce liver and meat with increased content of both vitamin $D_3$ and 25-hydroxyvitamin $D_3$.

**25-Hydroxyvitamin $D_3$: Vitamin $D_3$: Pig feed: Activity: Status**

Vitamin D deficiency increases the risk of bone fracture due to osteoporosis and decreases muscle strength. Recent investigations show a relationship between vitamin D deficiency and other afflictions such as cancer, reduced immune defence and CVD[1–3]. During the summer period, the primary source of vitamin D for man exposed to sunlight is the metabolism of 7-dehydrocholesterol to pre-vitamin $D_3$ in the skin by UV B radiation (290–315 nm), whereas vitamin D in food is the secondary source. In winter oral intake of vitamin D may be the primary source, as absorption through the skin is limited at latitudes above 35°, e.g. for 4 months in Boston, USA (42°N) and for 6 months in Bergen, Norway (61°N)[4]. Similarly, oral intake of vitamin D is the primary source all year round for people not exposed to sunlight due to confinement indoors or clothing.

Estimation of dietary intake of vitamin D is essential for investigating the influence of vitamin D on health parameters in a population as well as in human intervention studies. Such calculations are based on dietary intake data from dietary surveys combined with the content of nutrients available in food composition tables. Until 10–15 years ago vitamin D data in food composition tables was mainly derived from biological assays, which used the ability of vitamin D to cure rickets in vitamin D-deficient rats[5,6]. For the last 10 years, food composition tables have included specific values for vitamin $D_3$ (vit$D_3$) and 25-hydroxyvitamin $D_3$ (25OH$D_3$)[7–12]. To calculate the total vitamin D content, the relative activity between 25OH$D_3$ and vit$D_3$ is required.

The studies conducted to assess this factor were performed 30–40 years ago in deficient rats, in which the estimated values were between 1·4 and 5[13–16]. However, to date, no consensus has been established[17].

The aim of the present study was to investigate the relative activity between vit$D_3$ and 25OH$D_3$ in pigs, as a model for man. The end-points were plasma 25OH$D_3$, and the content of vit$D_3$ and 25OH$D_3$ in the pork loin and liver. Vitamin $D_{total}$ throughout the paper is defined as the sum of vit$D_3$ and 25OH$D_3$.

## Materials and methods

### *Pigs*

The twenty-four pigs selected for the present study were a subgroup of 3225 healthy pigs used in a feeding-trial conducted to investigate whether the productivity of the pigs was affected when vit$D_3$ was replaced by 25OH$D_3$. The feeding-trial was conducted at an ordinary Danish farm in stables with partially slatted floors and cover. A computer-controlled system

**Abbreviations:** 25OH$D_3$, 25-hydroxyvitamin $D_3$; vit$D_2$, vitamin $D_2$; vit$D_3$, vitamin $D_3$.

* **Corresponding author:** Dr Jette Jakobsen, fax +45 72 34 74 48, email jja@food.dtu.dk

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distributed the feed to the separate double pens. Each double pen contained forty-five pigs[18]. The pigs were fed via a tube feeder with nipple drinks as well as drinking bowls. The pigs were raised without exposure to sunlight. The stable lighting was produced with lamps (F36W/T8/33-630; Osram, Sylvania, MA, USA).

### *Experimental design*

The study was performed as a supplementation study and consisted of a parallel trial with three treatments of vitamin D. Vitamin D was given at equal levels as $vitD_3$, as a mixture of $vitD_3$ and $25OHD_3$, or as $25OHD_3$, from weaning at an age of 5 weeks to slaughter at an age of approximately $5\frac{1}{2}$ months. The feedstuff used was produced by DSM Nutritional Products (Copenhagen, Denmark) and DLG (Dansk Landbrugs Grovvareselskab, Copenhagen, Denmark). Detailed information of the content of the feed is given elsewhere[18].

### *Sampling*

The amount fed to each pig was calculated per pen for each of the three periods: 6–7 weeks, 8–12 weeks, 13–23 weeks (weight of 32·5 kg, and until slaughter at approximately 100 kg). For the same periods feed was sampled under the principle of the theory of sampling, and analysed for $vitD_3$ and $25OHD_3$[19].

The day before slaughter a blood sample was drawn, and processed to EDTA–plasma and stored at −80°C until analysis. At slaughter the liver was sampled and the next day boneless loin with rind was separated. The loin was subsequently carefully dissected into lean meat without any subcutaneous fat (lean meat), subcutaneous fat without any lean meat (fat), and skin without any subcutaneous fat (skin). All the samples were packed in plastic bags and frozen at −20°C until analysis, which was performed within 8 months. Before analysis the liver, lean meat and fat were slowly thawed and separately ground in a homogenizer (1094 Homogenizer; Tecator, Paris, France) for 2 min, while the skin was slowly thawed and manually cut into pieces of 10–15 $mm^2$.

### *Vitamin $D_3$ and 25-hydroxyvitamin $D_3$ in the feed*

DSM Laboratory (Basel, Switzerland) carried out analyses of $vitD_3$ and $25OHD_3$ in the feed. For quantification of $25OHD_3$, 10 g feed was added to 500 ng $d_6$-hydroxyvitamin $D_3$ (synthesized by Prof. Mourino, University of Santiago di Compostela, Spain[20]) as the internal standard, and 60 ml water. The sample was gently swirled into a slurry and sonicated at 50°C for 10 min. The vitamins were extracted with 40 ml *tert*-butyl methyl ether by shaking and sonication for 5 min followed by centrifuging for 3 min at 3000 rpm. Supernatant (10 ml) was evaporated and the residue dissolved in 2 ml mobile phase for preparative HPLC (2-propanol–ethyl acetate–isooctane, 1:10:89). For clean-up 100 µl were injected into a preparative HPLC system equipped with a silica-column (Si60, 3 µm, 150 × 4·6 mm; Hypersil, Shandon Products, Runcorn, UK). The fraction of $25OHD_3$ and the internal standard with a retention time of 14–16 min was collected. Subsequently, the organic solvent was evaporated and dissolved in 700 µl methanol and 300 µl water. The quantitative determination was performed by injection of 90 µl into the HPLC–atmospheric pressure chemical ionization–MS equipment (Agilent 1946C LC/MSD single-quadrupole mass specific detector equipped with an atmospheric pressure chemical ionization unit; Agilent Technologies AG, Basel, Switzerland). Additionally, the HPLC system consisted of a C18 column (Aquasil C18 (Aquasil, Thermo Fisher Scientific, Waltham, MA, USA), 3 µm, 2·0 × 100 mm) and the mobile phase was a gradient of methanol–water (99·95 : 0·05).

The quantification of $vitD_3$ in the feed was determined by using vitamin $D_2$ (vit $D_2$) as the internal standard according to EN12821[21].

### *Vitamin $D_3$ and 25-hydroxyvitamin $D_3$ in the meat*

The analytical method and the equipment used to determine $vitD_3$ and $25OHD_3$ in the meat are previously described[22]. Minor modifications were made as 25-hydroxyvitamin $D_2$ (Sigma-Aldrich, Buchs, Switzerland) was used as the internal standard for $25OHD_3$ similar to the utilization of $vitD_2$ as the internal standard for $vitD_3$. Briefly, the internal standards of $vitD_2$ and 25-hydroxyvitamin $D_2$ were added to the meat samples and saponified with ethanolic potassium hydroxide. The unsaponifiable matter was extracted with diethyl ether–petroleum ether (1 : 1). The solution was then purified on a silica solid-phase extraction column and further cleaned by preparative HPLC equipped with silica and amino columns. Analysis of the liver samples included an extra preparative HPLC procedure, which consisted of a cyano column (Luna, Cyano, 3 µm, 150 × 4·6 mm) from Phenomenex (Torrance, CA, USA), and a mobile phase of 2-propanol–*n*-heptane (1·5 : 98·5). The fraction of $vitD_2$ and $vitD_3$ co-eluted with a retention time of 5 min at a flow rate of 1 ml/min. This fraction was collected and evaporated to dryness using a gentle stream of nitrogen, and finally dissolved in methanol–acetonitrile (20 : 80). Another fraction containing 25-hydroxyvitamin $D_2$ with retention time at 16 min as well as $25OHD_3$ with retention time at 21 min was collected in the same vial, evaporated and dissolved in acetonitrile–water (90 : 10). These two fractions were injected into the analytical HPLC system described earlier[22].

### *Content of fat in the meat*

Content of fat in the meat was determined by the gravimetric method following a modified Schmid–Bondzynski–Ratslaff method[23]. Briefly, the sample was boiled with hydrochloric acid followed by the addition of ethanol and extraction of the lipids with diethyl ether–petroleum ether (1 : 1). After evaporation of the solvent, the fat was weighed.

### *Plasma 25-hydroxyvitamin D*

The quantification of $25OHD_3$ in plasma was performed by the HPLC method described previously[24]. Briefly, plasma proteins were precipitated with ethanol and the supernatant was cleaned by a MFC18 solid-phase extraction. The $25OHD_3$ in the solution was separated, detected and measured by analytical HPLC equipped with a diode array detector (220–320 nm) and a UV detector (265 nm) and external calibration.

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*Data analysis*

Based on previously assessed variation of content of $vitD_3$ and $25OHD_3$ in meat, six pigs should be included in each feeding group to detect a relative activity of 1·5 for $25OHD_3$ compared to $vitD_3$ with a power of 80 % and a significance level of 5 %[7].

To test the effect of the content of $25OHD_3$ and $vitD_3$ in feed on $25OHD_3$ in plasma and on $25OHD_3$ and $vitD_3$ in meat and liver, regression analysis was performed. In the regression model, $25OHD_3$ in plasma, meat and liver, and $vitD_3$ in meat and liver were dependent variables, and the total content of vitamin D in feed (vitamin $D_{total}$) and the difference between $25OHD_3$ and $vitD_3$ (vitamin $D_{diff}$) were independent variables. Furthermore, ANOVA was performed with feed as an independent fixed variable to test and estimate differences between feeding groups. Association between determinants and variables were assessed with Pearson's correlation coefficients. Data are expressed as means and their standard errors. SAS version 9.1 (SAS Institute, Cary, NC, USA) was used for all statistical analyses, with a significance level of 0·05.

## Results

*Pigs performance*

The vitamin D in each of the three diets was given as the same vitamin D source(s) but due to the different feed and consumption levels during the growth period, the mean daily intake differed. $vitD_3$ and $25OHD_3$ in each of the three feeding periods are presented in Fig. 1. Carcass weights are presented in Table 1. No significant difference was detected between the diets or between the subgroup and the pigs included in the main feeding study ($n$ 3225).

*Effect of 25-hydroxyvitamin $D_3$ and vitamin $D_3$ as vitamin D source*

Results for vitamin D status assessed as plasma $25OHD_3$, and the contents of $25OHD_3$ and $vitD_3$ in the liver, and in the three separated parts of the loin including the content of fat are presented in Table 1.

The results show that $25OHD_3$ in the plasma, in the liver and in the three separated parts of loin did not depend on the vitamin D source, but on the daily intake of vitamin $D_{total}$, as content of $25OHD_3$ in the separated cuts was significantly associated with vitamin $D_{total}$ ($R$ 0·42–0·56; $P<0·05$).

In contrast, $vitD_3$ in the liver and in the three separated parts of loin depended on the vitamin D source ($P<0·001$), and showed no association with vitamin $D_{total}$. However, content of $vitD_3$ was significantly associated with $vitD_3$ in the feed ($R$ 0·65–0·89; $P<0·001$).

## Discussion

Previous work has shown that $vitD_3$ and $25OHD_3$ in meat is positively associated with the content of fat[7]. Therefore it is essential that comparison of meat derived from pigs fed different diets does not differ in the content of fat. No significant difference was shown for subcutaneous fat, lean meat and skin. The present results show that the intention to produce three similar separated cuts from each pig was fulfilled regarding the content of fat.

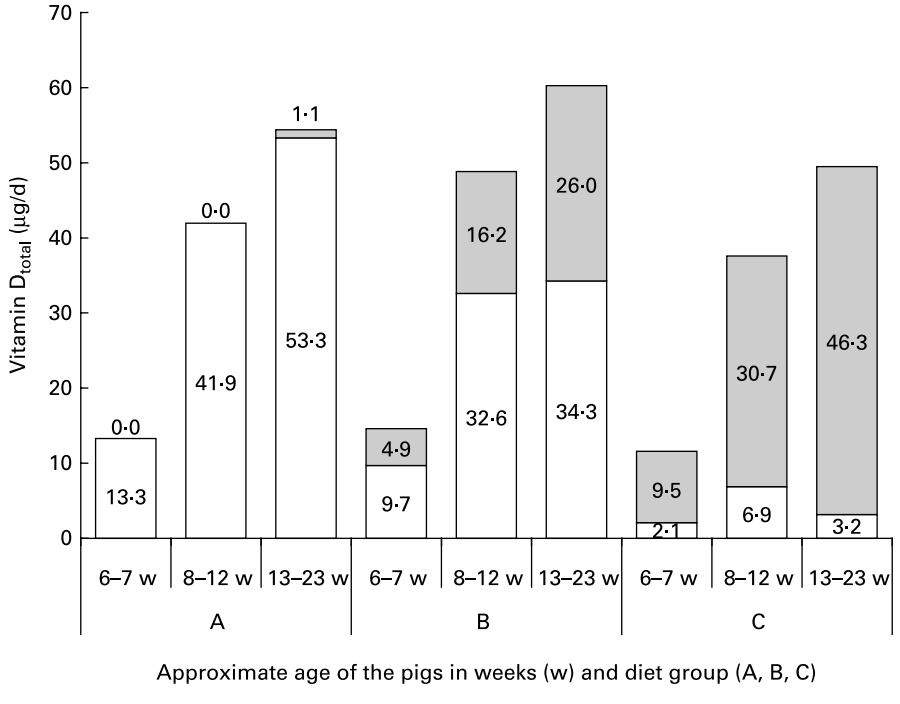


**Fig. 1.** The mean daily amount of vitamin D (Vitamin $D_{total}$) in each of the three diet groups (eight pigs in each) and divided into the three different age groups. Vitamin D source in the feed was either 25-hydroxyvitamin $D_3$ ($25OHD_3$, ▨) or vitamin $D_3$ ($vitD_3$, □). The diet groups A, B and C are $vitD_3$, a mixture of $vitD_3$ and $25OHD_3$, and $25OHD_3$, respectively.

**Table 1.** Weight of the carcass and the effect of the diet groups on 25-hydroxyvitamin $D_3$ ($25OHD_3$) in the serum, liver and separated parts of the loin and vitamin $D_3$ ($vitD_3$) in the liver and separated parts of the loin at slaughter*

(Mean values with their standard errors)

| | Diet A | | Diet B | | Diet C | |
|---|---|---|---|---|---|---|
| | Mean | SEM | Mean | SEM | Mean | SEM |
| Weight (kg) | 81·9[a] | 1·6 | 82·4[a] | 2·1 | 81·6[a] | 2·2 |
| Serum | | | | | | |
| $25OHD_3$ (ng/ml) | 18·1[ab] | 0·9 | 21·4[b] | 2·0 | 16·6[a] | 1·3 |
| Liver | | | | | | |
| $vitD_3$ (μg/kg) | 2·67[a] | 0·16 | 1·06[b] | 0·16 | 0·28[c] | 0·04 |
| $25OHD_3$ (μg/kg) | 3·95[a] | 0·36 | 5·22[b] | 0·40 | 3·64[a] | 0·30 |
| Loin – subcutaneous fat | | | | | | |
| Fat (%) | 79·1[a] | 1·0 | 76·8[a] | 1·4 | 77·0[a] | 1·2 |
| $vitD_3$ (μg/kg) | 7·47[a] | 0·49 | 3·51[b] | 0·29 | 0·57[c] | 0·05 |
| $25OHD_3$ (μg/kg) | 1·87[a] | 0·13 | 2·44[b] | 0·16 | 1·86[a] | 0·13 |
| Loin – lean meat | | | | | | |
| Fat (%) | 5·3[a] | 0·5 | 4·3[a] | 0·3 | 5·0[a] | 0·4 |
| $vitD_3$ (μg/kg) | 1·11[a] | 0·11 | 0·45[b] | 0·04 | 0·06[c] | 0·01 |
| $25OHD_3$ (μg/kg) | 0·89[a] | 0·08 | 1·10[b] | 0·09 | 0·81[a] | 0·12 |
| Loin – skin | | | | | | |
| Fat (%) | 10·8[a] | 1·1 | 9·8[a] | 1·8 | 11·5[a] | 1·2 |
| $vitD_3$ (μg/kg) | 2·99[a] | 0·31 | 2·07[b] | 0·17 | 1·04[c] | 0·15 |
| $25OHD_3$ (μg/kg) | 3·18[a] | 0·28 | 4·48[b] | 0·28 | 3·17[a] | 0·26 |

[a,b,c] Mean values within a row with unlike superscript were significantly different ($P<0·05$).

* Diets A, B and C are $vitD_3$, a mixture of $vitD_3$ and $25OHD_3$, and $25OHD_3$, respectively.

The significant effect of the feeding level of vitamin $D_{total}$ on plasma $25OHD_3$ in these pigs was in line with the positive association between dietary intake of vitamin $D_{total}$ and serum $25OHD_3$ shown in women and men[25,26].

The present study was originally designed to investigate whether the productivity of the pigs was affected when the $vitD_3$ was replaced by $25OHD_3$ in the feed. No difference was found in the present study which included 3225 pigs[18]. As the twenty-four pigs selected for the present nutritional study did not differ from the whole group concerning weight, and growth rate, the mean daily intake of vitamin D calculated from the whole group is applied.

Plasma 25OHD determined as the sum of plasma 25-hydroxyvitamin $D_2$ and plasma $25OHD_3$ is accepted as the biomarker for vitamin D intake in the absence of sun exposure[27]. The observed effect that plasma 25OHD achieves a steady state if supplemented at the same level for an adequate time period has previously been used to study differences between natural and synthetic $vitD_2$, $vitD_3$ in fortified bread, juice and supplement, as well as different levels of $vitD_3$ supplement[25,28–30]. In human intervention studies supplementation levels of 5–10 μg/d for 4 weeks was shown to be adequate to reach a steady state for vitamin D status (J Jakobsen, unpublished results). The half-life of vitamin D in man is 1 month[31]. Though the differences in the rate of metabolism between pigs and man are unknown for vitamin D, the applied period of 16 weeks to reach a steady state is assumed to be adequate. As the metabolism in pigs and man regarding fat-soluble vitamins is rather similar, it is assumed that the vitamin D status and vitamin D in the liver and in the meat at slaughter was not influenced by the feed given in the earliest stage of growth up to an age of 12 weeks.

To our knowledge, this is the first study investigating the effect of $25OHD_3$ and $vitD_3$ in healthy mammals.

Thirty to 40 years ago the difference between $25OHD_3$ and its parent $vitD_3$ was tested in vitamin D-deficient rats either by testing the effect on intestinal calcium absorption measured by the everted gut sac technique, serum calcium and body weight, or by the ability to cure rickets. By the everted gut sac technique an equal effect of the two compounds was shown after 24 h, though $25OHD_3$ acted more rapidly[13,32,33]. In 1973, $25OHD_3$ was shown to be five times as active as $vitD_3$ in the maintenance of serum calcium and growth[16]. In the ability to cure rickets $25OHD_3$ had an effect 1·4–2 times the activity of $vitD_3$ in three different studies, but in another study the effect was estimated to be 5 times as active[13–16].

Today, the factor of 5 for the activity between $25OHD_3$ and $vitD_3$ is widely used in recommended dietary allowances as well as its implementation in food composition tables[10–12]. However, the documentation for the factor of 5 seems limited due to the non-standardized methods used[34].

The data obtained for the relative activity between $25OHD_3$ and $vitD_3$ in pigs need to be verified in a similar study in man, as well as further investigation of the possible difference of the effect of vitamin D derived from pork and from supplements. For mushrooms, no difference was shown between natural $vitD_2$ and $vitD_2$ given as a supplement in the ability to increase vitamin D status, investigated in a human intervention study[28]. However, the vitamin D activity in meat may not be reflected only by $vitD_3$ and $25OHD_3$. The content of 1,25-dihydroxyvitamin $D_3$ and other dihydroxyvitamin $D_3$ compounds are unknown, but may contribute to the vitamin D activity of meat.

The effect of $25OHD_3$ and its parent form $vitD_3$ on plasma $25OHD_3$ should be regarded equal in the diet for pigs. However, for the nutritional value of pork meat, $25OHD_3$ in pig feed should be regarded as rather low compared with $vitD_3$, as the content of $vitD_3$ depended on the vitamin D source. The use of $25OHD_3$ only in the feed instead of $vitD_3$ produced meat and liver with significantly lower content of $vitD_3$. That the pigs fed solely on $25OHD_3$ did not produce meat and liver with $vitD_3$ is not surprising, as $vitD_3$ is not synthesized in the pigs. The hydroxylation of $vitD_3$ to $25OHD_3$ by 25-hydroxylase is not a reversible reaction.

Additionally, the present study shows that the concentration of $vitD_3$ as a vitamin D source in the feed determines the concentration of $vitD_3$ in meat and liver even at small differences in the feeding levels, which was previously shown in pigs fed super nutritional levels at 1000 μg $vitD_3$/kg[35]. Additional feeding trials are necessary to investigate fully the possibility of pork meat bio-fortified with vitamin D.

Presently, a human intervention study is being conducted to evaluate whether human subjects respond to supplements of $vitD_3$ and $25OHD_3$ in a similar fashion to pigs. However, more research on the relative bioactivity of vitamin $D_{total}$ from animal products compared to supplements of $vitD_3$ is an important issue for the calculation of dietary vitD intake.

## Conclusion

The findings of the present study showed that $25OHD_3$ and $vitD_3$ equally affect $25OHD_3$ in plasma, meat and liver.

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However, for the benefit of human nutrition, $25OHD_3$ in pig feed should be regarded as lower than $vitD_3$, as meat and liver produced by feeding the pigs exclusively $25OHD_3$ had a significantly lower content of $vitD_3$. Regardless of the vitamin D source the present study identified a dose–response effect between vitamin $D_{total}$ in the meat and in the liver with vitamin $D_{total}$ in the feed, which for meat and liver indicate the possibility to produce meat and liver bio-fortified with vitamin D.

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