Studies on κ-Casein of Bovine Milk. IV. The binding of calcium to α_S-, and κ-casein.

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Summary Reactions between Ca and casein were investigated at various Ca concentrations ranging from 0 to 10mM. α_S-Casein began to precipitate at 3 mM and was completely stabilized by κ-casein. κ-Casein tended to aggregate randomly in the presence of 5 mM Ca. $S_{20,w}$ values were 14.4 and 1.8 for κ-casein and α_S-casein, respectively, in the absence of Ca. Caseins were found in gel filtration to be brought into polymerization at such low Ca concentrations that no aggregated particles were visible. α_S-κ Complex did not grow as fast as α_S-casein did in the presence of Ca. The amounts of Ca bound to caseins were determined at pH 7 and 8 in the presence of 1 to 10 mM Ca using ; equilibrium dialysis, gel filtration and centrifugation. The minimum amount of Ca necessary for the initiation of α_S-casein precipitation is posited to be about 12 moles per mole casein. Weak adsorption of Ca, due to some structural factor, was indicated since the amounts of bound Ca obtained by centrifugation were remarkably lower than those determined by the other two methods. The state of bound Ca were studied by gel filtration of ^{45}Ca-caseinates with an eluant containing no Ca. More Ca was bound to the α_S-κ complex in the early stage of the reaction than to the same amount of α_S-casein only. But the amount of Ca bound to the complex did not vary with reaction time, while that bound to α_S-casein increased greatly, accompanied by the progress of polymerization. The intensity of Ca binding to κ-casein was so weak that only a small amount of Ca was eluted together with κ-casein in the gel filtration of Ca-κ-caseinate.

α_S-Casein begins to precipitate at a neutral pH when the Ca concentration is about 3 mM. But if κ-casein is present in an amount of more than one tenth of the α_S-casein by weight, most of the α_S-casein is prevented from sedimenting and maintains a stable colloidal state. The degree of this stabilizing ability was once used as a criterion of the purity of κ-casein.[1] It has been already established that κ-casein forms a complex with α_S-casein prior to displaying its stabilizing ability.[2,3] This complex is often referred to as micelle in the field of milk protein study. The mechanism of the micelle formation has been investigated using ultracentrifugation,[2,3] chemical modification of amino acid residues in the caseins,[4,5] measurement of various physical and chemical properties etc.[6,7,8]; however, little datum has been accumulated as yet. The present experiments were performed to obtain information on changes in molecular size of caseins due to Ca and on both the quantity and quality of Ca bound to them. Methods used were the stabilization test of α_S-casein, ultracentrifugation, gel filtration using a buffer with and without Ca, electric condu-

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ctivity measurement, equilibrium dialysis and centrifugation.

Experimental methods

1. *Preparation of α_S-casein and κ-casein.* α_S-Casein and κ-casein were prepared from acid casein according to the methods of Zittle and Custer.[9,10] They were shown to be reasonably pure by starch gel electrophoresis and disc electrophoresis.

2. *Stabilization of α_S-casein by κ-casein.* General procedures were similar to those described by Zittle.[11] This test was performed at low concentrations of Ca, ranging from 0 to 10 mM, in order to study the state of α_S-casein and Ca around the critical concentration where visible micelles are initiated to form.

3. *Ultracentrifugation.* Ten mg of κ-casein was dissolved in 2 ml of tris-HCl buffer (0.01M, pH 8.0) and left overnight at 5°C. Calcium chloride was added to 1 ml of the κ-casein solution to make the concentration 5 mM. The same buffer, containing no Ca, was added to the other κ-casein solution. κ-Casein solutions with and without Ca were warmed to 30°C for 15 minutes, and then were simultaneously centrifuged at 48,000 r.p.m. (180,000×g) for 25 minutes. In another series, an α_S-casein solution (0.5%) and a mixture solution of α_S- and κ-caseins (3 : 1) were prepared in the above buffer. After allowing them to stand at 30°C for 30 minutes, their calcium concentrations were made to 0.5 mM. Then, they were centrifuged at the same time under the above conditions for 90 minutes. Temperature was kept at 25°C during the centrifugation. $S_{20,w}$ values were calculated in the usual way.

4. *Gel filtration of α_S- and κ-caseins.* Two tenths percent solution of α_S-casein, κ-casein and an equal mixture of them were prepared in tris-HCl buffer (0.01M, pH 7.0). They were shaken for 30 minutes in a water bath kept at 37°C after being made to a given concentration of Ca ; 0, 1, and 2mM. Thereafter, 1 ml of each solution was applied to a column of Sephadex G-200(1.2×17cm) equilibrated with the same buffer containing Ca at the same concentration as the sample used. Elution was made at room temperature with a fraction of 1.7g. In another series, 0.6 percent solution of α_S-casein and 0.2 percent solution of κ-casein were prepared in tris-HCl buffer (0.01M, pH 7.0). One α_S-casein solution of 0.5 ml was mixed with 0.5 ml of the above buffer. Another solution of 0.5 ml was also mixed with equal volume of κ-casein solution. After warming them at 40°C for 45 minutes, 0.1 ml of 11 mM calcium chloride labeled with ^{45}Ca was added to each solution. Solutions were left in a water bath (30°C) for another 30 minutes or 120 minutes before being applied to a column of Sephadex G-200(1×19 cm). Elution was carried out at a constant flow rate of 0.4 ml per minute with a fraction volume of 2.5 ml. UV absorption at 280 mμ and radioactivity were measured for each fraction.

5. *Measurement of electric conductivity of the casein solution in the presence of calcium chloride.* Ten mM calcium chloride solution and 3 percent α_S-casein solution were prepared using 0.01M tris-borate buffer, pH 7 and 9. A solution of the α_S- and κ-casein mixture was prepared in such a way that 3 percent α_S-casein and 0.45 percent κ-casein were contained in it. The electric conductivity of a buffer solution containing no casein was measured at 15°C by repeatedly dropping 0.4 ml of 10 mM

$CaCl_2$ solution into a vessel which originally contained 8 ml of the tris-borate buffer. By replacing the vessel with another containing 8 ml of the casein solution, the conductivity of the casein solution was measured in the same way. When casein started to precipitate, measurement was stopped. In a few cases, time dependent changes of conductivity were measured until 30 minutes after 0.4 ml Ca solution was added at some steps.

6. *Equilibrium dialysis of* ^{45}*Ca-caseinate.* Four test tubes were prepared. The first contained 1ml of tris-HCl buffer, pH 7.0. The second contained 12 mg of α_S-casein in 1 ml of the same buffer, the third 12 mg of κ-casein and the last tube 3 mg of κ-casein and 9 mg of α_S-casein in 1 ml of the tris-HCl buffer. After being kept at 37°C for 60 minutes, calcium chloride solutions labeled with ^{45}Ca were added so that the Ca concentration became 1 mM. The same type of experiment was carried out by changing the pH to 8 or the Ca concentration to 3 and 10 mM. These four test tubes were shaken at 30°C in a water for 30 minutes. Each was put into a dialysis bag with one side open, and was dialysed against 40 ml of the above buffer containing cold calcium chloride at the same concentration as the casein solution. Dialysis was carried out at 15°C with continuous shaking. 0.5 ml of dialysate was used to measure the radioactivity at specified intervals. Finally when 300 minutes had passed from the beginning of dialysis, radioactivity of the casein solution inside the bag was also measured. By subtracting the count of the dialysate, the amounts of Ca adsorbed by caseins were calculated.

7. *Gel filtration of* ^{45}*Ca-caseinate.* Three milligrams of α_S-casein, 1 mg of κ-casein and a mixture of them were, respectively, dissolved in 1 ml of tris-HCl buffer, pH 7 and 8. These were kept at 37°C for 60 minutes, then, 0.1 ml of 11mM and 33mM calcium chloride solution labeled with ^{45}Ca was added to each. These solutions were allowed to stand at 30°C for 30 minutes. Thereafter they were subjected to gel filtration on Sephadex G-75 using as an eluant the above buffer containing exactly the same amount of active and cold Ca. 2.5 ml fractions were collected and their radioactivities were measured. The amount of Ca bound to caseins was calculated by converting the increased radioactivity of the casein fractions into a quantity of Ca in reference to the radioactivity of the eluant which was set at a known concentration of Ca. In another gel filtration series, caseins which reacted with exess amounts of $CaCl_2$ labeled with ^{45}Ca filtered with Sephadex G-75 using as an eluant the same buffer containing no Ca. In one case, 3 mg of α_S-casein, 3 mg of κ-casein and a mixture of 3 mg of α_S-casein and 1 mg of κ-casein were, respectively, dissolved in 1 ml of tris-HCl buffer at pH 7, 8 and 9, and were kept at 30°C for 45 minutes. Then, as soon as 0.1 ml of 11mM calcium chloride labeled with ^{45}Ca was added to each of them, they were applied to a Sephadex G-75 column (1×19 cm) and were eluted using 0.01M tris-HCl buffer without Ca. UV absorption at 280mμ and radioactivity were measured for each fraction of 2.5 ml. The reaction was also carried out between Ca and caseins at 30°C for 30 minutes and 120 minutes before gel filtration in order to investigate the time dependent progress of the Ca binding reaction. For α_S-casein only, other special experimental conditions were used to examine detailed

elution patterns of Ca near α_S-casein, using different ionic strengths of tris-HCl buffer at pH 9, different Ca concentrations and different reaction temperatures from those given above.

8. *Centrifugation of ^{45}Ca-α_S-caseinate.* Three milligrams of α_S-casein was dissolved in 0.01M tris-HCl buffer, pH 7 and 8. Then, ^{45}Ca labeled calcium chloride was added until its concentration reached a desired value. The α_S-casein solutions were left at 15°C for 15 minutes before being centrifuged at 12,000 r.p.m. for 20 minutes. Decreases in radioactivity in the supernatant solutions were measured in comparison with the radioactivity of the original calcium chloride solution. The amount of the precipitated α_S-casein was also determined by UV absorption. The molar ratio of bound Ca to precipitated α_S-casein was calculated from these measurements.

Results and Discussion

1. *Stabilization of α_S-casein by κ-casein in the presence of calcium.*

Although this stabilization test is usually performed in the presence of 0.02M calcium chloride, the behavior of α_S-casein at lower Ca concentrations was investigated in this experiment. Within the limits of the conditions used, α_S-casein was completely soluble up to 1mM Ca at pH 7, and started to precipitate at 3 mM Ca. Fig. 1 shows the solubility of α_S-casein depended upon pH mainly, although temperature, time, ionic strength, and concentration are involved in the factors affecting the solubility. The critical Ca concentration where α_S-casein becomes comletely unstable was around 8 mM at pH 7 and seemed slightly higher at pH 8. Attention was mainly focused on this range of Ca concentration in all the present experiments.

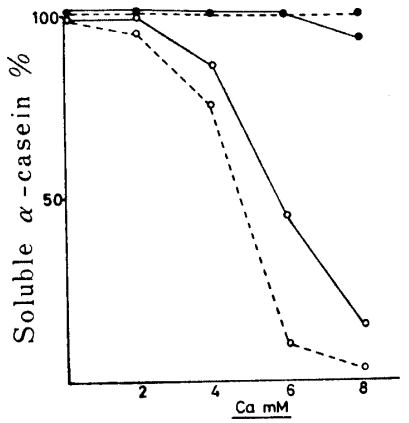

Fig. 1. Solubilty of α_S-casein & its stabilization by κ-casein in the presence of calcium.

	α_S	κ	
mg/3ml	0.01M tris-borate buffer		pH
···○···	3	0	7
—○—	3	0	8
···•···	3	1	7
—•—	3	1	8

Casein soln. was centrifuged at 3000×g after 30 min reaction at 30°C.

2. *Ultracentrifugation.*

Sedimentation behaviors of caseins shown in Fig. 2 showed that κ-casein in pH 8 buffer without urea had an $s_{20,w}$ value of 14.4 which was close to the value reported by Mckenjie and Wake.[12] The symmetrical peak was not so broad as had been assumed from the polydispersity shown by κ-casein in starch gel electrophoresis, indicating that polydispersity is attributable not only to molecular size but also to molecular charge. A much larger $s_{20,w}$ value was obtained in the presence of 5 mM Ca, which suggests that κ-casein polydispersed due to random polymerization so that the sedimentation peak disappeared in 25 minutes or so. This polymerization proceeded up to a Ca concentration of at least 10 mM. It is not possible to explain

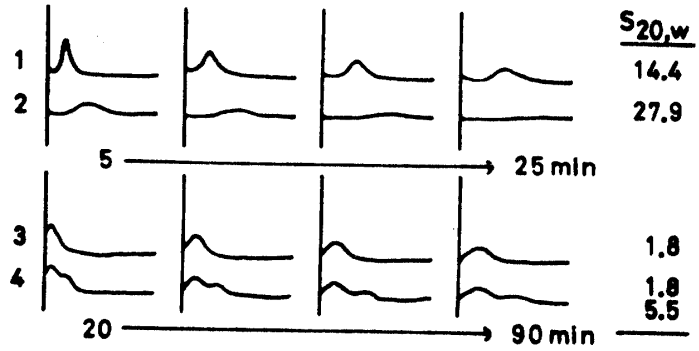

Fig. 2. Ultracentrifugation of α_S-casein, κ-casein and their mixture with and without calcium.
In 1 ml of 0.01M tris-HCl buffer, pH 8.0 : 1 ; κ-casein 5mg, 2 ; κ-casein 5mg & Ca 5mM, 3 ; α_S-casein 5mg & Ca 0.5mM, 4 ; α_S-casein 5mg, κ-casein 1.7mg & Ca 0.5 mM. Centrifugation was performed at 180,000 x g, 25°C

why this finding contradicts that reported by Chiba et al.[13] It does not seem reasonable that the inconsistency is due to the use of different concentrations of Ca. However, the decrease in the stabilizing ability of κ-casein which is caused by the presence of Ca in the κ-casein prior to its being mixed with α_S-casein is more easily accounted for in reference to the present data. When α_S-casein and κ-casein were mixed at 30°C in the presence of 0.5 mM Ca, they formed a new 1 : 1 comlex, leaving excess α_S-casein completely free as judged from the same $s_{20,w}$ value(1.8) as that of free α_S-casein, the $s_{20,w}$ value of which was calculated to be 5.5.

3. *Sephadex G-200 gel filtration of Ca-caseinates.*

Fig. 3 shows that the mixture of α_S-casein and κ-casein is in all the cases more highly polymerized than each casein. This tendency is particularly remarkable when the temperature is high enough to maintain the structure of the mixed complexes. Normally, the degree of polymerization increased as the Ca concentration went up. As Waugh et al.[3] reported, complex formation between α_S-casein and κ-casein occurred at 37°C even without Ca. It is not evident how much larger the α_S-κ complex was, because the largest portion was eluted at the void volume of the Sephadex column used. However, when changes in the elution patterns accompanying those of Ca concentration are checked, it seems that the α_S-κ complex is a complex between greatly polymerized α_S-casein and slightly polymerized κ-casein because the difference in elution patterns between the α_S-κ mixture and α_S-κ complex was not surprisingly

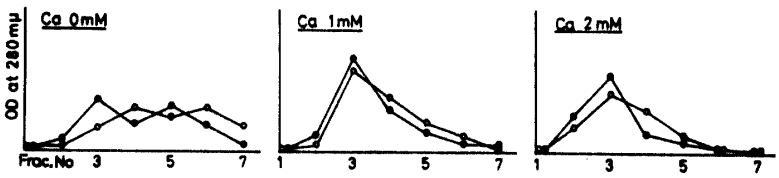

Fig. 3. Gel filtration of α_S- and κ-caseins on Sephadex G-200 at various Ca concentrations.
—○— Average curve of α_S- and κ-caseins.
—●— Curve of their mixture. One ml of 0.2% soln. in 0.01M tris-HCl buffer, pH 7, was applied to a column of Sephadex G-200 (1.2×17cm).

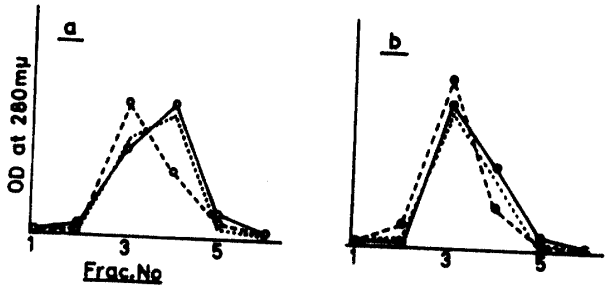

Fig. 4. Gel filtration of α_S- and κ-casein on Sephadex G-200 column (1×19cm).
Reaction time

	a 0.6% α_S-casin in 0.01M tris-HCl buffer. pH 7.	b 0.6% α_S-casein & 0.2% κ-casein in the same buffer.
——	30 min 30°C 1mM Ca.	30 min 30°C 1mM Ca.
----	120 min 30°C 1mM Ca.	120 min 30°C 1mM Ca.
·····	180 min 10°C 1mM Ca.	180 min 10°C 1mM Ca.

large. Gel filtration of κ-casein only was carried out in order to obtain the average elution pattern of α_S-κ mixture. It suggested the polymerization of κ-casein is probably due to the presence of Ca, which was also shown to occur by the above ultracentrifugation. Fig. 4 shows that polymerization of α_S-casein induced by 1 mM Ca advanced at 30°C with passage of time, but did not so eminently at 10°C. On the other hand, α_S-κ complex hardly showed any tendency to time dependent polymerization even at 30°C, not mention at 10°C.

4. *Electric conductivity in the presence of various concentrations of Ca.*

The electric conductivity produced on adding 10 mM calcium chloride solution to 8 ml casein solution was compared with the result of an addition of the same calcium chloride solution to an equal volume of tris-HCl buffer at pH 7 and 9. As unknown factors are involved, it is impossible to know the real nature of the Ca-protein interaction. But, a decrease in the slope of the titration curve in Fig. 5 indicates some interaction took place between Ca and casein. Based on this assumption, α_S-casein reacted with Ca to the greatest degree, followed in decreasing order by the α_S-κ complex, and κ-casein. It is difficult to explain changes in conductivity in a range of over 2 mM Ca because of the precipitation of casein. Possibly structural changes in casein occur at pH 7 since both the titration changes and the time dependent changes of conductivity were more evident at pH 7 than at pH 9.

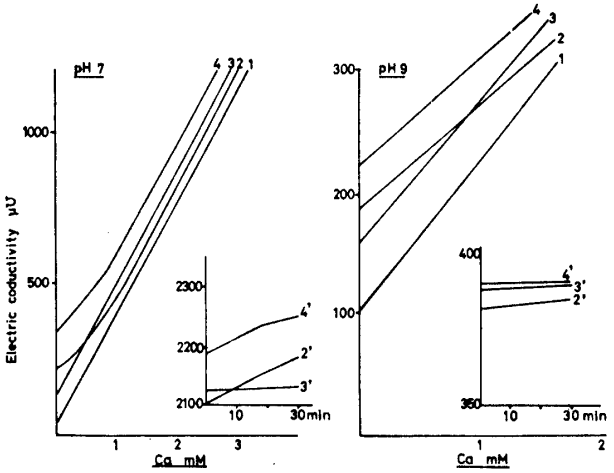

Fig. 5. Electric conductivity of casein solutions in the presence of various amounts of calcium at 15°C. 1 : without casein, 2 : α_S-casein, 3 : κ-casein, and 4 : κ-, & α_S-caseins preincubated at 30°C for 30 minutes. 2′, 3′and 4′ show time dependent change of conductivity.

5. *Equilibrium dialysis of ^{45}Ca-caseinates.*

The binding of Ca to α_S-casein, κ-casein and their complex was determined by equilibrium dialysis using several concentrations of Ca at pH 7 and 8. Representative data from which the binding values were calculated are given in Fig. 6 and Table 1. The order of the ability to adsorb Ca ion was α_S-casein $>\alpha_S$-κ complex$>$ κ-casein by weight. In the early step of dialysis, no difference was observed between α_S-casein and α_S-κ complex. The apparent difference in the later step indicates

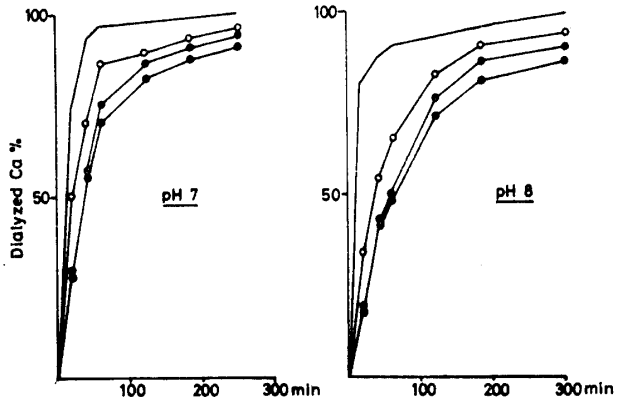

Fig. 6. Equilibrium dialysis of Ca-caseinates.

Inside a dialysis bag ; 1ml of 0.01M tris-HCl buffer and :	α_S mg	κ mg
——	0	0
-○-	0	12
-●-	12	0
-◉-	9	3

Casein solns were preincubated with 1mM $CaCl_2$ (^{45}Ca) at 30°C for 30min before dialysis.

Table 1. Calculation of calcium bound to caseins: determined by equilibrium dialysis. Experimental conditions are shown in Fig. 6.

pH	Casein type	Casein moles $\times 10^{-7}$	Ca in soln. mM	Ca in soln. cpm/41 ml	Ca bound %	Ca bound moles $\times 10^{-7}$	Ca bound calcd. $\times 10^{-7}$	Ca/casein molar ratio
7	α_s	4.3	1	130512	8.6	35.3		8.2
7	κ	6.0	1	134397	4.7	19.3		3.2
7	α_s + κ	3.3 + 1.5	1	132253	7.9	32.4	26.3	
7	α_s	3.6	3	142977	3.7	45.5		12.6
7	κ	2.0	3	138711	0.7	8.6		4.3
7	α_s + κ	2.7 + 0.5	3	140537	3.8	46.7	36.4	
8	α_s	4.3	1	129113	11.9	48.8		11.4
8	κ	6.0	1	130266	6.1	25.0		4.2
8	α_s + κ	3.3 + 1.5	1	130138	10.3	42.2	42.9	
8	α_s	4.3	10	115711	1.4	56.0		12.9
8	κ	6.0	10	115598	0.6	24.5		4.1
8	α_s + κ	3.3 + 1.5	10	115899	1.7	69.7	48.1	

that time dependent polymerization of Ca-α_s-caseinate progressed faster than that of the α_s-κ complex. The complex caseins bound more Ca than the expected amount which had been calculated from the amount of Ca bound by each component of the complex. This phenomenon suggests that additional Ca, other than that bound to casein through electrostatic binding, may be involved in micelle formation. Little difference between the experimentally obtained value and the calculated value at pH 8 and 1 mM Ca indicates that few micelles were formed under these conditions. It is probable that more Ca is necessary for the micelle formation at pH 8 than at pH 7. The molar ratios of bound Ca to caseins are also listed in Table 1. A molar ratio of 12 or 13 is probably a critical value for the initiation of α_s-casein precipitation and the ratio of 4 is perhaps the highest value for κ-casein.

6. *Sephadex G-75 gel filtration of ^{45}Ca-caseinates.*

The binding of Ca to α_s-casein and their complex was determined by gel filtration with two concentrations of Ca at pH 7 and 8. Representative data are shown in Fig. 7 and Table 2. The amounts of Ca bound to caseins were calculated from the increase of ^{45}Ca in the casein fractions ; normally the third and the fourth fractions. Another calculation could have been made from the decrease of ^{45}Ca in fractions following those

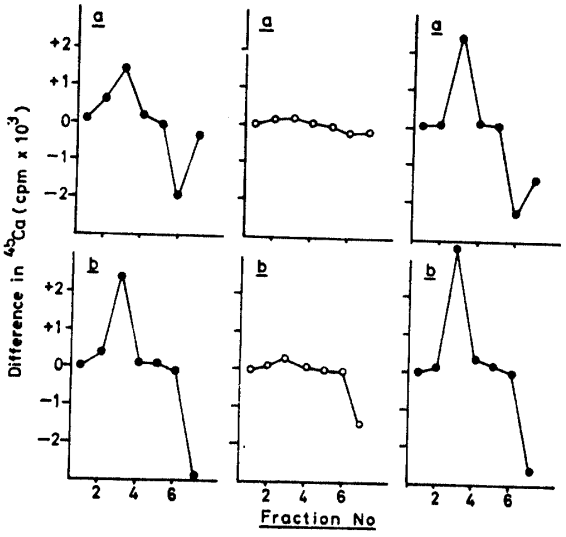

Fig. 7. Gel filtration of Ca-caseinates on Sephadex G-75 column (1×19cm).

	α_s	κ	other conditions
—●—	3mg	0mg	• Ca : 1mM
—○—	0	1	• buffer : 0.01M tris-borate a ; pH 7, b ; pH 8
—◉—	3	1	• fraction : 2.5ml

Casein solutions were incubated with Ca at 30°C for 30 minutes before gel filtration with Ca containing buffer.

Table 2. Calculation of calcium bound to caseins; determined by gel filtration. Experimental conditions are shown in Fig. 7.

pH	Casein type	Casein moles $\times 10^{-7}$	Ca in soln. mM	Ca in soln. cpm/0.5 ml	Ca bound total cpm	Ca bound moles $\times 10^{-7}$	Ca bound calcd. $\times 10^{-7}$	Ca/casein molar ratio
7	α_s	1.07	1	2500	3000	6.0		5.6
7	κ	0.50	1	2500	22	0.04		0.1
7	α_s + κ	1.07 + 0.50	1	2500	4245	8.5	6.04	
7	α_s	1.07	3	2533	1962	11.6		10.8
7	κ	0.50	3	2533	197	1.2		2.4
7	α_s + κ	1.07 + 0.50	3	2533	2630	15.6	12.8	
8	α_s	1.07	1	2133	4748	11.1		10.4
8	κ	0.50	1	2133	442	1.0		2.0
8	α_s + κ	1.07 + 0.50	1	2133	6645	15.6	12.1	

of casein. But the first method for calculation of the bound Ca was used because the partial specific volume of casein is small enough to neglect. The molar ratios of bound Ca to casein are generally smaller than those obtained by the dialysis method. These differences, ranging from 1 to 3, are probably due to the difference in reaction time employed in the two methods. Dialysis continued up to 300 minutes after Ca had been added to the casein solutions. On the other hand, gel filtration was finished within 10 minutes so that neither turbidity nor precipitation appeared in this case. Therefore, the additional bound Ca necessary for casein to visibly aggregate should be within 3, based on the molar ratio from values obtained by gel filtration. The ability of casein to adsorb Ca is of an order similar to that determined by dialysis on the molar basis ; α_S-κ>α_S->κ-casein. Here again, the α_S-κ complex bound more Ca than the total amount bound to each casein of the same quantity.

7. *Centrifugation of ^{45}Ca-α_S-caseinate.*

Results of the calculation of Ca bound to α_S-casein determined by centrifugation are listed in Table 3. Distinctly lower ratios of Ca to α_S-casein were obtained than those ratios determined by the other two methods. The low value may be ascribed to the absence of structural binding of Ca. When Ca-α_S-caseinate is centrifuged, it is strongly pressed to the cell bottom and excludes weakly bound Ca out of the outer binding region of α_S-casein aggregates. It is probable that there are at least two kinds of Ca binding ; one is an electrostatic binding with definitely fixed ion pairs, the other a structural binding with fairly moving ion clouds. Although there is little information on this type of problem, it is interesting to apply the concept of P and ψ bindings of small ions to polymers to the study of the nature of Ca bound to casein.

Table 3. Binding of calcium to α_S-casein; determined by centrifugation. Three mg of α_S-casein was incubated with various amounts of Ca at 30℃ for 30min in 1.5ml of 0.01M tris-HCl buffer before centrifugation.

pH	Ca mM	^{45}Ca in the sup. cpm/0.5 ml	Ca bound x 10^{-7}mole	Casein ppt. x 10^{-7}mole	Ca/casein molar ratio
7	0	11689	---	---	---
7	4	10394	4.5	0.86	5.2
7	8	10856	5.7	1.00	5.7
8	0	53384	---	---	---
8	5	53336	---	0.00	---
8	10	51830	10.6	1.00	10.6

8. *Sephadex G-75 gel filtration of ^{45}Ca-caseinates with buffer containing no Ca.*

The results of gel filtration of ^{45}Ca-caseinates with an eluant of 0.01M tris-HCl buffer containing no Ca are shown in Fig. 8. They supposedly show the relative intensity of Ca binding by casein. When compared with the elution volume of free Ca, some

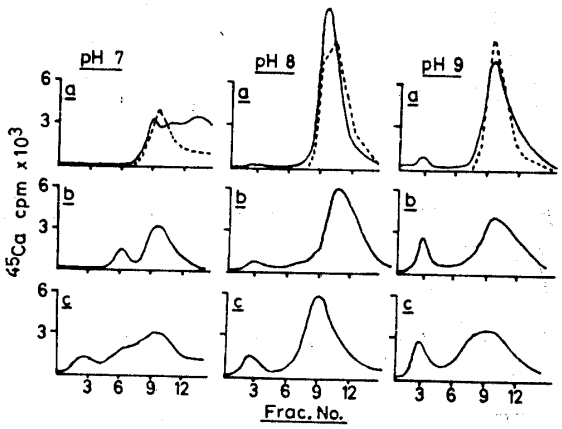

Fig. 8. Sephadex G-75 gel filtration of Ca-caseinates.

In 1ml of 0.01M tris-HCl buffer

..... $CaCl_2$ 1mM

—— a κ-casein 3mg, $CaCl_2$ 1mM

—— b α_S-casein 3mg, $CaCl_2$ 1mM

—— c α_S-casein 3mg, κ-casein 1mg, $CaCl_2$ 1mM

One ml of casein solution was incubated with 0.1ml of 11mM $CaCl_2$ at 30°C for 30 minutes before gel filtration with a buffer containing no Ca. Column : 1×19cm, frac. Vol. : 2.5ml.

calciums are seen to be eluted earlier. This type of Ca is considered to have been bound to proteins in some way. An assumtion was made that those calciums eluted together with casein are probably the most strongly adsorbed calciums. This assumption made it possible to survey the relative state of bound Ca, although the elution pattern does not reflect the real state of Ca in casein solution. There is a general tendency for caseins to adsorb more Ca, more strongly, at pH 9 than at pH 7 or 8. The tendency is particularly noticeable for α_S-casein. In the α_S-κ casein complex, not only the Ca in the protein fractions but also that in the second peak is of greater quantity than the simple total of each casein. This is consistent with the above stated increase of bound Ca. It is very interesting that the Ca binding intensity of κ-casein is very weak. In addition to the low values of Ca bound to κ-casein, the weakness of this binding has some connection with the high stability of κ-casein in the presence of Ca. Fig. 9 shows time dependent changes of gel filtration patterns on Sephadex G-75. As the pH became more alkaline, as already noted, more calciums were restricted in mobility in the casein solution. As reaction time became longer, α_S-casein continued to polymerize fixing a greater quantity of Ca. From data in Fig. 10 we suggest that α_S-casein split into at least two components ; one which was able to adsorb Ca and another which could not. Even when various changes were made in elution conditions, there was no difference in the results. These results indicate that the reaction between casein and Ca is much more complicated than imagined.

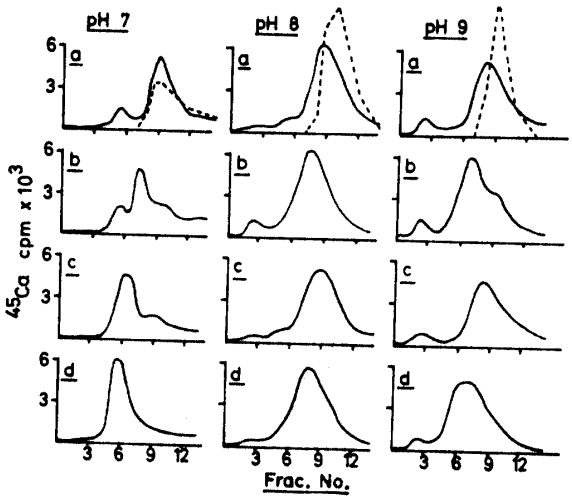

Fig. 9. Sephadex G-75 gel filtration of Ca-caseinates.

	In one ml of 0.01M tris-HCl buffer			
	Ca	α_S	κ	reaction time at 30°C
----	1mM	0mg	0mg	
—— a	1	3	0	30min
—— b	1	3	0	120
—— c	1	3	1	30
—— d	1	3	1	120

Other conditione are shown in Fig. 8.

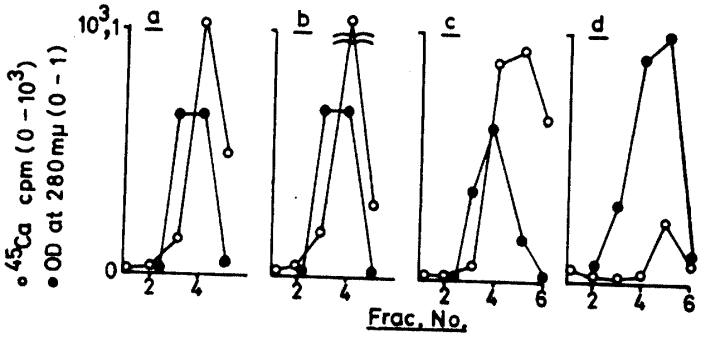

Fig. 10. Sephadex G-75 gel filtration of Ca-α_S-caseinates in various conditions.

	a	b	c	d
α_S-casein, mg	3	3	3	3
tris-HCl buffer, pH 9, M	10^{-2}	10^{-2}	10^{-3}	10^{-2}
$CaCl_2$, mM	1	0	1	1
$^{45}CaCl_2$ soln., ml	0.1	0.1	0.1	0.1
preincubation, T°C	30	30	30	10
time (min.)	30	30	30	30

Other conditions are shown in Fig. 8.

要旨：Caとカゼインとの反応をCa濃度0～10mMの範囲で調べた。3mM 濃度で沈殿し始めるα_S-カゼインはκ-カゼインによって完全に安定化された。一方，κ-カゼイン自身もCa の存在下で会合を起こし，多分散状態が著るしくなったが，沈殿はしなかった。Ca が存在しない場合，κ-カゼインと α_S-カゼインの $s_{1,0,w}$ は各々14.4と1.8であったが，Ca濃度の増加と共に会合していく様子が超遠心分析とゲルろ過によって確認された。Caの存在下で α_S-カゼインは時間と共に会合度を増し，ついには沈殿するがα_S-κ-カゼイン複合体や，κ-カゼイン単独の場合は時間的変化が極めて緩慢であった。pH 7と8におけるカゼインによる Caの吸着量を，Ca濃度1～10mM の範囲で調べた。α_S- カゼインの沈殿に必要な吸着 Ca 量はカゼイン1モル当り約12モルであった。一般的にいって，Caとの反応でα_S-κ-カゼイン複合体のCa吸着能はα_S-，κ-カゼイン単独の場合の吸着能から推定されるより強かった。一方，κ-カゼインのCa吸着能は著るしく弱いことが明白となった。その他吸着の時間的変化についても調べた。

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