

INFLUENCE OF HYDROGEN AND CALCIUM ION CONCENTRATIONS, TEMPERATURE, AND OTHER FACTORS ON THE RATE OF AGGREGATION OF CASEIN

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SUMMARY

The rates of aggregation of 1% whole casein, α -casein, mixtures of α - and β -casein, and pepsin-paracasein, produced by the addition of calcium chloride at pH 6.3, have been studied at 25, 30, and 35°; the rates of aggregation of β -casein alone have been studied at 30 and 31°. Temperature coefficients for the rate of aggregation are quite high (> 14 per 10°). Similar studies were performed with casein to which phosphate was added, with casein plus citrate, with a mixture of α - and β -casein (3:1), and with casein aggregated without calcium chloride at pH 5.5. In addition, the aggregation of whole casein by CaCl₂ has been studied from pH 7.9 to 5.5, at concentrations of casein of 0.2 to 1.5%, and the effect of several concentrations of sodium chloride on the aggregation determined.

A study (17) of the effect of temperature on the precipitation of casein with calcium chloride has shown that the development of turbidity is time-dependent and largely reversible. In the present report, the initial stages of casein aggregation, implemented by calcium chloride or by hydrogen ions (pH 5.5), have been studied turbidimetrically at 25 to 35°. Effect of concentrations of the components, effect of buffer type and concentration, effect of sodium ion, phosphate ion, and citrate ion also have been studied. The caseins studied have been whole casein, α -casein, β -casein, and pepsin-paracasein.

MATERIALS AND METHODS

Whole casein. The casein was precipitated from skimmilk by acidification of the milk to pH 4.5 with *N* HCl. The precipitate was washed four times with water, and redissolved. The precipitation and washing were repeated twice (6). The precipitated casein was dried with absolute ethanol and ether.

α -Casein and β -casein. These caseins were prepared from whole casein by fractionation in aqueous urea solutions (7).

Pepsin-paracasein. Paracasein was prepared from whole casein with pepsin. A 100-ml. portion of 2% sodium caseinate solution, pH 6.5, containing 15.0 mM CaCl₂ per liter, was treated with 2 mg. crystalline pepsin at 30°. A clot formed at the end of 5 min. The suspension was heated for 15 min. at 100° to inactivate the pepsin. The casein clot was washed at pH 4.7 to reduce the calcium concentration and finally dissolved at about pH 7 and reprecipitated several times at pH 4.7. The paracasein was finally dried with absolute ethanol and ether.

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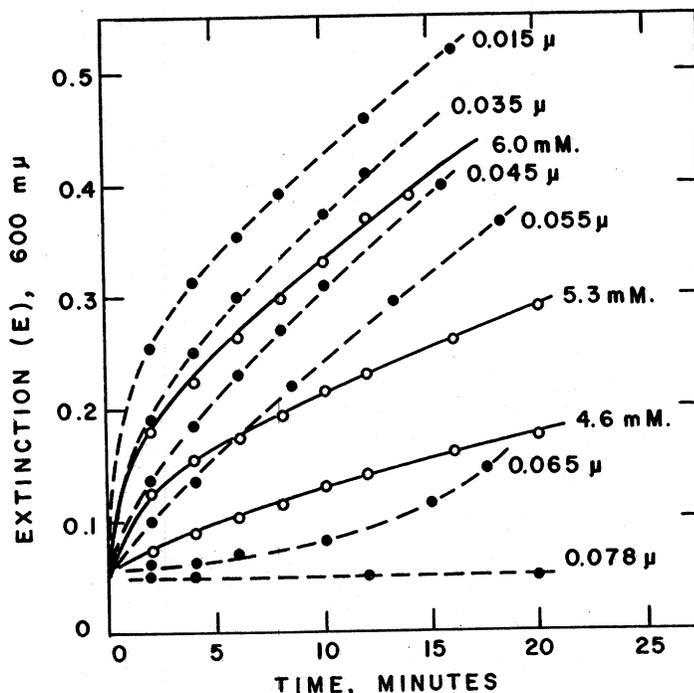


FIG. 1. The rate of aggregation of sodium caseinate (1.0%) in cacodylic acid (0.05 *M*)-NaOH (0.030 *M*) buffer, pH 6.28, by the addition of calcium chloride as measured by the extinction at 600 $m\mu$ at 29.8° (solid lines). The numbers indicate the concentrations of calcium chloride in *mM* per liter. Also shown (dashed lines) is the effect of sodium chloride concentration on the aggregation, all at 6.0 *mM* CaCl₂ per liter, with the buffer one-half as concentrated (0.015 *M*). Numbers indicate ionic strength. The lowest (0.015) contains buffer only, the others contain NaCl in addition.

the effect of temperature on this aggregation. This aggregation is inhibited by NaCl (Figure 5), just as is the CaCl₂ aggregation (Figure 1).

Phosphate is present in the natural calcium-casein complex in milk. Further, it has been found to prevent or greatly retard (17, 18) the reversal at low temperatures of the aggregation of calcium caseinate brought about at high temperatures. Accordingly, the influence of phosphate on the rate of aggregation was of interest. The results are shown (in Figure 5) for temperatures of 25, 30, and 35°. The phosphate not only reduces the amount of aggregation obtained in a given time, as one would expect, by the binding of calcium ions (5) (at pH 6.3, less than 30% of the phosphate is present as divalent phosphate, the ion which binds the calcium), but it also changes the shape of the rate curve, and greatly reduces the effect of temperature on the aggregation. These changes are probably due to the formation of the calcium phosphate-calcium caseinate complex. The effect of sodium citrate on the aggregation was also studied for comparison, for the result presumably would be due solely to the reduction in the calcium concentration by the binding to the citrate. The results are shown (in Figure 6)

All of the caseins were stored in a desiccator at a relative humidity that maintained the moisture content at 10%. The concentrations given are for moisture-free products.

Preparation of solutions. In most instances, the casein solutions were freshly prepared for each experiment. However, it has been found that a stock 4% solution of sodium caseinate, pH 6.5, remained stable for at least five days, kept at 7°. In preliminary aggregation experiments with unbuffered solutions, the rate curve was difficult to reproduce. Satisfactory reproducibility was obtained when the solutions were buffered. Solutions were freshly prepared by dissolving the casein in buffer, or the stock solution of casein was diluted with an appropriate amount of buffer, as described for each experiment. Because of the strong solubilizing effect of sodium ions in comparative systems, for example where NaOH was used to adjust the pH, it was necessary to keep the sodium ion concentration constant, which was done by adding sodium chloride.

Turbidity measurement. The extinction of light caused by the turbidity was read in a Beckman Model B spectrophotometer² at a wave length of 600 m μ in glass tubes 18 mm. O.D., 16 mm. I.D., selected for optical uniformity. The temperature of the cell was maintained by circulating water maintained at the specified temperature \pm 0.2. Measurements in this instrument with a suspension of polystyrene latex, diameter 0.13 μ , which superficially resembles the casein aggregates, were linear with concentration for extinction values up to 1.3.

RESULTS

The rates of aggregation of whole casein at 30.0°, pH 6.30, in cacodylate buffer, ionic strength (μ) of 0.030, with several concentrations of CaCl₂, are shown in Figure 1 (solid lines) as extinction (E) values. Also shown in this figure is the strong effect of NaCl in reducing aggregation (dash lines). The rates of aggregation are not described by any simple rate law, but with 4.5 to 7.0 mM CaCl₂ per liter the curves are parabolic and, when E² is plotted against time, straight lines are obtained. At lower concentrations of calcium chloride, there is an initial lag period in the aggregation similar to that shown at an ionic strength of 0.065. The rates of aggregation were also determined for a range of casein concentrations with several calcium chloride concentrations. The results are given in Figure 2 as derived rates; namely, the change in E² with time. The effect of temperature on the aggregation of whole casein is shown in Figure 3. This figure shows also the effect of temperature on the rate of aggregation of a 3:1 mixture of α - and β -caseins.

The rates of aggregation obtained at different pH values are shown in Figure 4. Overlap of the pH values with the three buffers used showed that there were no marked specific buffer effects. In view of the effect of pH on calcium aggregation, the aggregation in the isoelectric region without calcium ion was measured. The results are shown (in Figure 5), together with data showing

² Mention of commercial products does not imply recommendation by the U. S. Department of Agriculture over similar products not mentioned.

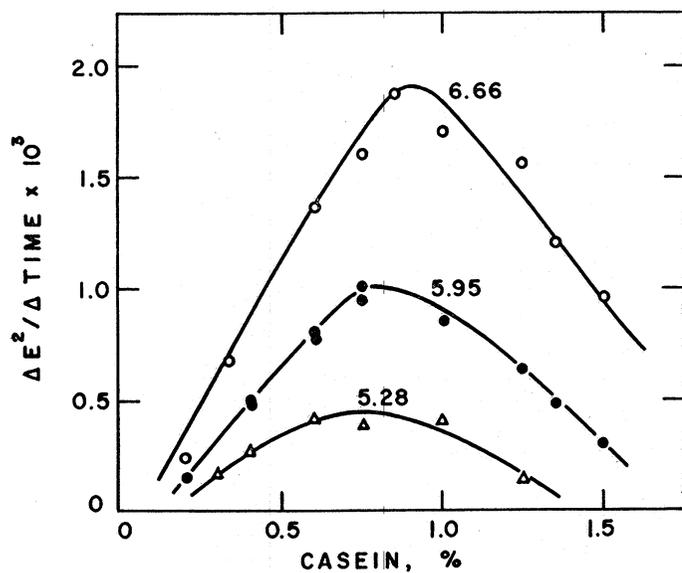


FIG. 2. The influence of casein concentration on the rate of aggregation ($\Delta E^2/\Delta \text{time}$) of casein by calcium chloride. The buffer was the same as for Figure 1. The numbers indicate the concentration of calcium chloride in mM per liter.

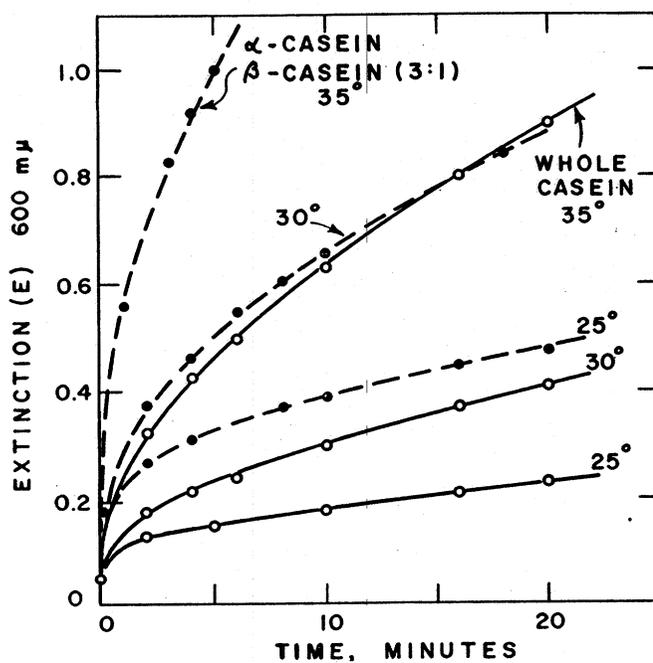


FIG. 3. The rate of aggregation of whole casein (solid lines) in cacodylate buffer, pH 6.33 at 25, 30, and 35° with 6 mM CaCl₂ per liter. Also shown is the rate of aggregation of a 3:1 mixture of α - and β -casein (dashed lines) under the same conditions.

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for 25, 30, and 35° and it is apparent that the effects are what one would expect from a reduction in the calcium concentration.

The aggregation of an α -casein preparation at 25, 30, and 35° in the presence of calcium chloride is shown (Figure 7). In the period of time studied, the aggregation is linear. Two concentrations of calcium chloride were used at 30°. A comparison of the larger of these, 4.5 mM per liter, with the 4.6 mM concentration in Figure 1, shows that the aggregation of α -casein is more sensitive to calcium concentration than is whole casein. A quantitative expression of the sensitivity has been chosen as the concentration of calcium chloride required to give an extinction of 0.5 in 30 min. at 30°. On this basis, the sensitivity of whole casein is 6.0. The α -casein preparation used in the present study had a sensitivity number of 4.1. Other preparations, differing as much as tenfold in the concentration of calcium chloride required to precipitate them,³ had sensitivity numbers much the same; namely, from 3.9 to 4.7. The differences in precipitation of α -casein are attributed to differences in content of K-casein (15).

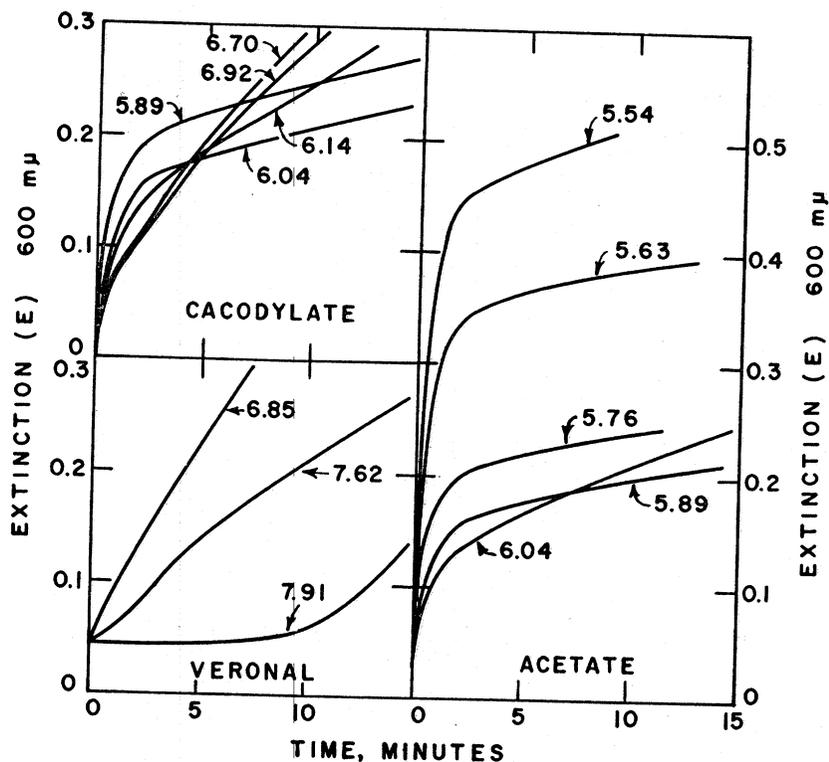


FIG. 4. The influence of pH on the aggregation of casein by calcium chloride (5.95 mM per liter) in veronal (0.05 M sodium salt plus HCl), cacodylate (0.05 M acid plus NaOH), and acetate (0.05 M sodium salt plus HCl) buffers at 29.7°. The total sodium ion concentration in each was 0.050 M; sodium chloride was added when necessary to attain this concentration. The numbers on the graph give the pH values.

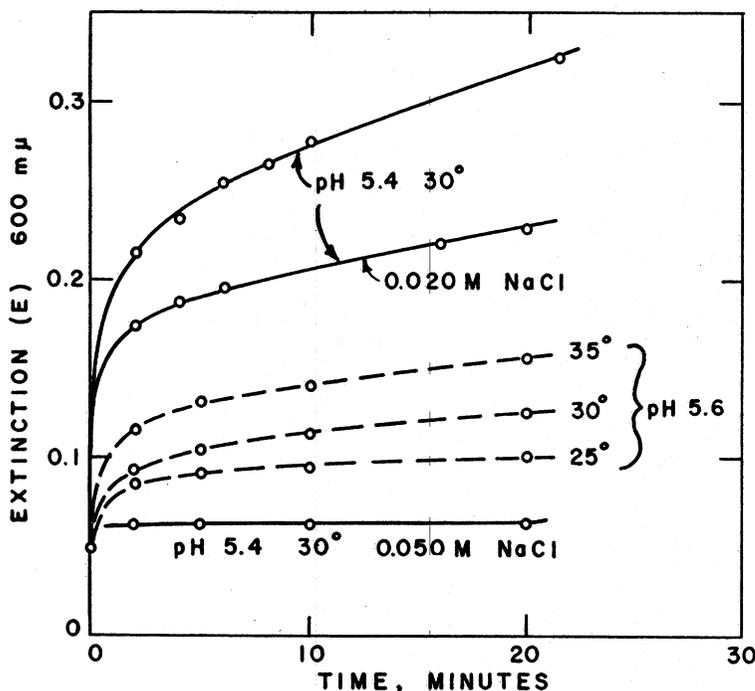


FIG. 5. The rate of aggregation of casein in the isoelectric region at pH 5.4 and 5.6 without calcium chloride, at 25, 30, and 35°. Also shown is the effect of NaCl on the aggregation. The low pH was obtained by adding 2.0 ml. of 0.1 *M* cacodylate buffer, pH 5.06 (obtained by acidifying 20.0 ml. of the 0.1 *M*, pH 6.53 cacodylate buffer with 1.10 ml. of 1.0 *N* HCl) to 4.0 ml. of the casein-containing solution in the spectrophotometer. The final pH of 5.6 was obtained when 0.33 ml. of 0.1 *N* NaOH was used to dissolve the casein (pH about 6.5); for the final pH of 5.4 only 0.23 ml. of NaOH was used.

The results of both precipitation and rate of aggregation experiments suggest that K-casein influences not the amount of aggregate formed but its stability.

β -Casein was difficult to study because of its extreme sensitivity to temperature and its dependence on calcium chloride concentration. Some of the results are shown (Figure 8). At 30° with 8 *mM* CaCl₂ per liter, the solution remained clear; with 10 *mM* the aggregation shown was obtained, and with 11 *mM* a dense white suspension with a gummy precipitate was obtained. Only a 1° temperature span could be studied quantitatively in the spectrophotometer. Any greater increase in temperature gave a white suspension and gummy precipitate. One curve is shown for whole casein (dash line), which at this particular temperature (30°) is more sensitive to CaCl₂ than is β -casein.

The aggregation of pepsin-paracasein by calcium chloride at 25, 30, and 35° is shown (Figure 9). Paracasein is more sensitive to calcium ions than is casein and in these experiments the concentration of CaCl₂ was 4.0 *mM* per liter, a concentration that gives a barely detectable aggregation of casein under the same conditions.

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DISCUSSION

The primary emphasis in the present study was to determine the rate at which casein aggregates in the presence of calcium ions or in the isoelectric region (pH 5.5). The size of the aggregates was of less interest; hence, a spectrophotometric method that would give comparative values was considered satisfactory. The aggregates studied are quite large and give an opalescent, milky appearance to the casein solution. Under the conditions studied, the aggregates are stable colloids, however, and no precipitation or sedimentation occurred during the period of study. Consideration of the results shows that in a general way the aggregations were of two types: (1) aggregation was almost instantaneous when CaCl_2 was added; (2) aggregation was gradual when CaCl_2 was added. β -Casein, pepsin-paracasein, casein plus phosphate, and casein plus acid (no CaCl_2) were of the first type, whereas whole casein, α -casein, and casein plus citrate were of the gradual type.

The aggregation rate curves obtained with whole casein and CaCl_2 are very similar to curves obtained by Dyachenko and Vlodayets (4). These authors prepared their casein (3) by solution at pH 3.0 and reprecipitation with NaOH .

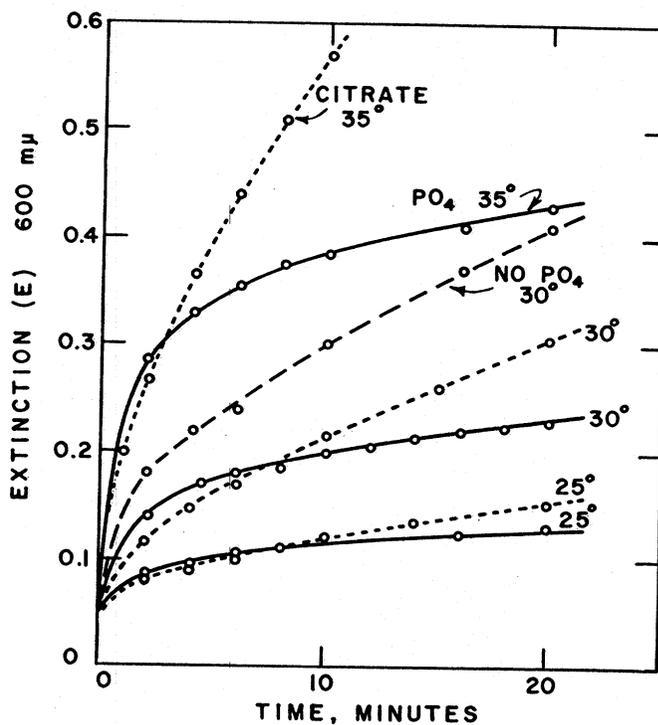


FIG. 6. Rate of aggregation of whole casein in cacodylate buffer, pH 6.33 at 25, 30, and 35° with 6 mM CaCl_2 per liter, in the presence of 5 mM of phosphate per liter. Also shown is the rate of aggregation in the same systems with 0.5 mM sodium citrate per liter replacing the phosphate. The aggregation of casein without phosphate or citrate at 30° is shown for comparison.

It was surmised that the procedure used would remove the β -casein from the α -casein (14), but unless the temperature was low (about 2°) and the casein solution dilute (0.2 to 0.3%), no separation of α - and β -caseins would occur. The shape of their aggregation curves suggests that they have whole casein and not α -casein.

Dyachenko and Vlodayets report (4) that there is an optimum CaCl_2 concentration for maximum aggregation and that concentrations of CaCl_2 greater than the optimum effect a reduction in the size of the aggregates. In unbuffered systems at pH 6.0, containing 0.018 mg. casein per milliliter at 40°, the optimum CaCl_2 concentration was 0.01 M. The whole casein used in the present studies with a cacodylate-buffered system, pH 6.3, containing 0.5 mg. casein per milliliter at 30°, showed no solubilization effect with concentrations of CaCl_2 of 0.1 to 1.0 M. This range of calcium concentrations covers the calcium:casein ratios showing the reported solubilization (4).

The principal function of the calcium in the casein aggregation is probably through binding to the casein to reduce the net charge, so that it is comparable to isoelectric casein. Calcium, however, may also have a specific contribution to the aggregation, perhaps through hydration, for at alkaline pH values, potassium salts of casein increase in solubility with increase in temperature, whereas the calcium salts decrease in solubility (19, 20).

The effect of various ions on the aggregation of casein is complex. Sodium

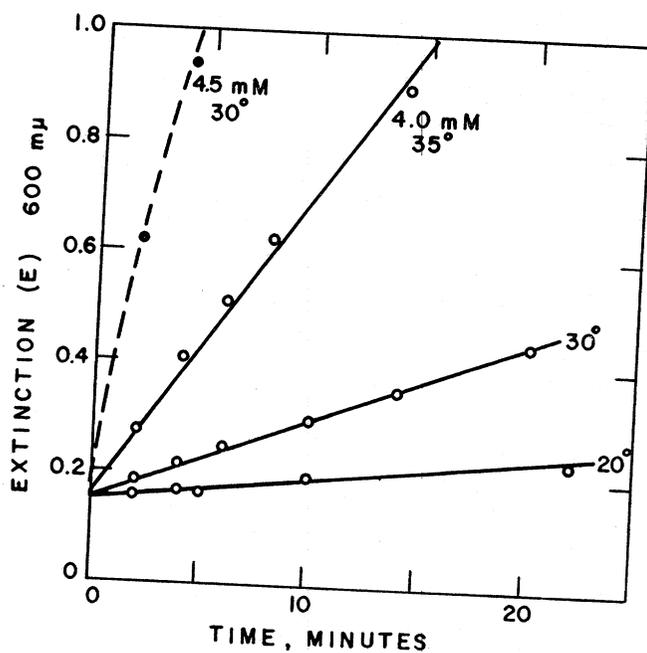


Fig. 7. Rate of aggregation of α -casein (1%) in cacodylate buffer at pH 6.33 at 25, 30, and 35°. At each temperature, the concentration of calcium chloride was 4.0 mM per liter. One curve is shown for 4.5 mM CaCl_2 per liter at 30°.

chloride decreases the activity of the calcium ion, and sodium ion probably is competitive with calcium ion (18). The effect of sodium chloride on the calcium-free, isoelectric aggregation shows also that salts must exert an electrostatic interaction with the charged protein ion, that is, a salting-in effect. This occurs also with the calcium aggregates. Cacodylate ion reduces the rate of aggregation somewhat, compared with chloride ion. This is apparent in Figure 1, for the experiments with 6 mM CaCl₂ per liter and 0.030 μ (the variable CaCl₂ experiments are all for μ of 0.030). With buffer, the E value is 0.33 for 10 min.; when one-half the buffer is replaced with NaCl, the E value is 0.37 at 10 min. A col-

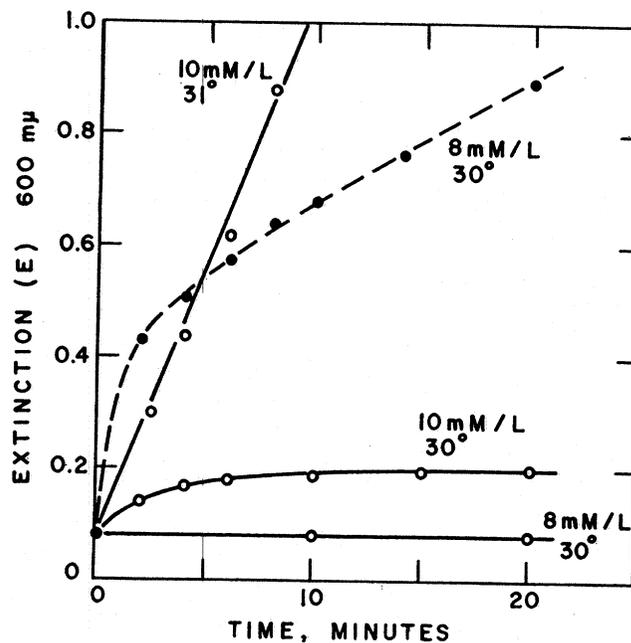


Fig. 8. Rate of aggregation of β -casein (1%) in cacodylate buffer at pH 6.33 at 30 and 31° with 10 mM CaCl₂ per liter. No aggregation is obtained with 8 mM per liter. The aggregation of whole casein (dashed line) with this concentration of CaCl₂ is shown for comparison.

lidine buffer (0.025 M, pH 7.0), on the other hand, exerted an enhancing effect on the aggregation, presumably due to the positively charged collidinium ion. The collidinium ion could not substitute for calcium ion, however, for even with a collidine concentration of 0.05 M, no aggregation occurred without CaCl₂. The effect shown by the collidinium ion may be exerted by other ammonia type ions, since it has been observed (9) that ammonium salts are less inhibitory to casein coagulation by pepsin than are sodium and potassium salts. The negatively charged veronal ion is slightly inhibitory to the aggregation, an effect which might be due to the binding of calcium ions.

The calcium: casein ratio is a factor which influences the rate of aggregation (Figure 2). This follows from the laws of mass action, which apply to the bind-

ing of calcium ions by casein (2). In solutions containing not more than 1% casein and 6.66 mM CaCl_2 per liter, the neutralization of charge on the casein molecule brought about by the binding of Ca^{++} is maximal, and under these circumstances, rate of aggregation is determined by the number of casein molecules per unit volume, *i.e.*, the concentration. With lower concentrations of calcium, the concentration of casein giving a maximum rate of aggregation is somewhat lower. Beyond this maximum, the rates decrease, presumably because the amount of calcium available to reduce the charge on each casein molecule becomes increasingly inadequate.

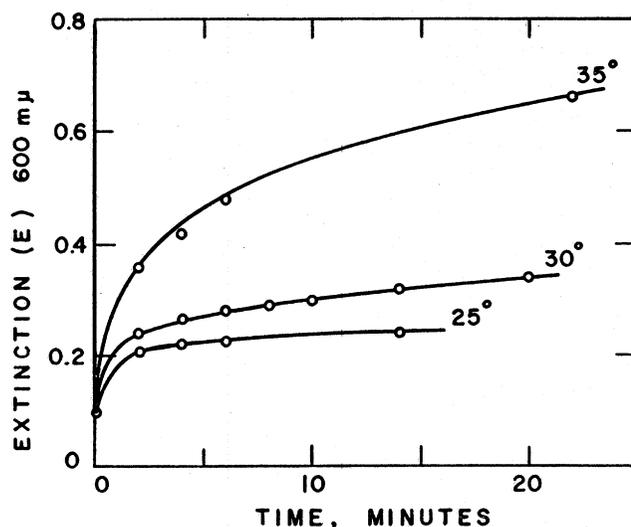


FIG. 9. Rate of aggregation of pepsin-paracasein in cacodylate buffer, pH 6.33 at 25, 30, and 35°, with 4.0 mM CaCl_2 per liter.

The effect of pH on the calcium aggregation of casein is quite complex. In the system studied (1% casein, 5.95 mM CaCl_2 per liter) at pH 7.9, there is an initial lag period in the aggregation. This is also observed at pH 6.3 when calcium concentration is low and in systems to which considerable NaCl is added (Figure 1). As the pH is lowered, the rate of aggregation increases rapidly and, for a limited range, the curves are parabolic. Below pH 6, however, the curves are rectilinear. Due to this change, it is difficult to compare the curves; however, a comparison of the times required to obtain an extinction of 0.2 shows that there is a minimum at pH 6.7, followed by a maximum at pH 5.9, and a continuous decrease at lower pH values. The nature of the pH influence is not understood, but it may reflect changes in hydration with the aggregate being least hydrated at low pH values.

The temperature coefficient of the aggregation of casein by CaCl_2 is of considerable interest. It is obvious from the behavior of β -casein that the temperature coefficient of aggregation is extremely high. The linear aggregation curves obtained with α -casein provide data for calculation of the temperature coefficient.

The temperature coefficient for the aggregation reaction can be calculated from the integrated form of the Arrhenius equation (12, 13):

$$R_2/K_1 = e^{\frac{\Delta H(T_2 - T_1)}{R(T_2 T_1)}}$$

K_2 and K_1 are the rate constants at two temperatures, ΔH a constant equivalent to the heat of activation of the reaction, R the gas constant of 1.99 calories per mole, and T_2 and T_1 the absolute temperatures. When the temperature range is relatively short, the product $T_2 T_1$ can be regarded as a constant and the temperature coefficient takes the form $K_2/K_1 = e^{k(T_2 - T_1)}$. This form was used by Berridge in studies on the effect of temperature on the clotting of paracasein (1). A plot of $\log K$ (rate) for α -casein versus temperature gave a straight line, the slope of which gave the k value. The value of e^k gives the temperature coefficient for a 1° interval; e^{10k} gives the value for a 10° interval. The 10° temperature coefficients obtained from e^{10k} are identical with the values of the ratios $K_{35^\circ}/K_{25^\circ}$. The 1° temperature coefficient for α -casein is 1.30; the 10° temperature coefficient is 14. Similar calculations with the whole casein data with the linear rates obtained from E^2 versus time plots gave 1 and 10° temperature coefficients of 1.35 and 19, respectively. Thus, these aggregations are characterized by high temperature coefficients.

Berridge (1) studied a related phenomenon, the clotting time of para-casein with calcium ions, and compared the inverse of the time required for clotting at several temperatures. The 1° temperature coefficients at 26 to 38° range from 1.3 to 1.6, depending on the sample of skimmilk used. The shape of the present aggregation curves of pepsin-paracasein does not permit a similar end-point comparison, but the 1° temperature coefficients of calcium ion aggregation of casein, in general, is of the same magnitude as Berridge's values. Pyne (10) has reported the 1° temperature coefficients of clotting of calcium caseinate to be 2.0/1°, without giving any experimental details. He also observed, analogously with the present observations, that the addition of phosphate reduces the temperature coefficient about 20%, to a value with phosphate of 1.6/1°. Berridge concluded from the high-temperature coefficients and a heat of activation of the magnitude associated with denaturation that the clotting was a denaturation phenomenon. There are many aspects of the clotting reaction, however, that make the denaturation hypothesis appear to be untenable. The aggregation reactions shown by the caseins other than paracasein are probably comparable reactions. In the case of β -casein, the heat of activation of the reaction is extremely high. Further, the aggregation is partly reversed by lowering the temperature, or it can be reversed by removing the calcium. If the phenomenon was a denaturation involving solely hydrogen cross-binding, as its reversibility suggests, one might expect an increase in temperature to decrease the aggregation, as is observed for gelatin (8). Casein in acid solution (pH 1.5) shows (3) an aggregation of this type; that is, the aggregation decreases as the temperature is raised (20 to 84°). The magnitude of this temperature effect was considered to be consistent with hydrogen bond aggregation. Thus, the clotting of casein probably resides in some phenomenon other than denaturation. The evidence suggests that in

clotting the usual aggregation of casein by calcium has been enhanced by the removal of a stabilizing protein by the action of rennin to give the less stable paracasein (16).

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