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Effects of porphyrins on encystment and excystment in ciliated protozoan *Colpoda* sp.Saori TSUTSUMI,¹ Tomoko WATOH,¹ Koji KUMAMOTO,² Hiyoshizo KOTSUKI² and Tatsuomi MATSUOKA^{1*}¹*Institute of Biological Science, Faculty of Science, Kochi University, Kochi 780-8520, Japan***SUMMARY**

Excystment of *Colpoda* sp. was found to be triggered by a brownish fraction obtained by high performance liquid chromatography (HPLC) of cereal infusion that was expected to contain porphyrins. A commercial porphyrin, chlorophyllin (coppered, sodium salt) induced excystment and prevented encystment, while protoporphyrin IX without metal, protoporphyrin IX bound to zinc, and riboflavin did not have any excystment-inducing effect. These results suggest that the porphyrins contained in cereal leaves might trigger excystment and prevent encystment. Green-colored chlorophyllin is expected to be useful as a molecular probe to visualize and isolate receptors for triggering the encystment and excystment processes.

INTRODUCTION

The processes of encystment and excystment in protozoans involves overall reconstruction at the cellular level (Grimes, 1973; Martín-González et al., 1992; Delmonte et al., 1996; Gutiérrez et al., 2001). These processes might be controlled by unknown intracellular signaling chains including gene expression and silencing that are activated by certain receptor molecules. One of the strategies for elucidating intracellular signaling chains leading to the gene expression or silencing required for encystment or excystment involves determining environmental signals for activating receptors so that these signals can be employed as a molecular probe for isolation of the receptors. The receptor-like protein isolated to date may be only a 40 kD surface protein (encystment stimulating protein, ESP) of soil amoeba that was screened by using immunological methods (Yang and Villemez, 1994).

It has previously been reported that vegetative cells of *Colpoda* sp., which had been isolated from cysts adhered to dried fallen leaves, encyst in response to an increase in the concentration of ions such as Ca²⁺, which may be a signal for forthcom-

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ing desiccation (Watoh et al., 2003; Yamaoka et al., 2004). In addition, encystment of the cells can also be induced by increasing cell density up to 1000~3000 cells/ml (our unpublished data). On the other hand, Ca^{2+} -induced encystment is prevented by certain molecules released from bacteria (*Enterobacter aerogenes*) suspended in a surrounding medium (Yamasaki et al., 2004).

Excystment of *Colpoda* is induced by certain components contained in hay extracts (Thimann and Barker, 1934) or cereal infusion (Watoh et al., 2003). In the present study, we focused on environmental factors initiating the excystment process, analyzing in particular what kinds of components contained in cereal leaves activate the signaling chains leading to excystment, and suggested that certain kinds of porphyrins could trigger excystment.

MATERIALS AND METHODS

Colpoda sp. was isolated from cysts adhered to dried fallen leaves on the soil in Kochi prefecture, and cultured in 0.1% (w/v) infusion of dried cereal leaves (autoclaved for 20 min) inoculated with bacteria (*Enterobacter aerogenes*) at 23°C in the dark. Bacteria were cultured on agar plates containing 1.5% agar, 0.5% polypeptone, 1% meat extract and 0.5% NaCl. The cysts employed in the present assays for excystment (Figs. 1-4) were prepared as follows: vegetative cells were collected by centrifugation (1,000 g, 1 min) and kept suspended at a cell density of 1,000~3,000 cells/ml for more than 1 month in 0.1 mM Tris-HCl (pH 7.2). For induction of excystment, the cysts were rinsed twice in 0.1 mM Tris-HCl (pH 7.2) containing tested components such as porphyrins, and 50~100 cysts were subsequently suspended in 1 ml of each test solution by transferring them using a thin glass pipette. The rate (%) of excystment or encystment was examined at 10 hr (Figs. 1b, 1c,

3d, 4, 5) or at 24 hr (Fig. 2d) after the onset of induction, and is expressed as a percentage of the total number of tested cells (50-100 cells). Points (columns) and attached bars correspond to the means of four identical measurements (50-100 cells per measurement) and standard errors.

Protoporphyrin IX zinc (II) (Aldrich) was dissolved in 0.1 mM Tris-HCl buffer (pH 7.2) as sodium salt, because original compound was less soluble in water; that is, the functional group - $\text{CH}_2\text{CH}_2\text{COOH}$ was replaced by - $\text{CH}_2\text{CH}_2\text{COONa}$ by adding an excess of NaOH, and subsequently neutralized with HCl. The excess Na^+ and Cl^- were removed by dialyzing with 500 Da cut-off membrane (Float-A-Lyzer, Spectrum Laboratories, Inc., USA) for 24 hr in a cooled 0.1 mM Tris-HCl buffer (pH 7.2) on ice. Other porphyrins and riboflavin (Aldrich) were simply dissolved in 0.1 mM Tris-HCl buffer (pH 7.2).

Cereal infusions (0.5%) with and without 0.1% trifluoroacetic acid (TFA) were fractionated by reverse-phase high performance liquid chromatography (HPLC) equipped with a column (Cosmosil 5C₁₈ AR-300) having a 0-100% acetonitrile gradient with or without 0.1% TFA. Fractionated samples (1 volume) were dried at room temperature using a rotary evaporator (Rotavapor, Sibata) and dissolved in 5 volumes of 0.1 mM Tris-HCl buffer (pH 7.2) for excystment assays. For the ninhydrin test, the fractions were concentrated by using a rotary evaporator and applied on a TLC plate (Funacel SF, Funakoshi, Japan). The plate was dried and immersed in 95% ethanol solution containing 0.2% ninhydrin.

The density of bacteria was spectroscopically determined in a diluted bacterial suspension whose value had been calibrated by comparison with the cell density obtained by directly counting the colonies on plates with the optical density value at 600 nm (OD_{600}).

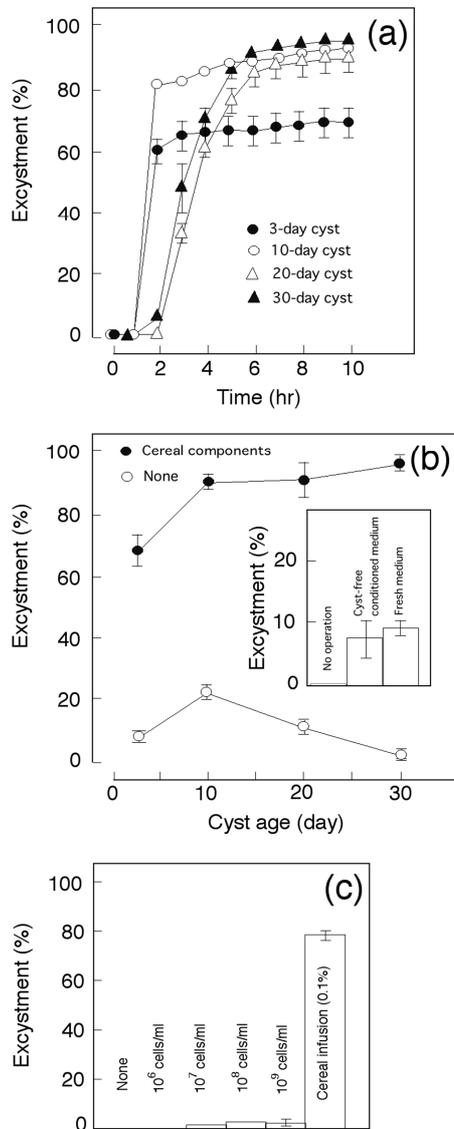


Fig. 1. Effect of cyst age on excystment of *Colpoda* induced by cereal infusion or on spontaneous excystment, and excystment-inducing effect by bacteria. (a) Time course of excystment induced by 0.1% cereal infusion containing 0.1 mM Tris-HCl (pH 7.2). The rate of excystment is expressed as a percentage of the total number of tested cells (50-100 cells). (b) The relationship between cyst age and excystment rate (%) induced by cereal components (closed circles) and the rate (%) of spontaneous excystment (open circles). The cysts were transferred from 0.1% Tris-HCl (pH 7.2) into a fresh 0.1 mM Tris-HCl (pH 7.2) buffer containing 0.1% cereal infusion (closed circles) or into a fresh 0.1 mM Tris-HCl (pH 7.2) buffer containing 0.1% cereal infusion (closed circles) or into a fresh 0.1 mM Tris-HCl (pH 7.2) buffer containing 0.1% cereal infusion (closed circles). Inset, the effect of cyst-free conditioned medium on excystment. Three-day-old cysts adhered to the bottom of a petri dish were scraped and transferred into cyst-free conditioned medium or a fresh 0.1 mM Tris-HCl buffer (pH 7.2). The label "No operation" indicates the spontaneous excystment rate of the cysts without mechanical touch (cysts remained adhered to the bottom of the petri dish); in this case, the cysts were randomly selected (50-100 cysts) in the field of the dissecting microscope and vacant cysts were counted. (c) Excystment induction by bacteria (*Enterobacter aerogenes*) suspended in the surrounding medium containing 0.1 mM Tris-HCl (pH 7.2).

RESULTS AND DISCUSSION

It has been reported that the rate of excystment of *Histiculus* is influenced by the age of the cysts (Nakamura and Matsusaka, 1991). The rate of excystment of *Colpoda* employed in the present study was also influenced by cyst ages. As shown in Fig. 1a, the rate (%) of excystment reached a plateau within 6 hr after the onset of induction. Therefore, the excystment rates at 8 hr after the

onset of induction were examined (Figs. 1b, 1c, 2d, 3d, 4). The young cysts (aged less than 20 days) tended to excyst when they were transferred into a fresh medium (0.1 mM Tris-HCl, pH 7.2) without cereal components (Fig. 1b, open circles) and the rate (%) of excystment tended to be lower in comparison with 30-day-old cysts (Fig. 1b). Therefore, in the present study, cysts aged more than 30 days were used. Such a spontaneous excystment of younger cysts might be triggered by

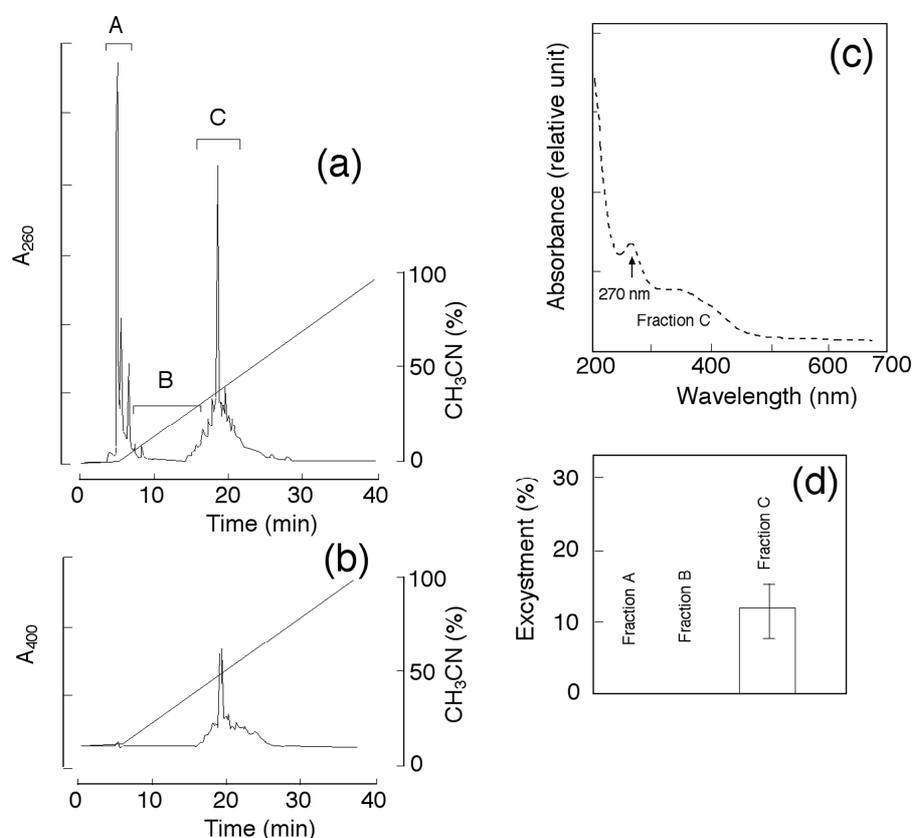


Fig. 2. Induction of excystment of *Colpoda* by cereal components. (a, b) Elution profiles of cereal infusion containing trifluoroacetic acid (TFA) by reverse-phase HPLC equipped with a column (Cosmosil 5C18 AR-300) having a 0-100% acetonitrile gradient containing 0.1% TFA; profiles were monitored by the absorbances at 260 (Fig. 2a) and 400 nm (Fig. 2b); in the present case, the monitored sensitivity was different between Figs. 2a and b. (c) Absorption spectrum of colored fraction (fraction C). (d) The rate of excystment induced by each fractionated component dissolved in 0.1 mM Tris-HCl buffer (pH 7.2). Their concentrations correspond to those contained in 0.1 % cereal infusion.

replacing the conditioned surrounding medium (0.1 mM Tris-HCl, pH 7.2) in which the cells had encysted and had possibly excreted certain components such as metabolic products, with a fresh medium; that is, it might be triggered by the removal of *Colpoda*-derived substances accumulated in the conditioned surrounding medium. When 3-day-old cysts were transferred into cyst-free conditioned medium using a glass pipette, the excystment rate was not significantly different from the

rate in a fresh medium (Fig. 1b, inset; $p > 0.05$, Mann-Whitney test). On the other hand, the cysts that were not transferred rarely excysted (Fig. 1b, inset, labeled “No operation”); there was a significant difference between “No operation” and “Cyst-free conditioned medium” ($p < 0.05$, Mann-Whitney test). These results indicate that mechanical stimulation by pipetting might induce excystment in younger cysts. Presumably, younger cysts are more likely to be influenced by a me-

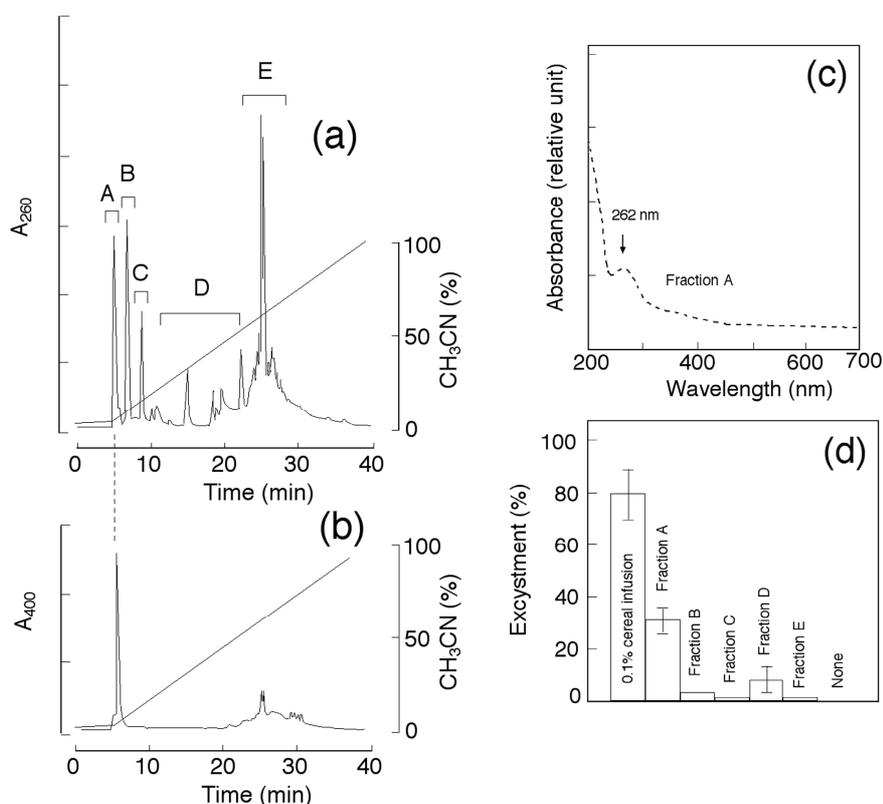


Fig.3. Induction of excystment of *Colpoda* by cereal components. (a, b) Elution profiles of cereal infusion without TFA by reverse-phase HPLC equipped with a column (Cosmosil 5C18 AR-300) having a 0-100% acetonitrile gradient without TFA; profiles were monitored by the absorbances at 260 (Fig. 3a) and 400 nm (Fig. 3b); note that the monitored sensitivity was different. (c) Absorption spectrum of colored fraction (fraction A). (d) The rates of excystment induced by fractionated components (their concentration corresponding to those contained in 0.1 % cereal infusion) that were dissolved in 0.1 mM Tris-HCl buffer (pH 7.2). The column labeled "None" indicates the excystment rate of cysts suspended in 0.1 mM Tris-HCl buffer without any components.

chanical touch because the cyst wall might be thinner than in older, mature cysts.

Excystment of *Colpoda* is triggered by certain molecules contained in cereal leaves (Watoh et al., 2003; Figs. 1a, 1b). In order to determine whether the excystment-inducing activity of cereal infusion could be due to bacteria in the infusion, the bacterial density in 0.1% cereal infusion containing cysts was measured spectroscopically. The density of proliferated bacteria was less than 10^5

cells/ml at 18 hr after the cysts were suspended in a sterilized fresh cereal infusion, a value which is at the lower limit of spectroscopic detectability. On the other hand, even a bacterial density of 10^9 cells/ml failed to show excystment-inducing activity (Fig. 1c). Therefore, the excystment-inducing effect of cereal infusion cannot be attributed to bacterial contamination, but to certain cereal components.

Figures 2a and 2b show the elution profiles

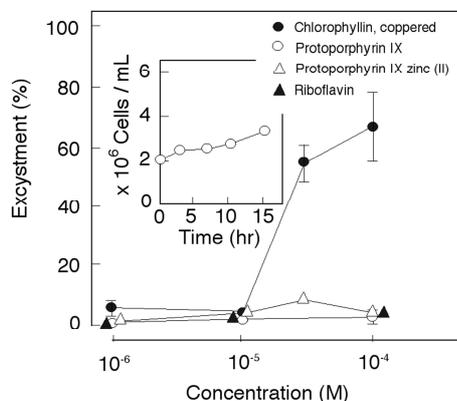
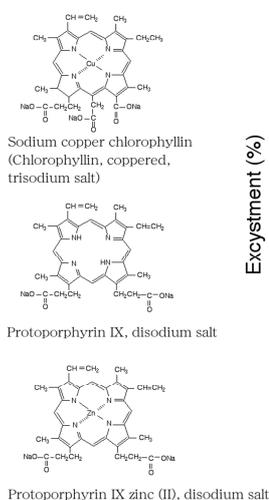


Fig. 4. Effects of porphyrins and riboflavin on *Colpoda* excystment. The porphyrins (sodium salts) or riboflavin was dissolved in 0.1 mM Tris-HCl buffer (pH 7.2). Inset, growth of bacteria (*Enterobacter aerogenes*) in a 0.1 mM Tris-HCl (pH 7.2) containing 10^{-4} M chlorophyllin.

of cereal infusion containing 0.1% TFA by reverse-phase HPLC monitored by the absorbances at 260 nm and 400 nm, respectively. Of fractions A-C, only fraction C (light brownish in color; absorption spectrum shown in Fig. 2c) showed excystment induction activity (Fig. 2d). In this case, the cells did not excyst within 10 hr after induction, but did excyst within 24 hr after induction. In addition, the excystment rate was lower, despite the fact that the concentration of components of fraction C corresponded to those contained in 0.1% cereal infusion (ref. Fig. 3d). It is possible that a large amount of colored components was lost as precipitates produced by TFA treatment. Fraction C was ninhydrin-positive, as was fraction A, and had an absorption ranging from 200 to 450 nm (Fig. 2c).

Figures 3a and 3b show the elution profiles of cereal infusion by reverse-phase HPLC analyses using solvents without TFA. Of these fractions, fractions A (absorption spectrum shown in Fig. 3c) and E were colored, and only fraction A showed a marked excystment-inducing effect (Fig. 3d). In this case, only the rate of excystment induced by fraction A was significantly different from the control (labeled "None"; excystment rate in 0.1 mM Tris-HCl without components) ($p < 0.05$, Mann-

Whitney test). Fractions B-E were slightly ninhydrin-positive while fraction A was strongly positive, suggesting that fraction A may contain compounds with amino groups and certain colored components such as porphyrins derived from chlorophyll or riboflavin.

We examined the excystment-inducing activity of commercially obtained porphyrins (chlorophyllin, coppered, trisodium salt; protoporphyrin IX, disodium salt; protoporphyrin IX zinc) and riboflavin. Chlorophyllin was found to have a marked excystment-inducing effect, while protoporphyrin IX, protoporphyrin IX bound to zinc and riboflavin had no effect (Fig. 4). The density of bacteria (*Enterobacter*) increased up to only 1.5 times at 10 hr in a 0.1 mM Tris-HCl buffer (pH 7.2) containing 10^{-4} M chlorophyllin (Fig. 4, inset). However, the excystment-inducing effect by chlorophyllin is not attributed to bacteria proliferated in chlorophyllin solution because even high density of bacteria (10^6 – 10^9 cells/ml) hardly induces excystment (Fig. 1c). These results imply that the three-dimensional porphyrin structure conformed by coordination to copper may be involved in the activation of receptors triggering excystment processes. It is also possible that the excystment-inducing activity of the chlorophyllin may be at-

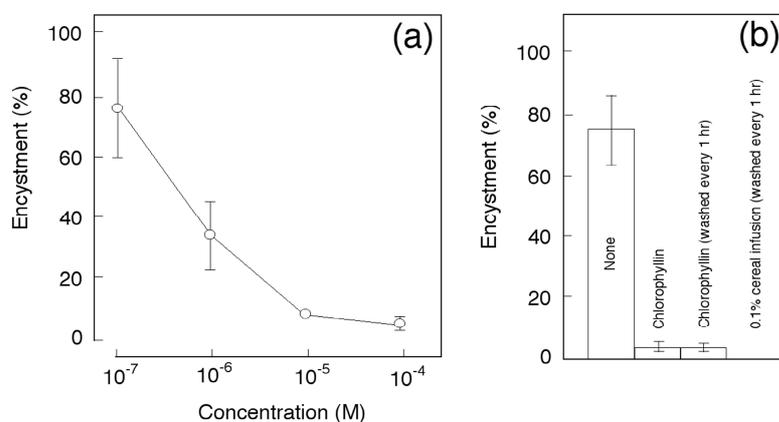


Fig. 5. Suppression of encystment of *Colpoda* by chlorophyllin or cereal components. Encystment was induced by suspending the vegetative cells (50-100 cells) in 1 ml of saline solution (1 mM CaCl_2 , 1 mM KCl, 5 mM Tris-HCl, pH 7.2) containing various concentrations of chlorophyllin or 0.1% cereal infusion. (a) Relationship between the encystment rate and chlorophyllin concentrations. (b) Effect of the elimination of bacteria on encystment suppression by chlorophyllin or cereal components. The cysts were transferred into fresh (sterilized) standard saline solutions containing 10^{-4} M chlorophyllin or 0.1% cereal infusion every hour (labeled "chlorophyllin, washed every hour" or "0.1% cereal infusion, washed every hour"). "None", saline solution (1 mM CaCl_2 , 1 mM KCl, 5 mM Tris-HCl, pH 7.2).

tributable to other elements such as functional groups characterized by chlorophyllin. Chlorophyllin (bound to copper) does not occur in nature. It is presumable, therefore, that excystment-inducing elements contained in cereal leaves may be chlorophyll-derived water-soluble porphyrins associated with magnesium.

Cereal infusion (0.1%) and chlorophyllin remarkably prevented Ca^{2+} -induced encystment (Fig. 5a). Such a suppression effect still remains to be attributed to bacteria contaminated and proliferated in these media. In the present experiments, therefore, the cells were transferred into the fresh media every hour. In this case, both cereal infusion and chlorophyllin strongly suppressed encystment (Fig. 5b).

The binding of antibody to the receptor-like protein (ESP) located on the cell surface of vegetative cells of soil amoeba induces encystment, while the binding of ESP antibody located on the cysts

suppresses excystment (Yang and Villemez, 1994). The results obtained by Yang and Villemez show that a common receptor is involved in both the induction of encystment and the suppression of excystment. The fact that chlorophyllin triggers the excystment processes of *Colpoda* and suppresses its encystment processes likewise implies that chlorophyllin may activate a common receptor located on the vegetative cells and cysts.

Chlorophyllin may be useful as a molecular probe to visualize and isolate receptor proteins to which chlorophyllin is bound and which activate signaling pathways for excystment processes. Further research is needed to isolate and characterize these receptors by using chlorophyllin.

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