

Inhibitory Effect of Marine Alga *Sargassum horneri* Extract on Bone Resorption in Tissue Culture *in Vitro*

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The effect of marine alga *Sargassum horneri* extracts on bone resorption *in vitro* was investigated. Femoral-diaphyseal and -metaphyseal tissues obtained from normal rats were cultured for 48 hr in Dulbecco's modified Eagle's medium (high glucose, 4.5%) supplemented with antibiotics and bovine serum albumin. The experimental cultures contained water-solubilized extracts (10, 25, and 50 µg/ml of medium) of *S. horneri*. The bone-resorbing factors parathyroid hormone (1–34) (PTH; 10^{-7} M) and prostaglandin E₂ (PGE₂; 10^{-5} M) caused a significant decrease in the diaphyseal and metaphyseal calcium content. The decrease in bone calcium content induced by PTH or PGE₂ was completely inhibited by water-solubilized extracts (10, 25, and 50 µg/ml) of *S. horneri*. In addition, these extracts (25 and 50 µg/ml) completely prevented the PTH (10^{-7} M)- or PGE₂ (10^{-5} M)-induced increase in medium glucose consumption and lactic acid production by bone tissues. Moreover, *S. horneri* extracts (10–50 µg/ml) blocked PTH (10^{-7} M)-increased acid phosphatase activity in the diaphyseal and metaphyseal tissues. These findings indicate that water-solubilized extracts of *S. horneri* have a direct inhibitory effect on bone resorption in tissue culture *in vitro*.

Key words — bone resorption, *Sargassum horneri*, marine alga, osteoporosis, rat femur

INTRODUCTION

Bone loss with increasing age induces osteoporosis.^{1–3)} This loss may be due to increased bone resorption and decreased bone formation. A decrease in bone mass leads to bone fracture. Osteoporosis is widely recognized as a major public health problem.⁴⁾ Food and nutritional factors can help to prevent bone loss with increasing age.⁵⁾

Recent studies have shown that isoflavones, which are contained large quantities in soybean, have a stimulatory effect on osteoblastic bone formation^{6–8)} and an inhibitory effect on bone resorption,^{9–11)} thereby increasing bone mass. Also, menaquinone-7, an analogue of vitamin K₂ which is essential for the γ -carboxylation of osteocalcin of a bone matrix protein, is abundant in fermented soybean (*natto*).¹²⁾ Menaquinone-7 has been demonstrated to stimulate osteoblastic bone formation¹³⁾ and to inhibit osteo-

clastic bone resorption¹⁴⁾ *in vitro*. The prolonged dietary intake with supplementation of isoflavones and vitamin K₂ has a preventive effect on bone loss induced by ovariectomy in rats, which is an animal model for osteoporosis.¹²⁾ Thus nutritional factors play a role in bone health and may be important in the prevention of bone loss with increasing age in human.^{15–17)}

The effect of marine algae on bone metabolism has not been fully clarified. Recently, it has been shown that of various marine algae (including *Sargassum horneri*, *Undaria pinnatifida*, *Eisenia bicyclis*, *Crytonemia scmitziana*, *Gelidium amansli*, and *Ulva pertusa* Kjellman), *S. horneri* has a unique anabolic effect on bone calcification *in vivo* and *in vitro*.¹⁸⁾ The anabolic effect of *S. horneri* extract on bone formation and mineralization may be based on a newly synthesized protein component.¹⁹⁾ The effect of *S. horneri* extract on bone resorption, however, is unknown.

The present study was undertaken to determine the effect of water-solubilized extracts of *S. horneri* on bone resorption in tissue culture *in vitro*. We found that the extract has a direct inhibitory effect on the

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bone-resorbing factor-induced bone resorption using rat femoral-diaphyseal and -metaphyseal tissues *in vitro*.

MATERIALS AND METHODS

Chemicals — Dulbecco's modified Eagle's medium and penicillin-streptomycin solution (5000 units/ml penicillin; 5000 $\mu\text{g}/\text{ml}$ streptomycin) were obtained from Gibco Laboratories (Grand Island, NY, U.S.A.). Bovine serum albumin (fraction V), synthetic human parathyroid hormone (1–34) (PTH), and prostaglandin E_2 (PGE_2) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Other chemicals were of reagent grade and were obtained from Wako Pure Chemical Industries (Osaka, Japan). All water used was glass-distilled.

Marine Alga Extracts — The marine alga *S. horneri* was seasonally gathered from the coast at Shimoda (Shizuoka prefecture, Japan), and was freeze-dried and powdered. The fresh marine alga gathered was homogenized in distilled water with a Physcotron homogenizer, and the homogenate was centrifuged at 5500 g in a refrigerated centrifuge for 10 min.¹⁷⁾ The 5500 g supernatant fraction was pooled for freeze-drying. Powder of the water-solubilized extract was dissolved in ice-cold distilled water to use in experiments.

Animals — Male Wistar rats (conventional) weighing 100–120 g (4 weeks old) were obtained from Japan SLC (Hamamatsu, Japan). The animals were fed commercial laboratory chow (solid) containing 1.1% calcium, and 1.1% phosphorus at room temperature of 25°C, with free access to distilled water.

Bone Culture — Femoral-diaphyseal and -metaphyseal tissues from 4 week-old male rats were removed aseptically. These tissues were then cultured in a 35-mm dish in 2.0 ml medium consisting of Dulbecco's modified Eagle's medium (high glucose; 4.5%) supplemented with 0.25% bovine serum albumin (fraction V) plus antibiotics, with either bone-resorbing factors (PTH or PGE_2) or vehicle (sterile distilled water) in the absence or presence of water-solubilized extracts (10, 25, and 50 $\mu\text{g}/\text{ml}$ of medium) of *S. horneri*. Cultures were maintained at 37°C in a water-saturated atmosphere containing 5% CO_2 and 95% air for 48 hr.

Bone Calcium — The bone tissues were dried for 16 hr at 120°C, weighed, and then dissolved in nitric acid solution.²⁰⁾ Calcium was determined by

atomic absorption spectrophotometry.²¹⁾ The bone calcium content was expressed as milligrams of calcium per gram of dry bone.

Medium Glucose and Lactic Acid — The concentration of glucose in the medium cultured with bone for 48 hr was determined by the colorimetric method using *o*-toluidine.²²⁾ Dry weight of the bone tissue was measured after extraction with 5.0% trichloroacetic acid, acetone, and ether. The medium glucose consumed by bone culture in 48 hr was expressed as milligrams of glucose per gram of dry bone tissue. Likewise, the medium lactic acid was measured by the enzymatic method.²³⁾ Data were expressed as milligrams of lactic acid per gram of dry bone tissue.

Bone Acid Phosphatase — Acid phosphatase activity in the bone tissues was determined by the method of Walter and Schutt.²⁴⁾ The bone tissues were immersed in 3.0 ml of ice-cold 6.5 mM sodium barbital buffer (pH 7.4), cut into small pieces, homogenized in a Potter–Elvehjem homogenizer with a Teflon pestle, and disrupted for 60 sec with an ultrasonic device. The supernatant fraction, centrifuged at 600 $\times g$ for 5 min, was used for measurement of the enzyme activity. The efficiency of the enzyme extraction was greater than 90%, and the enzyme analysis was reproducible. The enzyme assay was carried out under optimal conditions in medium (1.2 ml) containing 50 mM citrate buffer (pH 4.8), 5.5 mM *p*-nitrophenylphosphate, and the supernatant solution of bone homogenate (13–20 $\mu\text{g}/\text{ml}$ of protein). Enzyme reaction was stopped by the addition of ice-cold 0.1 N NaOH (4 ml). Enzyme activities were expressed as nanomoles of *p*-nitrophenol liberated per minute per milligram of protein. Protein was determined by the method of Lowry *et al.*²⁵⁾

Statistical Methods — Data are expressed as means \pm S.E.M. Statistical differences were analyzed using Student's Paired *t*-test. *p* Values of less than 0.05 were considered to indicate statistically significant differences.

RESULTS

Effect of *S. horneri* Extract on Bone-Resorbing Factor-Decreased Bone Calcium Content *in Vitro*

The effect of water-solubilized extracts of *S. horneri* on the bone-resorbing factors-induced decrease in calcium content in the femoral tissues obtained from normal rats was examined *in vitro*. Femoral-diaphyseal or -metaphyseal tissues were

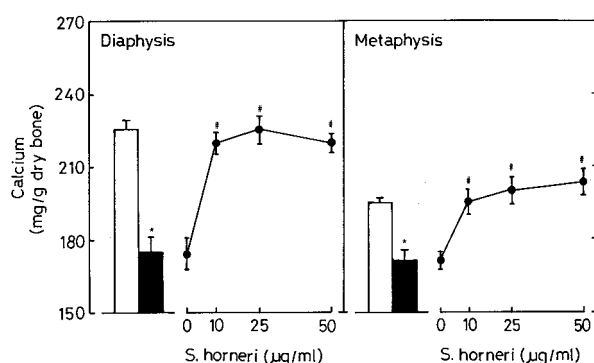


Fig. 1. Effect of *S. horneri* Extract on PTH-Decreased Bone Calcium Content in Rat Femoral-Diaphyseal and -Metaphyseal Tissues *in Vitro*

Femoral-diaphyseal or -metaphyseal tissues obtained from normal rats were cultured for 48 hr in a medium containing either vehicle or PTH (10^{-7} M) in the absence or presence of water-solubilized extract (10, 25, and 50 μ g/ml of medium) of *S. horneri*. Each value is the mean \pm S.E.M. of six rats. * $p < 0.01$, compared with the control (none) value. # $p < 0.01$, compared with the value for PTH alone. White bars, control (none); black bars, PTH alone.

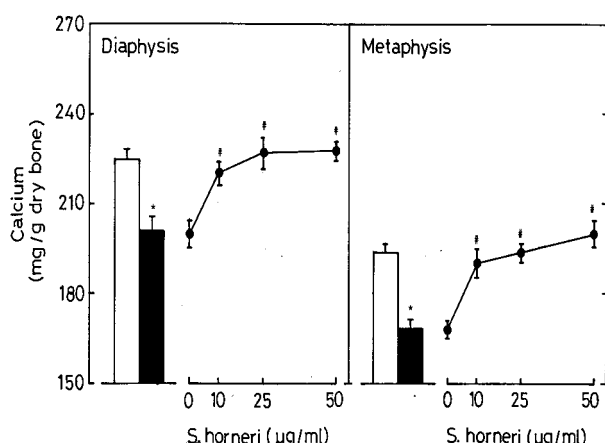


Fig. 2. Effect of *S. horneri* Extract on PGE₂-Decreased Bone Calcium Content in Rat Femoral-Diaphyseal and -Metaphyseal Tissues *in Vitro*

Femoral-diaphyseal or -metaphyseal tissues obtained from normal rats were cultured for 48 hr in a medium containing either vehicle or PGE₂ (10^{-5} M) in the absence or presence of water-solubilized extract (10, 25, and 50 μ g/ml of medium) of *S. horneri*. Each value is the mean \pm S.E.M. of six rats. * $p < 0.01$, compared with the value for PGE₂ alone. White bars, control (none); black bars, PGE₂ alone.

cultured for 48 hr in a medium containing either vehicle, PTH (10^{-7} M) or PGE₂ (10^{-5} M) in the absence or presence of water-solubilized extracts (10, 25, and 50 μ g/ml of medium) of *S. horneri*, as shown in Figs. 1 and 2, respectively. Diaphyseal and metaphyseal calcium content were significantly decreased in the presence of PTH (Fig. 1) or PGE₂. These decreases were completely prevented by water-solubilized extracts (10, 25, and 50 μ g/ml of medium)

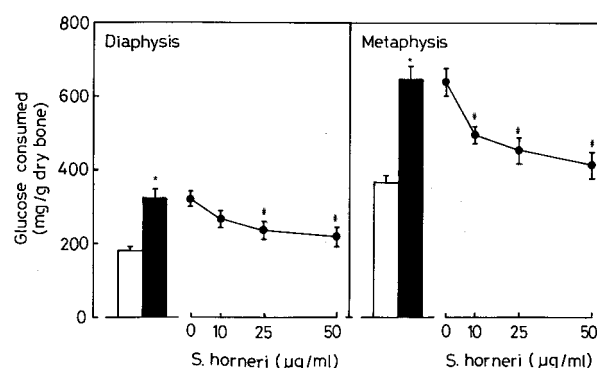


Fig. 3. Effect of *S. horneri* Extract on PTH-Stimulated Glucose Consumption by Rat Femoral-Diaphyseal and -Metaphyseal Tissues *in Vitro*

Femoral-diaphyseal or -metaphyseal tissues obtained from normal rats were cultured for 48 hr in a medium containing either vehicle or PTH (10^{-7} M) in the absence or presence of water-solubilized extract (10, 25, and 50 μ g/ml of medium) of *S. horneri*. Each value is the mean \pm S.E.M. of six rats. * $p < 0.01$, compared with the control (none) value. # $p < 0.01$, compared with the value for PTH alone. White bars, control (none); black bars, PTH alone.

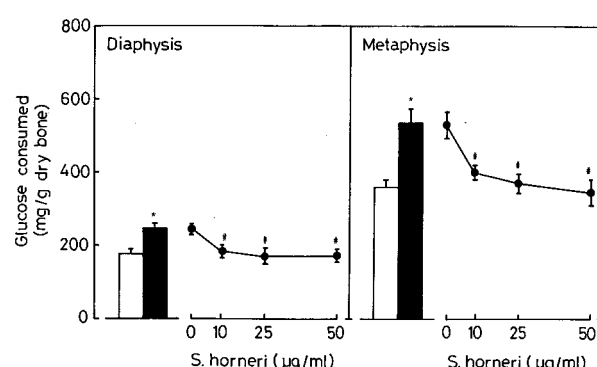


Fig. 4. Effect of *S. horneri* Extract on PGE₂-Stimulated Glucose Consumption by Rat Femoral-Diaphyseal and -Metaphyseal Tissues *in Vitro*

Femoral-diaphyseal or -metaphyseal tissues obtained from normal rats were cultured for 48 hr in a medium containing either vehicle or PGE₂ (10^{-5} M) in the absence or presence of water-solubilized extract (10, 25, and 50 μ g/ml of medium) of *S. horneri*. Each value is the mean \pm S.E.M. of six rats. * $p < 0.01$, compared with the control (none) value. # $p < 0.01$, compared with the value for PGE₂ alone. White bars, control (none); black bars, PGE₂ alone.

of *S. horneri*, as shown in Figs. 1 and 2, respectively.

Effect of *S. horneri* Extract on Bone-Resorbing Factor-Stimulated Glucose Consumption by Bone Tissues *in Vitro*

The effect of water-solubilized extracts of *S. horneri* on the PTH or PGE₂-induced stimulation of medium glucose consumption in the femoral-diaphyseal and -metaphyseal tissues obtained from normal rats is shown in Figs. 3 and 4. The presence of PTH

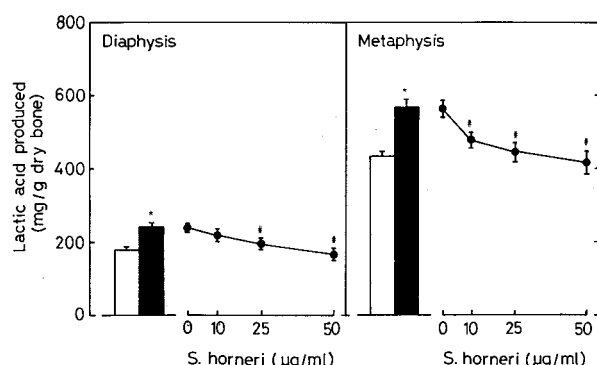


Fig. 5. Effect of *S. horneri* Extract on PTH-Stimulated Lactic Acid Production by Rat Femoral-Diaphyseal and -Metaphyseal Tissues *in Vitro*

Femoral-diaphyseal or -metaphyseal tissues obtained from normal rats were cultured for 48 hr in a medium containing either vehicle or PTH (10^{-7} M) in the absence or presence of water-solubilized extract (10, 25, and 50 μ g/ml of medium) of *S. horneri*. Each value is the mean \pm S.E.M. of six rats. * $p < 0.01$, compared with the control (none) value. # $p < 0.01$, compared with the value for PTH alone. White bars, control (none); black bars, PTH alone.

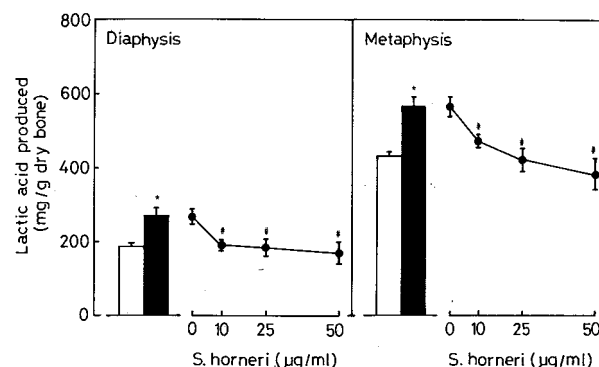


Fig. 6. Effect of *S. horneri* Extract on PGE₂-Stimulated Lactic Acid Production by Rat Femoral-Diaphyseal and -Metaphyseal Tissues *in Vitro*

Femoral-diaphyseal or -metaphyseal tissues obtained from normal rats were cultured for 48 hr in a medium containing either vehicle or PGE₂ (10^{-5} M) in the absence or presence of water-solubilized extract (10, 25, and 50 μ g/ml of medium) of *S. horneri*. Each value is the mean \pm S.E.M. of six rats. * $p < 0.01$, compared with the control (none) value. # $p < 0.01$, compared with the value for PGE₂ alone. White bars, control (none); black bars, PGE₂ alone.

(10^{-7} M) or PGE₂ (10^{-5} M) caused a significant increase in medium glucose consumption by the femoral-diaphyseal or -metaphyseal tissues when the bone tissues were cultured for 48 hr. These increases were completely prevented in the presence of water-solubilized extracts (25 or 50 μ g/ml) of *S. horneri*.

Effect of *S. horneri* Extract on Bone-Resorbing Factor-Increased Lactic Acid Production by Bone Tissues *in Vitro*

The effect of water-solubilized extracts of *S. horneri* on the PTH- or PGE₂-induced increase in lactic acid production in the femoral-diaphyseal and -metaphyseal tissues obtained from normal rats is shown in Figs. 5 and 6. Bone tissues were cultured for 48 hr. The production of lactic acid by the femoral-diaphyseal or -metaphyseal tissues was significantly increased in the presence of PTH (10^{-7} M) or PGE₂ (10^{-5} M), as shown in Figs. 5 and 6, respectively. PTH- or PGE₂-induced increase in bone lactic acid production was completely prevented in the presence of water-solubilized extracts (25 or 50 μ g/ml of medium) of *S. horneri* (Figs. 5 and 6).

Effect of *S. horneri* Extract on PTH-Increased Bone Acid Phosphatase Activity *in Vitro*

The effect of water-solubilized extracts of *S. horneri* on the PTH-induced increase in acid phosphatase activity in the femoral-diaphyseal and -metaphyseal tissues obtained from normal rats is shown in Fig. 7. Bone tissues were cultured for 48 hr in the

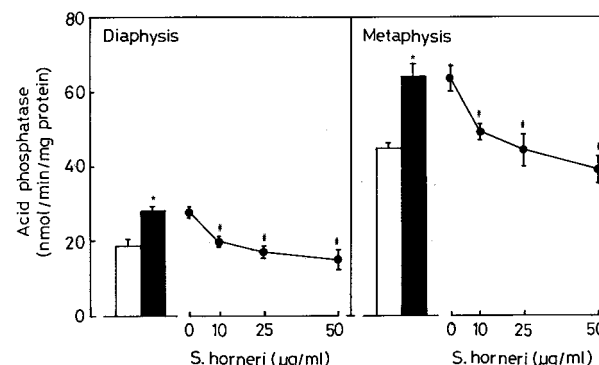


Fig. 7. Effect of *S. horneri* Extract on PTH-Increased Acid Phosphatase Activity in Rat Femoral-Diaphyseal and -Metaphyseal Tissues *in Vitro*

Femoral-diaphyseal or -metaphyseal tissues obtained from normal rats were cultured for 48 hr in a medium containing either vehicle or PTH (10^{-7} M) in the absence or presence of water-solubilized extract (10, 25, and 50 μ g/ml of medium) of *S. horneri*. Each value is the mean \pm S.E.M. of six rats. * $p < 0.01$, compared with the control (none) value. # $p < 0.01$, compared with the value for PTH alone. White bars, control (none); black bars, PTH alone.

presence of PTH (10^{-7} M). Acid phosphatase activity in the femoral-diaphyseal and -metaphyseal tissues was significantly increased in the presence of PTH. These increases were completely prevented in the presence of water-solubilized extracts (10, 25 μ g/ml of medium) of *S. horneri* (Fig. 7).

DISCUSSION

A decrease in bone mass with increasing age induces osteoporosis. Food and nutritional factors may play a role in the prevention of bone loss with increasing age. Isoflavones and menaquinone-7 (vitamin K₂) in fermented soybean (*natto*) have been demonstrated to have an anabolic effect on bone mass in rats.^{12,26–28)} More recent studies have shown that marine alga *S. horneri* extract has an anabolic effect on bone formation and bone calcification *in vivo* and *in vitro*.^{18,19)} The present study was also undertaken to determine the effect of *S. horneri* extract on bone resorption in tissue culture *in vitro*. It was found that water-solubilized extracts of *S. horneri* can inhibit the bone-resorbing factor-induced bone resorption in tissue culture *in vitro*.

It has been reported that PTH and PGE₂ have a stimulatory effect on bone resorption in a culture system *in vitro*.^{29,30)} The presence of PTH (10⁻⁷ M) and PGE₂ (10⁻⁵ M) clearly stimulated bone resorption in rat femoral-diaphyseal and -metaphyseal tissues cultured for 48 hr, when bone resorption was estimated by a decrease in bone calcium content. It has been shown that the concentration of bone-resorbing factors used can reveal a maximum effect on bone resorption in tissue culture *in vitro*.³¹⁾ The present data coincided with studies reported previously.³¹⁾ The effect of bone-resorbing factors (PTH or PGE₂) to stimulate bone resorption using femoral-diaphyseal and -metaphyseal tissues was completely inhibited in the presence of water-solubilized extracts of *S. horneri*. Thus *S. horneri* extract had an inhibitory effect on bone resorption in the tissue culture system *in vitro*.

PTH and PGE₂ caused a remarkable increase in glucose consumption and lactic acid production by rat femoral-diaphyseal and -metaphyseal tissues. The production of lactic acid from bone tissues may be related to the augmentation of glucose consumption. Presumably, PTH- and PGE₂-stimulated lactic acid production by bone tissues can induce a decrease in bone calcium content, since the stimulatory mechanism of PTH on bone resorption is related to the extracellular release of acid by bone cells (osteoclasts).³²⁾ *S. horneri* extract completely blocked the PTH- or PGE₂-induced increase in both glucose consumption and lactic acid production by bone tissues. These findings suggest that the inhibitory effect of *S. horneri* extract on bone resorption is partly related to the prevention of lactic acid production by bone tissues.

The presence of PTH caused a significant increase in acid phosphatase activity in rat femoral-diaphyseal and -metaphyseal tissues. This result is in agreement with other reports.³³⁾ The PTH-increased acid phosphatase activity was completely blocked in the presence of water-solubilized extracts of *S. horneri*. The result suggests that the inhibitory effect of *S. horneri* extract on bone resorption is partly involved in the restoration of bone acid phosphatase activity increased by PTH.

Osteoclasts, bone-resorbing cells, are formed from bone marrow cells.³⁴⁾ It is unknown whether *S. horneri* extract inhibits osteoclastic cell formation and mature osteoclasts. The cellular mechanisms by which an active component in water-solubilized extracts of *S. horneri* inhibits bone resorption remain to be elucidated.

Water-solubilized extracts of *S. horneri* had a stimulatory effect on bone formation and bone mineralization in tissue culture using rat femoral-diaphyseal and -metaphyseal tissues *in vitro*.^{18,19)} Also, *S. horneri* extract could inhibit bone resorption in tissue culture. *S. horneri* extract may induce an increase in bone mass due to stimulated osteoblastic bone formation and inhibited osteoclastic bone resorption. The intake of dietary *S. horneri* is a useful tool in the prevention of osteoporosis with increasing age.

In conclusion, it has been demonstrated that water-solubilized extracts of *S. horneri* have a direct inhibitory effect on bone resorption in tissue culture using rat femoral-diaphyseal and -metaphyseal tissues *in vitro*.

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