HEAT STABILITY OF CASEIN IN THE PRESENCE OF CALCIUM AND OTHER SALTS [1]

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SUMMARY

The extent of calcium binding and the heat stability of colloidal suspensions of freeze-dried casein in calcium hydroxide solution, with added phosphates, citrates, and chlorides in some tests, was studied. The calcium-binding power of freshly prepared freeze-dried casein was greater (pK 4.5) than that reported for other casein preparations. However, the pK value decreased during storage of the casein at room temperature and within 1 or 2 mo. attained a value (pK = 3.0 ± 0.3) in agreement with published values.

The heat stability of simple casein caseinate suspensions was inversely related to the extent to which the casein was saturated with calcium, and this relation appeared to be linear at calcium levels sufficient to give over 80% saturation. The heat stability of more complex suspensions was inversely related to the ratio of soluble or ionic calcium to soluble phosphate in the suspension.

Chlorides and phosphates of sodium, potassium, and ammonium displaced calcium from caseinate to about the same extent, but ammonium ion appeared to have a specific depressant effect on heat stability.

In an earlier paper (14), it was suggested that the heat stability of milk varied with a multiplicity of factors which might differ in relative importance between samples. If so, identification of these factors will be greatly facilitated by use of simple model systems containing milk proteins. Colloidal suspensions of acid casein, which do not involve extensive modification or fractionation of the casein itself, probably provide the most suitable system. Studies of the heat stability of such suspensions were, therefore, undertaken.

The binding of calcium by casein in calcium hydroxide-casein mixtures has been studied extensively (2, 3, 7, 19, 20), but data on the heat stability of calcium casein solutions are quite limited (6, 15, 17, 18). The present paper reports on the interrelations between calcium binding and heat stability in colloidal suspensions of casein in calcium hydroxide, with and without added chlorides, phosphates, and citrates.

MATERIALS AND METHODS

Acid casein was precipitated from skimmilk by the addition of hydrochloric acid to pH 4.6, washed several times with water, frozen, and dried under vacuum. The dried product was ground with a pestle and stored at room temperature. Several batches of freeze-dried casein were used in this work; differences between batches were less than the experimental error plus the effect of aging (vida infra). Except where otherwise stated, casein preparations were from 1 to 4 mo. old.

For dispersion of the casein, measured amounts of saturated calcium hydroxide solution, filtered through two thicknesses of Whatman No. 12 paper (and stored under oil if not used immediately) were mixed with a weighed amount of casein and the mixture shaken for 15 to 18 hr. Aliquots of this colloidal suspension were adjusted to the desired pH with 1 N hydrochloric acid or sodium hydroxide, and to the desired chloride, phosphate, and citrate content, and diluted to contain 2.8% casein (w/v, corrected for 6% moisture).

Calcium was determined by a turbidimetric method (9); total calcium by analysis of an acidified aliquot of the freshly filtered calcium hydroxide solution, soluble calcium by analysis of ultrafiltrates prepared as previously described (16). Ionic calcium was determined with murexide (16), by comparison with standards of the same ionic strength. Ionic strength varied between 0.008 and 0.05, except in a few tests at high chloride concentrations. Total (trichloracetic acid soluble) and soluble (ultrafiltrate) inorganic phosphate were determined colorimetrically (10). Heat stability was measured as the time at 140° C. required to develop visible particles in milk flowing down the wall of the test-tube (16).

Bound calcium was calculated as the difference between total and ultrafiltrate calcium. However, this calculation was not permissible when total and soluble inorganic phosphate values differed, indicating that calcium phosphate had precipitated. Although Zittle et al. (20) observed binding of phosphate itself, in the present tests differences between total and ultrafilterable phosphate were never observed in mixtures in which the estimated $[Ca^{++}]^3[PO_4^{\equiv}]^2$ and $[Ca^{++}]$ $[HPO_4^{=}]$ products were below those expected to induce precipitation of a calcium phosphate.

<div align="center">RESULTS</div>

Binding of calcium. In the absence of other salts, the extent to which calcium was bound to casein was a function of the concentration of calcium present and the pH. At fixed pH, the reciprocal of the moles of calcium bound per 10^5 g. casein (r) at room temperature was a linear function of the reciprocal of moles of free calcium present (A) (cf. 8). Extrapolation to the axes gave a dissociation constant for a fresh batch of freeze-dried casein of pK = 4.36 ± 0.10 at pH 6.5, and of pK = 4.52 ± .12 at pH 6.95. Caseins that had been stored at room temperature for 1 to 10 mo. had pK values from 2.66 to 3.80 at pH 7. The variability in these tests, inclusive of variation between batches of casein, was higher than suggested by the standard deviations quoted above, but nevertheless the data indicated that the pK for calcium caseinate decreased by 0.6 unit or more during the first month of storage at room temperature, with a further slow decline to pK = ca 3.0 ± 0.3. Published values (3, 19) vary from 2.39 to 3.34.

The maximum amount of calcium bound by 10^5 g. of fresh casein was 38.5 ± 0.5M at pH 6.95, and 33.1 ± 0.4 at pH 6.5. Older caseins (1 to 10 mo.) bound from 33 to 36 $M/10^5$ g. at pH 7, with no significant trend with time of storage. The effect of pH on the maximum amount of calcium bound by casein was similar to that reported by Zittle et al. (20).

If the calcium concentration was less than that required to saturate the casein at room temperature, more calcium was bound at 93° C. than at room temperature (Table 1). The increase was small under all conditions tested, but was sufficient to cause the 1/r vs. 1/A relation to deviate sharply from linearity (Figure 1).

TABLE 1

Effect of temperature on the amount of calcium bound by casein
(Averages of 3 tests, pH 6.5)

Calcium added (mM/liter)	Calcium in ultrafiltrate (mM/liter)		Calcium bound (M/10⁵ g.)	
	25° C.	93° C.	25° C.	93° C.
7.5	0.5	0.1	25.1	26.2
7.8	0.6	0.2	25.8	27.2
9.2	1.1	0.8	28.6	29.6
12.2	3.1	3.1	32.2	32.4

Addition of chlorides or phosphates of sodium, potassium, or ammonium to colloidal casein–calcium hydroxide suspensions in which the casein was not saturated with calcium, displaced calcium from the casein (Figure 2). Added citrates sequestered calcium and thus greatly reduced the amount bound by casein (Figure 2). These effects appeared to be independent of the cation present.

Heat stability. At pH 6.5, 7.0, or 7.5, and calcium levels above 7.5 mM/liter of colloidal casein–calcium hydroxide suspension, the heat stability was inversely related to the extent to which casein was saturated with calcium (Table 2).
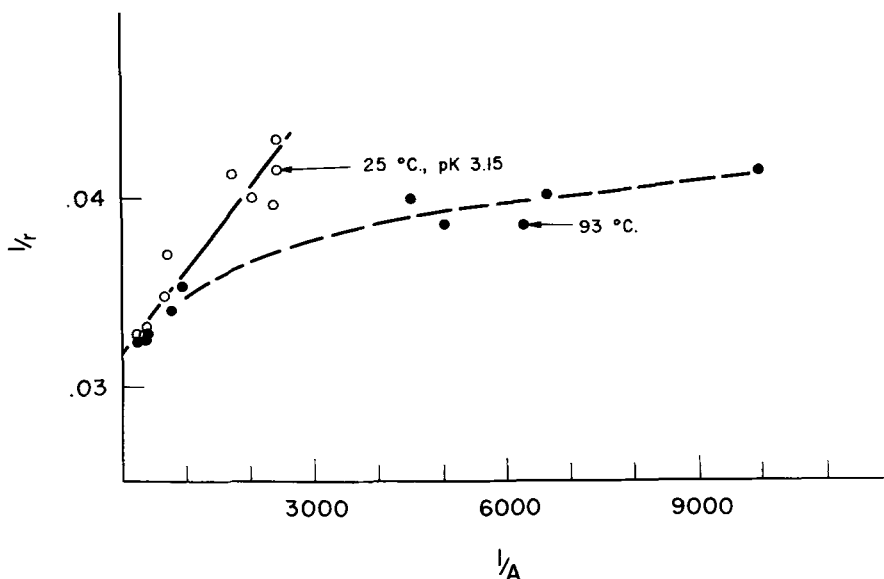

Fig. 1. Effect of temperature on the binding of calcium by casein. Data are from same tests as in Table 1, but not averaged: pH 6.5; O—25° C., ●—93° C.
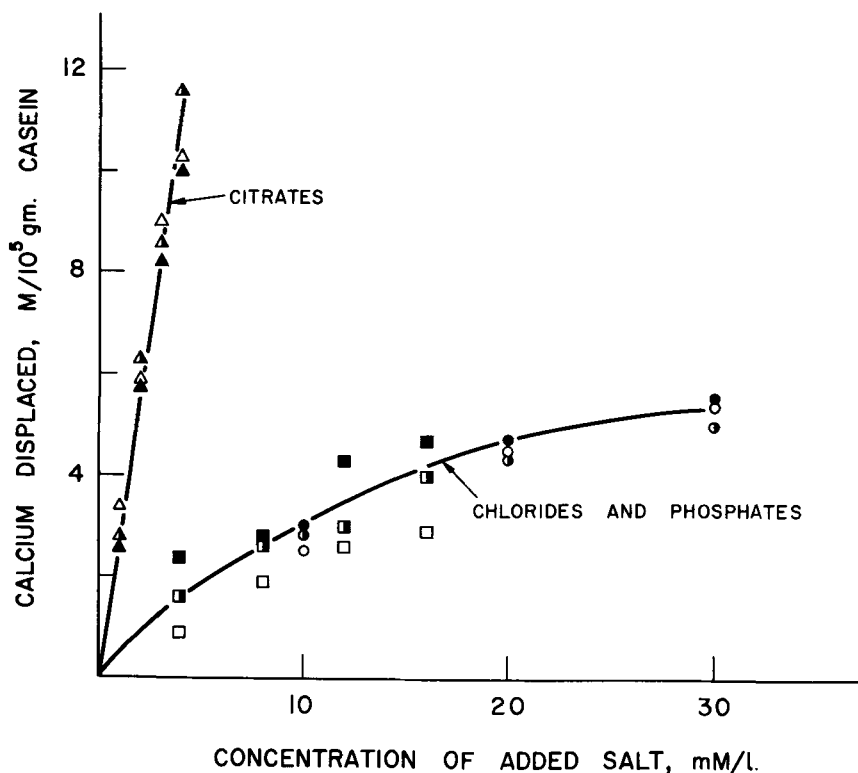
FIG. 2. Displacement of calcium from casein by sodium, potassium, and ammonium salts (i.e., bound calcium of control minus bound calcium of sample with added salt). Calcium concentration 7 to 8 mM/liter, pH 6.5, temperature 25° C.

O, ◑, ●—Na, K, and NH₄ chlorides, respectively

□, ◨, ■—Na, K, and NH₄ phosphates, respectively

△, ▲, ▲—Na, K, and NH₄ citrates, respectively

At fixed calcium level, heat stability decreased with decreasing pH, becoming essentially zero at pH 6.0 (Table 2). The heat stability of colloidal casein–calcium hydroxide suspensions also decreased with increasing age of the casein preparation; e.g., casein 80% saturated with calcium had a heal stability of 12 min. when 2 to 6 wk. old, and of only 8 min. when 20 to 24 wk. old (average of three tests in each period). With very fresh casein a heat stability of 20 min. at 80% saturation was observed (single test).

When other salts were added to the colloidal suspension of casein in calcium hydroxide, the heat stability was influenced both by the anion and by the cation (Figure 3). Sodium and potassium phosphates greatly increased the heat stability; ammonium phosphate did not. The heat stability of suspensions containing ammonium citrate was also lower than that of similar suspensions containing sodium or potassium citrate (Figure 3). Addition of 10 mM/liter sodium or potassium chloride did not significantly affect the heat stability of casein–calcium hydroxide suspensions; higher levels (20 and 30 mM/liter) de-

TABLE 2

Effect of saturation of casein with calcium, and of pH, on heat stability of colloidal
suspensions of casein in calcium hydroxide

pH	Total calcium (mM/liter)	Bound calcium (M/10⁵ g.)	Saturation [a] (%)	Heat stability (min.)
7.5	7.8	26.3	70.9	26
	9.8	30.5	82.3	16.5
	12.6	36.0	97.1	1
	16.0	36.9	99.4	0.5
	19.5	36.4	98.0	nil
7.0	7.8	26.0	76.6	21
	9.8	30.5	90.0	9
	12.6	33.7	99.4	0.5
	16.0	33.7	99.4	0.25
	19.5	32.9	96.9	nil
6.5	7.8	24.4	85.2	7.5
	9.8	27.3	95.6	0.75
	12.6	27.6	96.3	nil
	16.0	27.6	96.3	nil
	19.5	28.1	98.1	nil
6.0	7.8	19.1	85.7	0.5
	9.8	18.6	83.3	0.25
	12.6	20.1	90.5	nil
	16.0	22.3	100	nil
	19.5	22.3	100	nil

[a] Per cent of maximum as determined by extrapolation to infinite calcium concentration.

creased it by about 25%. Ammonium chloride decreased heat stability approximately 30, 60, and 80% when present at 10, 20, and 30 mM/liter concentrations (single test).

Since the heat stability of colloidal suspensions containing only casein and calcium hydroxide was related to the per cent saturation of casein with calcium (Table 2), and calcium binding, and hence per cent saturation of casein with calcium was related to the reciprocal of the free calcium present at room temperature (cf. 8), heat stability of these simple mixtures was also related to $1/Ca_{sol}$. However, this relation was not apparent when phosphate or citrate ($1/Ca^{++}$ substituted for $1/Ca_{sol}$ in the presence of citrate) was also present.

The heat stability of colloidal suspensions containing casein, calcium, and phosphate was inversely and linearly related to the Ca/P ratio in the ultra-filtrate. Data for 29 tests, including some in which calcium phosphate precipitated, gave a correlation between heat stability and the Ca_{sol}/P_{sol} ratio of -0.96 (Figure 4). However, the per cent saturation of casein with calcium was also related to the Ca_{sol}/P_{sol} ratio. These two factors could not be separated experimentally, but their individual effects can be approximated by standard statistical methods (partial correlation analysis). Among the 29 tests, there were 14 in which no calcium phosphate precipitated (hence, bound calcium could be estimated), and statistical analysis of the data from these yielded the following results:

Crude (apparent) correlation between
Ca_{sol}/P_{sol} and heat stability -0.97

FIG. 3. Effect of added citrate (0 to 4 mM/liter) and phosphate (0 to 16 mM/liter) on the heat stability of colloidal suspensions of casein. Calcium concentration 7 to 8 mM/liter, pH 6.5, temperature 25° C.

○—Na or K phosphate   ●—NH₄ phosphate

□—Na or K citrate   ■—NH₄ citrate

The linear relation above 80% saturation (no points shown) was established by several tests with casein–calcium hydroxide suspensions without added salts.

The corresponding crude regression coefficient of heat stability on Ca_{sol}/P_{sol} -70.0 ± 7.0

The same correlation corrected for the effect of per cent saturation on Ca_{sol}/P_{sol} -0.83

The corresponding corrected regression coefficient of heat stability on Ca_{sol}/P_{sol} -48.7 ± 9.9

It is thus apparent that the correlation and regression are inflated by the underlying relation between per cent saturation and Ca/P, and the screening out of this relation gives lower and more realistic values.
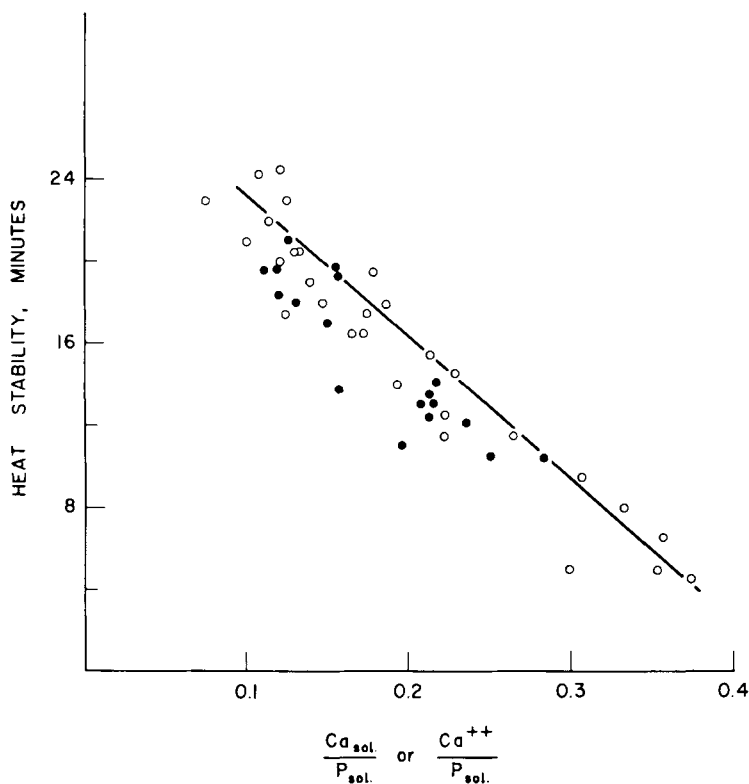
FIG. 4. Relation between the heat stability of colloidal suspensions of casein and the ratio of soluble (or ionic) calcium to soluble phosphate. Calcium concentration varied from 7 to 18 mM/liter, pH 6.5.

O—casein-calcium-phosphate suspensions
●—casein-calcium-phosphate-citrate suspensions

The heat stability of colloidal suspensions containing citrate in addition to casein, calcium, and phosphate was also inversely related to the Ca^{++}/P_{sol} ratio (Figure 4). There was no apparent relation between the heat stability of these suspensions and the $Ca_{sol}/P_{sol} + Citrate_{sol}$ ratio.

DISCUSSION

Freshly prepared freeze-dried casein had a greater affinity for calcium (pK = 4.5) than had the commercial (3) or solvent-dried (19) caseins tested by other authors. However, freeze-dried caseins stored 1 to 10 mo. at room temperature had pK values (ca 3.0 ± 0.3, range 2.7 to 3.8) that did not differ significantly from previously reported values (3, 19). The high value for freshly prepared freeze-dried casein suggests that native casein has a greater affinity for calcium than indicated by published data on acid casein.

The maximum amount of calcium bound by freeze-dried casein varied from 33 to 36 $M/10^5$ g. casein at pH 7.0 (38 for freshly prepared casein). Assuming

a molecular weight of 33,000, the calcium bound is 11 to 12 M/M casein. This value is lower than that obtained by Chanutin et al. (3) [cf. also (7)], but is in reasonable agreement with a value of 10 M/M estimated from the data of Zittle et al. [(20) Figure 4].

Heat stability of the colloidal suspensions of casein in calcium hydroxide appeared to be largely dependent upon (a) the per cent to which the casein was saturated with calcium, and (b) the ratio of soluble calcium to soluble phosphate in the suspensions. The amount of calcium bound to casein in milk has been estimated by assuming a constant Ca/P ratio in the colloidal calcium phosphate (4, 5, 13) but, in view of the variability in composition of calcium phosphate precipitates (1), these assumptions are difficult to justify. Pyne and McGann (11) have recently introduced an acidification-dialysis technique for preparing milk containing no colloidal calcium phosphate, but this method has not been applied to a study of the casein-bound calcium in a range of milks. At present, therefore, it is not possible to decide whether the degree of saturation of casein with calcium in normal milks can be a factor in determining their heat stability. On the other hand, the relation between the Ca_{sol}/P_{sol} ratio and the maximum heat stability of normal milk (14) was similar to that observed in the present tests. Colloidal calcium phosphate is thought to be a major factor controlling the heat stability of normal milks (12), but precipitation of calcium phosphate in the colloidal suspensions of calcium caseinate did not alter the relation between the Ca_{sol}/P_{sol} ratio and the heat stability.

Added salts probably affect the heat stability of colloidal suspensions of casein by displacing calcium from the casein and, when phosphate is present, by affecting the soluble (or ionic) calcium/soluble phosphate ratio. However, ammonium ion displaced calcium from casein to the same extent as did sodium or potassium, but did not increase heat stability correspondingly. Ammonium ion probably had a specific depressant effect on the heat stability of these colloidal suspensions.

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