

# PRECIPITATION OF CALCIUM CASEINATE BY HEAT AND SUBSEQUENT REVERSAL

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The precipitation of calcium caseinate by heat (90° C. for 30 to 60 minutes) in a range of pH values and calcium concentrations was measured. Changes in viscosity and clarity of the heated solutions were determined. The effect of cream on the re-solution of the casein precipitates is described. *Editor.*

A study of the effect of heat on calcium caseinate might be expected to contribute to an understanding of the physical changes in heated milk, since casein is a major component of milk. The relatively few studies of this type that have been done have been limited in scope and will be discussed later. In the present investigation the precipitation of calcium caseinate by heat (90° C. for 30 to 60 minutes) in a range of pH values and calcium concentrations has been measured. Changes in viscosity and clarity of the heated solutions have also been determined. Subsequent re-solution of the precipitates at 25° C. has been observed and the extent of this re-solution measured. The effect of cream on the re-solution of the casein precipitates is described.

## MATERIALS AND METHODS

*Casein.* The casein was precipitated from skimmilk by acidification to pH 4.5 with *N* HCl. The precipitate was washed four times with water and twice dissolved and reprecipitated with acid (2). The casein was finally dried with ethanol and ether. The casein was stored in a desiccator at a relative humidity that maintained the moisture content at 10.0%. The concentration given in the results are for the moisture-free product.

*Calcium hydroxide.* A standard solution of calcium hydroxide was prepared at 20 to 25° C. The concentration was determined by titration with standard HCl and found to be 0.045 *N*.

*Preparation of casein solutions.* The casein solutions were prepared and used within 1 to 3 days. When not in use the solutions were stored at 7° C. A stock 4% sodium caseinate solution at pH 6.8 was prepared by dissolving 1.0 g. (moisture-free) of the isoelectric casein in water, with 5.8 ml. of 0.1 *N* NaOH, in a final volume of 25 ml. A 4% calcium caseinate solution at pH 7.0 was prepared

Received for publication June 22, 1956.

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by dissolving 1.0 g. of casein in water, with 15.0 ml. of 0.045 *N* Ca(OH)<sub>2</sub>, in a final volume of 25 ml. The concentration of calcium in this solution was 0.0135 *M*. Other pH values were obtained by the addition of more or less NaOH or by the addition of HCl. The sodium caseinate-calcium chloride solution is considered approximately equivalent to calcium caseinate. The binding of the calcium ion to casein, however, will be lessened somewhat by the presence of sodium ions (unpublished studies).

*Heating.* Four milliliters of the solution to be tested was placed in a 12-ml. Pyrex centrifuge tube. A smaller tube containing ice water was suspended within the centrifuge tube to prevent loss of water by evaporation. The solutions were heated for various periods at 90° C. in a constant level water bath. The pH values were measured before and after heating; in most cases the values were identical. After heating, the solutions were immediately brought to 25° C. by placing the tubes in water at that temperature.

*Centrifuging and sampling.* The heated samples after variable periods of time (2 minutes to 3 hours) at 25° C. were centrifuged for 10 minutes at 3,000 × *G*. The top 0.5 ml. of the solution was withdrawn for analysis, taking care not to disturb the remainder of the solution. The amount of sample taken was kept constant since when the precipitates are colloidal a concentration gradient was apparent after centrifuging.

*Determination of casein.* (a) The casein in the supernatant fluid from the centrifuged samples in some instances was estimated from the nitrogen content, determined by the Kjeldahl procedure. (b) The casein was also estimated from the light absorption at 280 *mμ*. The heated solutions were clarified by mixing 0.15 ml. of 0.1 *M* disodium versenate, adjusted to pH 8.0 with 0.1 *N* NaOH, with 0.5 ml. of the calcium caseinate solution. Water was added, usually to a final volume of 25 ml., to give a dilution of the casein solution of 1:50, for the light absorption measurements at 280 *mμ*. An absorbance of 0.85 in a 1-cm. cell is equivalent to 1.0 mg. of whole casein per 1.0 ml. The absorbance factor for α-casein is 1.03 and for β-casein is 0.48 (2). Hence if a fractionation of the casein occurred in the heat precipitation, the protein estimations would be somewhat in error. There is, however, no evidence that a separation of α- and β-casein occurs.

*Viscosity and opacity determination.* The viscosity was determined at 30° C. in a Bingham type viscometer with a variable pressure head. For this measurement a volume of 6.0 ml. was heated in a 1-inch diameter test tube. The opacity or whiteness of these solutions was estimated from the apparent light absorption at 600 *mμ*. Details of the viscosity and opacity measurements are described in a previous paper (7).

## RESULTS

*Effect of calcium concentration on viscosity of unheated and heated sodium and calcium caseinates at pH 7.0.* The effect of calcium chloride concentration on the viscosity of 2.0% solutions of sodium and calcium caseinates at pH 7.0 is shown in Figure 1. The calcium caseinate curves (unheated and heated) are

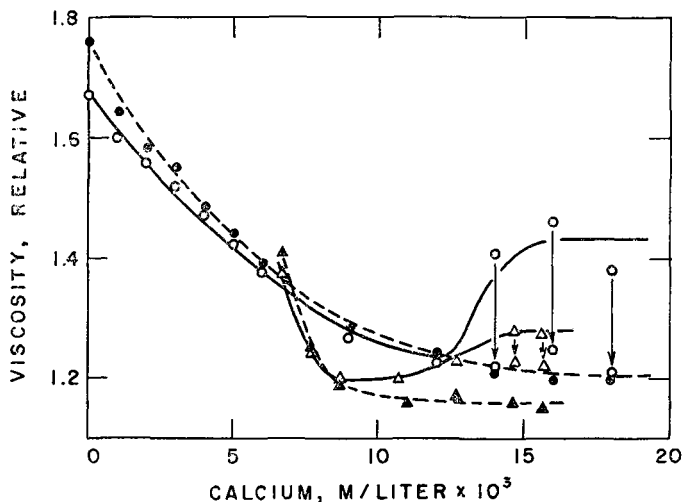


FIG. 1. The viscosities of 2% sodium and calcium caseinate solutions at pH 7.0 with increasing calcium concentrations, before heating and after heating for 30 minutes at 90° C. and cooling. Calcium was added to both caseinates as calcium chloride. Calcium concentration given is total calcium.

Sodium caseinate:                    unheated ●-----●;                    heated ○———○  
 Calcium caseinate:                    unheated ▲-----▲;                    heated △———△

The vertical arrows indicate the drop in viscosity of the heated solutions after 1 hour at 30° C.

plotted in terms of the total calcium concentration, that is, the graphs begin with the calcium concentration (0.0067 *M*) of the calcium caseinate solution.

Small concentrations of calcium decrease the viscosity of the casein solutions, but at total concentrations of calcium above 0.012 *M*, the viscosities of both heated sodium and heated calcium caseinates are greater than their viscosities before heating. This increase in viscosity is largely reversible and is due to the formation of precipitates of colloidal calcium caseinate which redissolve at lower temperatures.

The appearance of these solutions is of interest since opacity or light scattering (measured by apparent absorbance at 600  $m\mu$ ) is indicative of aggregation. The unheated sodium caseinate remains clear (absorbance of 0.05 or less) with concentration of calcium chloride up to 0.005 *M*; with greater concentrations of calcium an opalescence appears, which increases considerably with time even at 25° C. For calcium concentrations of 6, 8, 10, 12 and 14  $\times 10^{-3}$  *M*/l., the respective absorbance readings at 25° C. within a few minutes of adding the calcium chloride are 0.15, 0.34, 0.59, 0.94 and  $> 2$ . The light scattering, that is the aggregation, is greatly reduced if the solutions are cooled below 25° C. As one might expect, the light scattering is increased by heating; this is quickly reversible to a considerable extent when the solutions are lowered to 25° C. With 0.005 *M* calcium the reversal is complete: before heating, the absorbance is 0.05; at 90° C. the solution is opaque (absorbance  $> 2$ ); when returned to 25° C., the absorbance is 0.15, and within 1 hour it is at its original value of 0.05. With

0.006 *M* calcium or greater absorbance is  $> 2$  at  $90^{\circ}\text{C}$ .; at  $25^{\circ}\text{C}$ . the absorbance drops (to 0.3 with 0.006 *M* calcium) and subsequently increases again.

*Precipitation of casein by calcium chloride and heat ( $90^{\circ}\text{C}$ .) and re-solution at  $25^{\circ}\text{C}$ .* Relatively large concentrations of calcium chloride are required to precipitate sodium caseinate as the calcium salt without heat; the extent is partially pH-dependent. For example 0.02 *M* calcium chloride in a 2.0% sodium caseinate solution precipitates 30% at pH 5.6, 20% at pH 6.0 and 6.6, and 8% at pH 7.6. In the calcium chloride range of 0.015 to 0.025 *M* the extent of precipitation is proportional at each pH to the concentration of calcium; for example, precipitation at pH 5.6 declines to 12% at 0.015 *M* calcium chloride concentration. The precipitation of sodium caseinate by calcium chloride is in-

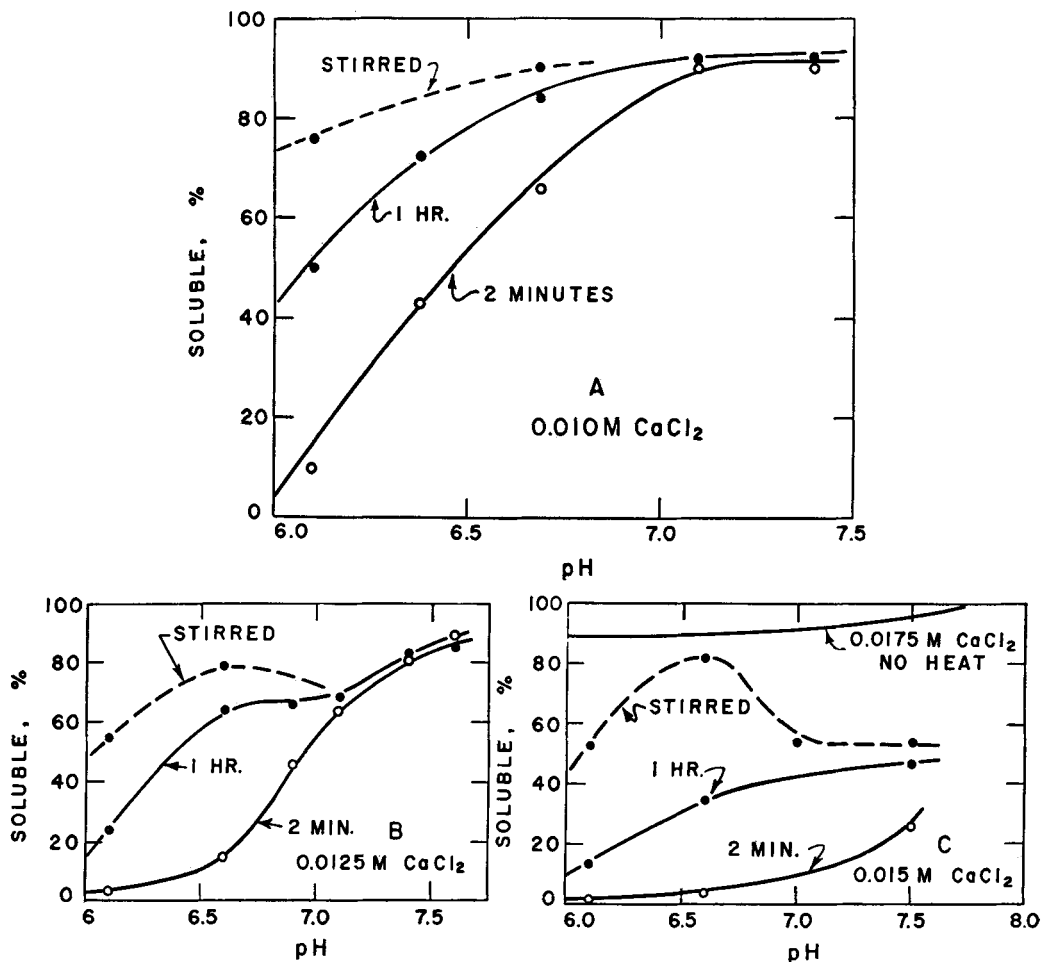


FIG. 2. Precipitation of 2% sodium caseinate solutions at pH 6.1 to 7.6 containing calcium chloride after heating for 1 hour at  $90^{\circ}\text{C}$ . The lower solid curves show the portion remaining in solution immediately after cooling to  $25^{\circ}\text{C}$ . (about 2 minutes required), and the upper curves the portion in solution 1 hour after bringing to  $25^{\circ}\text{C}$ . The dashed lines indicate the solubility attained when the precipitates are stirred every 10 minutes for 1 hour.

creased by heating at 90° C. Much of this precipitation reverses at 25° C., but in no case did the solubility return to the value before heating. The effect of pH and calcium concentration on this heat-produced precipitation is shown in Figures 2-A to -C. Similar experiments were also done with 0.0075 *M* CaCl<sub>2</sub>, a concentration that gave a precipitate (80% remained soluble) only at pH 6.1. The percentage in solution is shown for 2 minutes (time required to cool to 25° C. and begin centrifugation) and 1 hour after heating. The values plotted are averages of 2 to 10 or more experiments. In a few instances the reversed (1 hour) values have been as much as 20% higher than the values shown. The occasional high value may be due to difficulty in centrifuging and sampling these colloidal precipitates. These data (shown by solid lines) were obtained with precipitates that were undisturbed during the 25° C. period. Much of the precipitate adheres to the walls of the tubes when the precipitates are flocculent, as with high calcium concentration and low pH. It was found, however, that if the precipitates were broken up and kept in suspension, a higher level of solubility was attained on standing at 25° C. For example, at pH 6.1 and 0.0125 *M* calcium chloride almost all of the casein was precipitated on heating; after 1 hour at 25° C. undisturbed, about 20% of the total casein was in solution, and this amount had not increased in 2 hours. If, however, the precipitate was broken up and kept in suspension, 55% was in solution in 1 hour and 59% in 2 hours. Data obtained at other pH and calcium values when the precipitates are kept suspended are shown in Figures 2-A to -C by dashed lines. The precipitation of the casein by heat at pH 6.1 is plotted in Figure 3 with the calcium concentration as the ordinate to emphasize the influence of this important variable. Also shown is the solubility curve of the unheated casein solution.

Factors other than pH and calcium concentration also influence the precipitation and re-resolution of the precipitates. The influence of the time of heating on the amount of precipitate at pH 6.6 and 0.0125 *M* calcium chloride is

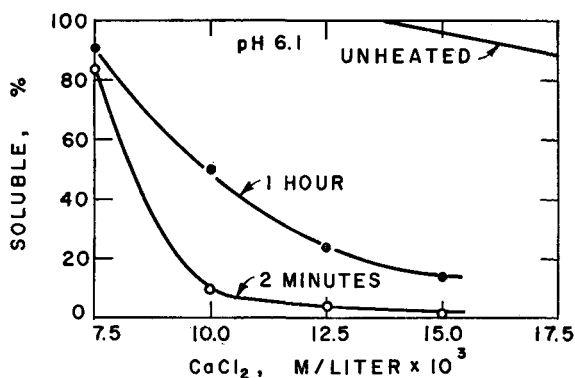


FIG. 3. Precipitation of 2% sodium caseinate solutions containing 0.0075 to 0.0175 *M* calcium chloride at pH 6.1 after heating for 1 hour at 90° C. The lower curve shows the portion remaining in solution immediately after cooling to 25° C. (about 2 minutes required) and the curve above this the portion in solution 1 hour after cooling to 25° C. The short upper right curve indicates the solubility of the unheated solution.

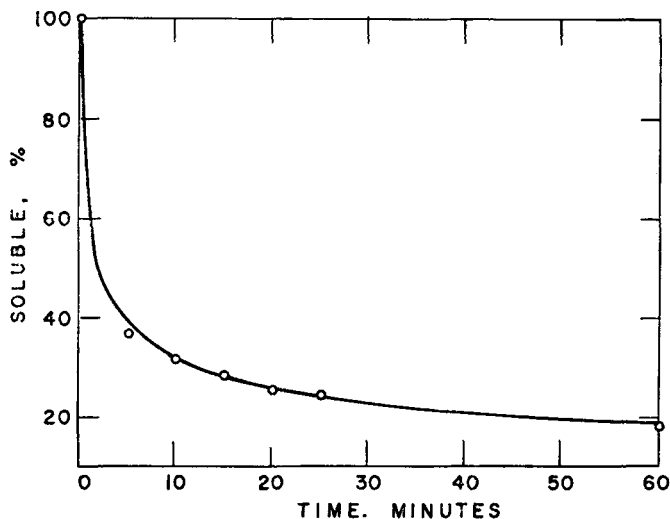


FIG. 4. Precipitation of 2% casein containing 0.0125 *M* calcium chloride at pH 6.5 after heating at 90° C. for various periods of time and bringing to 25° C.

shown in Figure 4. The appearance of the precipitate also changes during the heating period. The initial precipitates are colloidal and become more flocculent and dense as the heating continues. A comparison of solutions at this pH and calcium concentration heated for 10 minutes and 60 minutes indicated that the rate of re-solution of the precipitates was about the same but the solution heated for 10 minutes reached a higher level of solubility at 25° C. Heated solutions permitted to stand at 25° C. took about 1 hour for re-solution. Heated solutions standing at 3° C. redissolved more slowly but about the same solubility level was attained. A comparison of the rate of re-solution at the two temperatures is

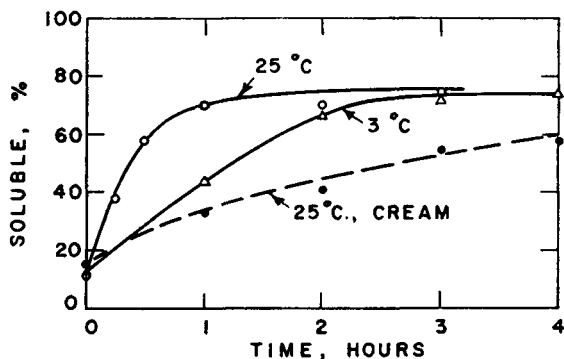


FIG. 5. The rate of re-solution of heated casein precipitates at 3° and 25° C., and the effect of cream at the latter temperature. The 2% casein solutions were at pH 6.5, contained 0.0125 *M* calcium chloride, and were heated for 1 hour at 90° C. The total casein value for the cream experiment was determined with the cream present on a centrifuged sample before heating. Cream (approximately 25% butterfat) was added to 2% by volume.

shown in Figure 5. This figure also contains data illustrating the influence of cream on the re-resolution of the calcium-casein precipitates.

The effect of cream on the heated casein solutions was studied to see whether the re-resolution of the precipitates would be hindered, perhaps by their being physically coated with the cream. The results in Figure 5 show that this actually occurs. It was anticipated that parallel changes in viscosity would occur. The viscosity measurements, however, were unsatisfactory for showing such an effect. A 2% solution of sodium caseinate containing no cream, at pH 6.6 and with 0.0125 *M* calcium chloride, had a relative viscosity of 1.22 before heating and 1.42 after heating, and within 30 to 50 minutes the viscosity had declined to 1.22. The same system containing 2 or 10% cream (approximately 25% butterfat) had an initial viscosity of 1.45, which did not increase when the mixture was heated. Apparently, although the casein has precipitated, parallel changes in the physical state of the cream prevent a change in the viscosity.

#### DISCUSSION

The reduction in viscosity of 2% calcium caseinate, pH 7.0, on the addition of calcium chloride confirms almost exactly results of Hankinson and Briggs (1) at pH 6.6. These authors, however, reported that sodium caseinate showed structural viscosity, and this has not been observed in the present studies. Viscosity measurements on 1% sodium caseinate with several concentrations of sodium chloride were only 1% lower than the data of Hankinson and Briggs, although these authors stated that their measurements were made with a pressure of 30 cm. of mercury to eliminate pressure dependence. Heating the sodium caseinate to 90° C. did not induce structural viscosity. Examination of different preparations of casein would be desirable to explain these divergent results.

The reduction in the viscosity of caseinate solutions on the addition of NaCl or CaCl<sub>2</sub> in part is due to the reduction in electroviscosity (1). It is apparent, however, that the calcium ion has also a specific effect, as shown by its ability to aggregate casein, since the opacity of the solutions increased with the addition of calcium. This aggregation may also contribute to the reduction in viscosity, perhaps by the formation of more compact and symmetrical aggregates, which might explain the fact that calcium caseinate is considerably less viscous than sodium caseinate. The ability of the calcium ion both to aggregate and to reduce viscosity was observed in heated  $\beta$ -lactoglobulin solutions (7), and aggregation of  $\beta$ -lactoglobulin by heat near the isoelectric point leads to a reduction in viscosity (unpublished studies).

The increase in viscosity on heating casein solutions containing more than 0.012 *M* calcium is due to the formation of a colloidal casein precipitate. When the calcium concentration is high enough, however, to give a precipitate of discrete floccules, viscosity measurements are lower and are, of course, meaningless. The precipitation of casein is greatest at high calcium concentration and low pH values, as was observed for  $\beta$ -lactoglobulin (7). With casein, however, even

at concentrations of calcium that almost totally precipitate the casein at 90° C., much of the precipitate will go back into solution at 25° C. The marked effect of stirring on the re-solution of the precipitates at pH 6.6 is not clear. It was expected that the unstirred precipitates would attain the same solubility as the stirred samples, but more slowly. Cessation of re-solution of the unstirred precipitate between 1 and 2 hours suggests that some factor, perhaps a coalesced film of precipitate, is hindering further solution.

The re-solution of calcium caseinate precipitates obtained with heat apparently has not hitherto been reported. Pyne (5) has observed that low calcium concentrations precipitated  $\beta$ -casein and that the precipitate redissolved on cooling. Although this appears to be an unusual solubility property of calcium caseinate, this increase in solubility at low temperature is characteristic of calcium phosphate and of some other inorganic salts. This behavior of inorganic salts is thought to be due to the formation of soluble hydrate at low temperatures and of less soluble anhydride at high temperature. Something similar may be true of calcium caseinate, perhaps through hydration at the phosphate groups.

The precipitation of casein by heat has been investigated by Howat and Wright (3). The treatment used was severe, up to 5 hours at 120° C. The precipitation of calcium caseinate (3.0% concentration at pH 6.5 to 7.0) at 120° C. was less than 10% in 1 hour. In 3 to 5 hours precipitation was about 90%, but under this severe treatment 80% of the phosphate was liberated from casein. The heat treatment used in the present studies (90° C. for 1 to 3 hours) does not precipitate calcium caseinate unless additional calcium chloride is added. This heat treatment will release little or no phosphate from casein (4), and it appears that heat processing of milk is not so severe that phosphate will be released from casein. Howat and Wright (3) found that only calcium caseinate precipitated, although both calcium and sodium caseinates released phosphate on heating. These authors also observed the enhanced precipitation of casein with additional calcium; if the calcium content of the calcium caseinate was increased 16% with calcium chloride, the precipitation in 1 hour at 120° C. was now about 30%.

The ability of cream to slow up the re-solution of the heated calcium caseinate precipitates may be of significance in the storage of heat-processed milk. The drop in viscosity is reminiscent of the drop in viscosity on storage of evaporated milk (6), although the latter takes several weeks for completion. The formation of colloidal casein precipitates during sterilization of milk and their slow re-solution on storage may account for the slow drop in viscosity on the storage of evaporated milk. This possibility, however, is made less likely by the finding that the addition of sodium phosphate (unpublished studies) makes the casein precipitates more dense and less colloidal, and retards or prevents re-solution of the precipitates. The slowing of the re-solution of heated casein precipitates by cream probably results from a physical coating of the colloidal calcium caseinate precipitates, which hinders access to the aqueous phase into which the precipitate can dissolve.



## SUMMARY

The addition of calcium chloride to 2% sodium or calcium caseinate solutions causes a drop in viscosity, perhaps through aggregation since the solutions become opalescent. If the total concentration of calcium is not greater than 0.012 *M*, heating to 90° C. for 1 hour does not change the viscosity, but the solutions become more opalescent and even opaque (white). The opalescence is completely reversible, at the lower calcium concentrations, when the solutions are cooled. At concentrations of calcium above 0.012 *M* the viscosity is increased by heating to 90° C.; on standing at lower temperatures the viscosity increase is partly reversed in 1 to 3 hours. The viscosity increase is due to the formation of a colloidal precipitate of casein which partly redissolves at lower temperatures. The amount of precipitate is increased by high calcium concentration and low pH values. Other factors that affect the precipitation and re-resolution are time of heating, stirring, and temperature after heating. Cream slows up the re-resolution of the colloidal casein precipitates.

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