Food Chemistry **42** (1991) 183–201

The Effects of Heating, Alone or in the Presence of Calcium or Lactose, on Calcium Binding to Milk Proteins

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(Received 6 August 1990; revised version received and accepted 13 November 1990)

ABSTRACT

The binding of calcium by different milk proteins and the effects of heat were investigated. The influences of calcium and lactose were also investigated. It was shown that the caseins of cow's milk have a high calcium binding capacity, when treated with calcium at 25°C for 30 min, pH 7·00, due to the high number of calcium binding sites. The order of binding capacities is α_{s_1}*-casein >* β*-casein >* κ*-casein. For whey proteins the order is* β*-lactoglobulin (*β*-lg) >* α*-lactalbumin (*α*-la). Lactose, in general, when present at 25°C, did not appreciably affect the calcium binding of the major milk proteins. Heating of whole casein at 80 or 95°C for 30 min caused conformational changes of the protein resulting in an increase in its calcium binding ability. The presence of lactose during heating with or without calcium reduced its calcium binding. The calcium binding of* α_{s_1}*-casein was not affected by heating, throughout the temperature range studied, either in the presence or in the absence of calcium, an indication that* α_{s_1}*-casein may undergo physical changes at high temperatures, but the accessibility and/or the number of calcium binding*

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Food Chemistry 0308-8146/91/$03·50

centres are not increased. β-Casein, when heated with calcium, was subject to conformational changes which were reflected in changes in the degree of calcium-binding. Heating of β-lg in the presence of calcium did not much affect the amount of calcium bound to it. However, in the absence of calcium an increase of 21·5% in calcium binding was observed after heating at 80°C and a decrease of 40·0% after heating at 95°C, compared to unheated samples.

INTRODUCTION

The detrimental effect of calcium ions on the stability of evaporated milk has been recognised since the beginning of this century (Sommer and Hart, 1919). Similarly, the stabilizing effect of casein-whey protein interactions against precipitation by heat has also been recognised for many years (Trautman & Swanson, 1958, 1959; Trautman, 1958; Beeby, 1966; Kannan & Jenness, 1961; Morr, 1965, 1967, 1969).

Forewarming of milk before evaporation is considered to be one of the vital factors affecting the stability of milk during processing and storage of the finished product (Deysher *et al.*, 1929; Rose, 1962; Hartman & Swanson, 1962; Sweetsur & White, 1974). How forewarming affects the stability of evaporated milk, is a question which is not yet fully answered.

The extent of the protein–protein interaction brought about by heating milk, as in forewarming, is affected by the intensity of heat treatment, the length of time, pH, the presence of divalent ions, mainly calcium and lactose.

Ntailianas (1959), using the solubility of trichloroacetic acid–protein derivatives in alcohol as an indication of protein–protein interaction, observed that calcium exerts some effect on these interactions. However, he did not carry out any quantitative determination on the amount of calcium bound to milk proteins and the influence of different parameters on the ability of proteins to bind calcium. In general, while the sensitivity of milk proteins to calcium ions has been the basis of many investigations (Zittle *et al.*, 1956; Ho & Waugh, 1965; Waugh *et al.*, 1970; Dickson & Perkins, 1971; Fox & Hoynes, 1975; Holt *et al.*, 1975; Imade *et al.*, 1977), the influence of calcium, lactose and heat on casein–whey protein interactions has not received enough attention.

The objective of this work was to study the heat-induced changes in major milk proteins, with special reference to the influence of calcium and lactose. These changes were investigated by studying the changes in their calcium binding and their electrophoretograms under various experimental conditions. In a subsequent work the interactions of milk proteins with special reference to the influence of calcium, lactose and heat will be reported.

MATERIALS AND METHODS

The preparation of whole casein

Fresh evening bulk milk, exclusively from British Friesian cows, was collected, brought to the laboratory and immediately separated at 40°C. To four litres of skimmed milk, diluted with three times the volume of water, a mixed solution of 0·5M HCl and 0·5M $CaCl_2$ was added dropwise with effective stirring until the pH had fallen to 4·6. The temperature during precipitation was kept between 33 and 40°C. Stirring was continued for another 30 min and the precipitate then left to settle. The supernatant was decanted and the residual suspension filtered through a Buchner funnel using a Whatman filter paper No. 50 placed over a No. 31 paper. The precipitate was washed by decantation *in situ* with distilled water until no protein could be detected in the supernatant when equal volumes of 20% TCA acid and filtrate were mixed. After washing, the casein was dispersed in one litre of distilled water and then heated in a water bath at 70°C for 30 min to destroy a proteolytic enzyme, which co-precipitates with casein and might hydrolyse it (Warner, 1944; Shahami, 1966; Kaminogawa *et al.*, 1972). The casein suspension was dialysed at 2–4°C, under constant stirring, three times against a dilute solution (1/50M) of HCl and finally distilled water to obtain a calcium-free product. At the end of dialysis the casein was freeze-dried and then ground to a fine powder. It was washed three times with peroxide-free petroleum ether to remove lipids.

The fractionation of whole casein

The separation of α_{s_1}-casein

α_{s_1}-Casein was separated from the whole casein by a combination of the first part of the method proposed by Thompson and Kiddy (1964) (the DEAE cellulose-urea-chromatographic purification step was omitted) and the method of Tripathi and Gherke (1970), slightly modified. (Step 1 was twice repeated, and the precipitate, consisting mainly of α_{s_1}- and κ-caseins, was washed 6–8 times with cold (2°C) water.)

Separation of β-casein

For the fractionation of β-casein the method of Aschaffenburg (1963) was applied with some minor modifications. The product was further purified by treatment with calcium phosphate gel as described by Green (1969).

Separation of κ-casein

κ-Casein was prepared from whole casein by the urea–sulphuric acid method of Zittle and Custer (1963). The ethanol precipitation step was

omitted, because κ-casein was further purified by treatment with calcium phosphate gel as described by Green (1969).

At the final stage of separation, the suspensions of α_{s_1}-, β- and κ-caseins were dialysed three times against distilled water until the dialysate was free from chlorides. They were freeze-dried, powdered, further dried over P_2O_5 under vacuum and stored, when not in use, at −20°C, in brown air-tight bottles.

Separation of whey proteins

Whey proteins β-lactoglobulin (β-lg) and α-lactalbumin (α-la) were prepared, from the whey resulting from precipitation of casein at its isoelectric point, following the method of Armstrong *et al.* (1967).

All batches of protein were checked for calcium (a) by the atomic absorption method and (b) complexiметrically (Ntailianas & Whitney, 1963), and for purity by polyacrylamine gel (PAG) electrophoresis. All of them were free from calcium and free from other proteins. The moisture of all protein samples was within the range 0·5–0·8%. No corrections were introduced for this low moisture content in subsequent calculations.

A full account of the methods for preparation and purification of proteins is given by Pappas (1979).

Experimental solutions

All volumetric apparatus used was of borosilicate glass and class A. All chemicals used were '*Pro analysi*' grade. Glass-distilled water was used throughout.

Standard calcium chloride solution

A weighed amount of $CaCO_3$ was dissolved in a minimum volume of dilute HCl to give a clear solution. Sodium barbital was added to make its final concentration 30mM. The pH of the solution was carefully adjusted to 7·00 ± 0·05 with dilute NaOH or HCl, as required. A digital Orion pH meter, Model 801A (Orion Research Inc., Cambridge, Mass., USA) was used. A few drops of toluene were added as a preservative (Warner, 1944). The concentration of calcium of this solution was determined using potassium permanganate after precipitation of calcium as oxalate (Vogel, 1962). This solution was diluted quantitatively with 30 mM sodium barbital buffer pH 7·00 to the required experimental calcium concentrations (3, 10, 15 and 20 mM).

Standard di-sodium di-hydrogen ethylenediamine tetra-acetate (Na_2H_2 EDTA) solution

Na_2H_2 EDTA . $2H_2O$ (12·060 g) was dissolved in water (12 pellets of NaOH

were added to assist solution (Ntailianas & Whitney, 1963)) and then 1·00 g $MgCl_2 . 6H_2O$ was added. This solution was diluted to 2 litres with distilled water and standardised against the standard $CaCl_2$ solution using Eriochrome black T indicator (Vogel, 1962).

Sodium barbital buffer 30 mM, pH 7·0
Sodium barbital (5,5-diethyl-barbituric acid disodium salt) was dissolved in distilled water to make a final concentration of 30 mM. The pH of the solution was adjusted to pH 7·00 ± 0·05 with dilute HCl or NaOH, as required.

Protein solutions
Proteins and lactose were generally used at concentrations close to those found in milk (Jenness, 1970). However, where the expected calcium binding capacity was low, as for κ-casein (Imade *et al.*, 1977), or when the protein concentration in milk is very low, as for α-la (0·07%), the protein concentrations used were higher than those found in milk. The concentrations (w/v) used in these experiments were: whole casein 2·50%, α_{s_1}-casein 1·37%, β-casein 0·62%, κ-casein 0·75%, β-lg 0·30%, α-la 0·30%. Lactose monohydrate was used at 5·00%.

The exact amount of protein(s) was accurately and quickly weighed and dissolved in 15 ml capacity borosilicate, stoppered test tubes in sodium barbital buffer solution. Lactose was added here when it was required. The pH of the solutions was adjusted to 7·00 ± 0·05 with N-NaOH under quick stirring. Finally, more buffer solution was added with a microsyringe to the weight corresponding to 5 ml volume of the solution. The samples were shaken on a continuous horizontal shaker, at a constant speed, until no visible sign of protein was present and then for a further 30 min. In those experiments in which the protein samples were heat-treated in the presence of calcium, the $CaCl_2$ solution was added to the protein sample at this stage of the experimental procedure before any heat treatment. When heat treatment was carried out in the absence of calcium, calcium was added at the end of heating, after the samples had been cooled to 25°C. Equal volumes (4 or 5 ml) of the protein and calcium chloride solutions of double the required concentration of protein and calcium were mixed together. After mixing, the samples were shaken for another 30 min. Heat treatments were carried out at 25, 80 or 95°C for 30 min by immersing the samples in a thermostatically controlled water bath or at 115°C for 10 min. Heating at 25, 80 or 95°C was done in borosilicate loosely stoppered tubes whereas at 115°C it was done in a small aluminium bath containing high boiling point mineral oil. For heating at 115°C the test tubes with the protein samples were kept hermetically closed with glass stoppers, with a simply constructed device using a high-vacuum silicone grease. After heating, the samples were cooled

to 25°C. Any quantity of water evaporated during heating at 25, 80 or 95°C was replaced at this stage.

The determination of calcium
Calcium concentration in ultrafiltrates was determined compleximetrically against the standard Na_2H_2 EDTA solution (Vogel, 1962), whereas in protein solution the method of Ntailianas and Whitney (1963) was followed.

Calcium binding studies

The calcium binding to different milk proteins was studied by applying the continuous ultrafiltration, UF, (diafiltration or equilibrium ultrafiltration) technique, using the multicell micro-ultrafiltration system Model MMC and the UF membranes type DIAFLO® UM10 of 25 mm diameter with a nominal molecular weight cut off of 10000 (Amicon Corp., Lexington, Mass., USA). A brief description of this technique is given below.

The 10 ml protein samples, of the same calcium concentration, were added to each of the eight cells of the micro-ultrafiltration system. The 800 ml capacity common reservoir was filled with calcium solution of the same calcium contentration as the protein samples. Reservoir and cell compartments were then equally pressurised by nitrogen gas. The system operated under the same stirring speed for all cells. The liquid in the cells, which was continuously discharged through the UF membranes was replaced by an equal volume from the reservoir. Thus, the volume of the sample in the cell remained essentially constant during the run. To find the total calcium binding capacity of the protein(s) the UF experiment allowed binding equilibrium to be reached, i.e. the point where the calcium concentration of the ultrafiltrates was equal to that of the feeding solution. From preliminary experiments it was found that binding equilibrium was established when five times the sample volume of ultrafiltrate (50 ml) had passed through. Therefore the first 50 ml of ultrafiltrates were rejected and the following 5 ml were collected for calcium determination.

The moles of calcium bound to the amount of protein in the sample solution were calculated by the difference of the concentrations of calcium in the protein sample (bound and free calcium) and in the ultrafiltrate.

A full description of the ultrafiltration technique was given by Pappas (1979).

Polyacrylamide gel electrophoresis

A combination of the discontinuous vertical polyacrylamide gel (PAG) electrophoresis method of Kiddy *et al.* (1972) and Sargent (1969) was conducted in tris-glycine electrode buffer pH 8·3–8·4, slightly modified.

When ultrafiltration was completed, 2 ml of the retentate were transferred to a 5 ml volumetric flask. Sucrose (0·4 g) was added to the flask and the mixture diluted to volume with electrode buffer. From this solution, 20 μl of α_{s_1}-, β-, or whole casein or 10 μl of κ-casein or 25 μl of β-lg or α-la were applied to each electrophoresis tube. The type SAE 2761 power unit with a vertical electrophoresis cell of eight tubes capacity (Shandon, London, England) was used.

A constant voltage of 100 V was applied for 1 h 20 min. Gels were stained for 30 min in a solution containing 1% amido black and 7% acetic acid. Destaining was done in 7% acetic acid. Details of the electrophoresis methods applied here are given by Pappas (1979).

RESULTS AND DISCUSSION

The binding of calcium by different milk proteins

The binding values of calcium by different milk proteins treated at 25°C for 30 min, pH 7·00, with calcium at varying equilibrium-free calcium concentrations, with and without lactose, are presented in Table 1.

Whole casein

Applying the calcium-binding data of Table 1 for whole casein and eqn (1) given by Klotz (1950):

$$\frac{1}{r} = \frac{1}{Kn} \cdot \frac{1}{(Ca^{2+})} + \frac{1}{n} \tag{1}$$

where

r = the number of moles of calcium bound to 10^5 g of protein;
K = the intrinsic binding constant;
(Ca^{2+}) = the concentration of free calcium ions in the protein solutions.

The maximum number (n) of moles of calcium bound to 10^5 g of protein at infinite calcium concentration can be estimated. Thus, plotting $1/r$ against $1/(Ca^{2+})$, the intercept of the ordinate gives $1/n$ from which n can be calculated. The above equation applies when this plotting gives a linear relationship. However, the data of Table 1 for whole casein deviate from linearity. This may be due to the electrostatic influence of the bound calcium on its subsequent binding and/or the variation in the type and number of calcium binding sites, which become available as the calcium binding builds up. This explanation is in accordance with suggestions given by Klotz (1950).

On the other hand, the binding data of whole casein obtained within the range of calcium concentrations of 10–20 mmoles/litre are in better

TABLE 1
The Binding of Calcium by Different Milk Proteins[a]

Protein solution	*Equilibrium-free calcium concentration (mmoles/litre)*							
	3		*10*		*15*		*20*	
	mmoles of calcium bound/litre of protein solution	*Moles of calcium bound/ 10^5 g of protein*	*mmoles of calcium bound/litre of protein solution*	*Moles of calcium bound/ 10^5 g of protein*	*mmoles of calcium bound/litre of protein solution*	*Moles of calcium bound/ 10^5 g of protein*	*mmoles of calcium bound/litre of protein solution*	*Moles of calcium bound/ 10^5 g of protein*
Whole casein, 2·50%	6·01	24·0	7·50	30·0	7·10	28·4	6·65	26·6
Whole casein, 2·50% + lactose, 5·00%	5·81	23·2	7·82	31·3	7·56	30·2	6·78	27·1
Change (%)	−3·3		+4·3		+6·5		+1·9	
α_{s_1}-Casein, 1·37%	4·26	31·1	4·75	34·7	5·76	42·0	6·26	45·7
α_{s_1}-Casein, 1·37% + lactose, 5·00%	—	—	4·28	31·2	5·66	41·3	6·28	45·8
Change (%)	—	—	−9·8	—	−1·7	—	+0·3	—
β-Casein, 0·62%	1·25	20·2	1·30	21·0	1·88	30·3	2·15	34·7
κ-Casein, 0·75%	—	—	0·78	10·4	1·33	17·7	1·67	22·2
κ-Casein, 0·75% + lactose, 5·00%	—	—	0·75	10·0	1·26	16·8	1·68	22·4
Change (%)	—	—	−3·8	—	−5·2	—	+0·6	
β-Lactoglobulin, 0·30%	0·56	18·6	0·74	24·6	0·98	32·6	1·06	35·3
β-Lactoglobulin, 0·30% + lactose, 5·00%	—	—	0·73	24·3	0·93	31·0	1·05	35·0
Change (%)	—	—	−1·3	—	−5·1	—	−0·9	—
α-Lactalbumin, 0·30%	—	—	0·70	23·3	0·80	26·6	0·93	31·0
α-Lactalbumin, 0·30% + lactose, 5·00%	—	—	0·68	22·6	0·74	24·6	0·94	31·3
Change (%)	—	—	−2·8	—	−7·5	—	+1·1	—

[a] The protein solutions were treated at 25°C for 30 min (a) with calcium at varying equilibrium-free concentrations (the concentrations of calcium in equilibrium with the calcium bound to protein); (b) with and without lactose.

agreement with linearity. Applying eqn (1) gives values of n of 24·1 and 24·7 for whole casein and whole casein + lactose, respectively. Values reported in the literature for whole casein without lactose are $n = 48$ (Chanutin *et al.*, 1942), $n = 22$ (Carr, 1953), $n = 29$ (Zittle *et al.*, 1958), $n = 33$–36 (Tessier & Rose, 1961) and $n = 16$ (Imade *et al.*, 1977). These differences in the value of n may be due to (a) variations in the methods of preparations of proteins; (b) the use of different buffers at different ionic strength, as increases in ionic strength raise the dissociation of calcium caseinates (Chanutin *et al.*, 1942; Klotz, 1950); and (c) variations in the experimental procedures.

In experiments using direct ultrafiltration instead of continuous ultrafiltration (diafiltration) and calcium concentrations of 10, 15 and 20 mmoles/litre, the values of n obtained were 22·7 and 22·3 for whole casein without and with lactose, respectively. These differences in n between the direct and continuous UF methods may be explained by the fact that in diafiltration the protein is continuously fed with $CaCl_2$ solution, so that the casein is almost saturated with calcium. This is not the case in the direct method where the protein is not necessarily saturated with calcium to the same degree. Table 1 shows that whole casein with or without lactose reaches its maximum binding capacity rapidly before the highest concentration of Ca^{2+} is reached, indicating that whole casein shows a high affinity for calcium. It also shows that, above a certain level of $CaCl_2$ (10 mmoles/litre), the binding capacity tends to decline, presumably due to physical alterations in the casein induced by the bound calcium (Klotz, 1950; Imade *et al.*, 1977; Dalgleish, 1973).

α_{s_1}-Casein

The value of n for α_{s_1}-casein, estimated from their calcium binding data presented in Table 1 is 44·6. Other values reported in the literature are $n = 36{\cdot}0$ (Dickson & Perkins, 1971), $n = 25{\cdot}0$ (Imade *et al.*, 1977), $n = 46{\cdot}5$ (Noble & Waugh, 1965) and $n = 42{\cdot}3$ (Bingham *et al.*, 1972). These differences may be attributed to the same reasons as for whole casein.

It was observed in these studies that macroscopic precipitation of α_{s_1}-casein did not occur at a calcium concentration of 3 mmoles/litre, but did occur at 10 mmoles/litre. Table 1 shows that binding of calcium to α_{s_1}-casein continued beyond the point of precipitation. This is in agreement with the findings of Dalgleish & Parker (1980). Ho and Waugh (1965), in a brief account of the interaction of calcium with α_{s_1}-casein, as shown by infrared spectroscopy, indicated that calcium binds first with the phosphate groups of this protein and that binding with these groups reaches completion before any precipitation occurs. Taking this into account and the fact that the calcium binding of α_{s_1}-casein in this study continued beyond its precipitation, i.e. when the interaction of calcium with phosphate groups has

reached completion, and that the amount of calcium bound is higher than would be expected if the binding were only to phosphate groups, it is concluded that other groups, possibly carboxylic groups of α_{s_1}-casein, are also involved in the binding of calcium. This is also supported by the fact that dephosphorylated α_{s_1}-casein is still precipitated by calcium (Pepper & Thompson, 1963; Bingham *et al.*, 1972). The maximum theoretical number of moles of calcium bound to one mole of α_{s_1}-casein B (mol. wt 23, 613; Mercier *et al.*, 1972) at pH 7·0 was calculated as 25·4 (Pappas, 1979).

β-Casein

The results for the calcium-binding capacity of β-casein are given in Table 1. The moles of calcium bound to 10^5 g of β-casein were found to be 20·2, 21·0, 30·3 and 34·7 for concentrations of calcium, 3, 10, 15 and 20 moles/litre, respectively. It is seen from these results that the ability of β-casein to bind calcium is lower than that of α_{s_1}-casein for the same calcium concentration.

κ-Casein

The moles of calcium bound to 10^5 g of κ-casein are 10·4, 17·7 and 22·2 for calcium concentration 10, 15 and 20 mmoles/litre, respectively. Dickson & Perkins (1971) found that the number of moles of calcium bound to 10^5 g of κ-casein was 11. The higher values of moles of calcium bound, found in these experiments, may be attributed to the larger concentrations of calcium used in this study, compared to those used by the authors, since the binding of small ions to proteins follows the law of mass action (McLean & Hastings, 1935; Klotz, 1946). This discrepancy may be also attributed to differences in methods and materials used.

β-Lactoglobulin

The moles of calcium bound to 10^5 g of β-lg were found to be 18·6, 24·6, 32·6 and 35·3 for calcium concentrations of 3, 10, 15 and 20 mmoles/litre, respectively. This protein has an appreciable calcium-binding capacity even at low calcium concentration. Since this protein contains no phosphorus (McKenzie, 1971) the calcium must be bound to the free carboxylic groups of aspartic and glutamic acids, which are 11 and 16 per molecule of β-lgA and 10 and 16 for β-lgB, respectively (Braunitzer *et al.*, 1973).

α-Lactalbumin

The moles of calcium bound to 10^5 g of this protein are 23·3, 26·6 and 31·0 for calcium concentrations of 10, 15 and 20 mmoles/litre, respectively. Since this protein contains no phosphorus but 9 and 8 free carboxylic groups of aspartic and glutamic acids per molecule, respectively (Brew *et al.*, 1970), it seems, again that these acids are the calcium binding centres.

Effect of lactose on calcium binding

The values for n of 24·7 and 24·1 calculated for whole casein with and without lactose, respectively, indicate that lactose does not affect the amount of calcium bound to whole casein under the experimental conditions.

Continuous PAG electrophoresis showed that lactose slightly increases the electrophoretic mobility of whole casein. This may be due to the interaction of lactose with proteins taking place even at room temperature (Goulden, 1956), which eliminates some of the positively charged free amino groups of whole casein, resulting in an increase in the overall negative charge of the protein at the operating pH value.

For α_{s_1}-casein, the presence of lactose slightly depresses the amount of calcium bound to this protein at lower calcium concentration. At higher concentrations of calcium, lactose exerts no effect (Table 1).

With respect to the effect of lactose on calcium binding by κ-casein, β-lg and α-la, it is shown that lactose has no effect. The data of Table 1 show that the caseins of cow's milk have a high binding capacity for calcium at pH 7·00. The order of binding capacities was found to be α_{s_1}-casein > β-casein > κ-casein, which is in agreement with the results of Dickson and Perkins (1971).

Heat-induced changes in individual milk proteins. The influence of calcium and lactose

The heat-induced changes in the binding of calcium by individual milk proteins heated with or without lactose at 25, 80 and 95°C for 30 min and at 115°C for 10 min in the presence or absence of calcium are presented in Table 2. The experiments were carried out applying the continuous UF technique. The concentration of calcium used in this series of experiments (3 mmoles/litre) is close to the ionic concentration of calcium in milk (Christianson *et al.*, 1954; Seekles & Smeets, 1954; Waugh, 1971).

Whole casein

From the data of Table 2 it is shown that, on heating whole casein alone at 80, 95 and 115°C in the presence of calcium, its ability to bind calcium increased slightly when heated at 80 and 95°C, while at 115°C it was almost the same as the original level of the unheated control sample (treated at 25°C). The same tendency, but to a lesser extent, was found when heating was carried out in the absence of calcium. However, when lactose was present during heating with or without calcium the degree of binding remained almost unchanged throughout the temperature range. The presence of lactose during heating decreased the calcium binding by whole casein, especially at 80 and 95°C. These data suggest that the increases of calcium binding caused by different heat treatments appear to be

TABLE 2

Heat-Induced Changes in the Binding of Calcium by Individual Milk Protein under Different Heat Treatments[a]

	Calcium absent							*Calcium present*[b]										
Temperature of treatment (°C)	*25*	*80*	*95*	*115*	*80*	*95*	*115*	*25*	*80*	*95*	*115*	*80*	*95*	*115*	*25*	*80*	*95*	*115*
Time of treatment (min)	*30*	*30*	*30*	*10*	*30*	*30*	*10*	*30*	*30*	*30*	*10*	*30*	*30*	*10*	*30*	*30*	*30*	*10*
Protein solution	*mmoles of calcium bound/litre of protein solution*				*Changes in calcium binding caused by different heat treatments (%)*			*mmoles of calcium bound/litre of protein solution*				*Changes in calcium binding caused by different heat treatments (%)*			*Changes in calcium binding caused by presence of calcium during heat treatments (%)*			
Whole casein, 2·50%	5·84	6·34	6·23	5·90	+8·6	+6·7	+1·0	6·01	6·42	6·57	5·78	+6·8	+9·3	−3·8	+2·9	+1·3	+5·5	−2·0
Whole casein, 2·50% + lactose, 5·00%	5·69	5·82	5·69	5·68	+2·3	0·0	−0·2	5·81	5·90	5·90	5·61	+1·5	+1·5	−3·4	+2·1	+1·4	+3·7	−1·2
Change caused by lactose (%)	−2·6	−8·2	−8·6	−3·7				−3·3	−8·1	−10·2	−2·9							
α_{s_1}-Casein, 1·37%	4·26	4·07	4·28	4·06	−4·5	+0·5	−4·7	4·26	4·16	4·22	4·19	−2·3	−0·9	−1·6	0·0	+2·2	−1·4	+3·2
β-Casein, 0·62%	1·28		1·00	0·99		−21·9	−22·6	1·26		1·50	1·50		+19·0	+19·0	+1·6		+50·0	+51·0
β-Casein, 0·62% + lactose, 5·00%	1·30	1·27	1·05	0·97	−2·3	−19·2	−25·4	1·26	1·38	1·48	1·46	+9·5	+17·4	+15·9	−3·1	+8·7	+40·9	+50·5
Change caused by lactose (%)	+1·5		+5·0	−2·0				0·0		−1·3	−2·6							
β-Lactoglobulin, 0·30%	0·65	0·79	0·39	0·68	+21·5	−40·0	+4·6	0·56	0·58	0·53	0·64	+3·5	−5·3	+14·2	−13·8	−26·6	+35·9	−5·9

[a] The treatments were carried out at the required temperature (a) in the absence and presence of calcium and (b) in the absence and presence of lactose. The results were obtained at an equilibrium-free calcium concentration of 3 mM.

[b] Initial total concentration of 3 mM.

counteracted by the decreases introduced by lactose. Our binding data indicate that whole casein may undergo conformational changes during heating at 80, 95 and 115°C. As the protein molecule unfolds more calcium-binding centres become unscreened and more calcium binds to the protein. The amount of calcium bound to whole casein, which had been heated at 80 and 95°C and then cooled, is greater than the calcium bound to intact unheated casein. This suggests that some of the heat-induced conformational changes in whole casein are irreversible and the protein does not return to its native state after cooling. The effect of lactose on decreasing the calcium binding to casein might be explained by a reduction of accessibility of calcium to binding sites as a result of the Maillard reaction of lactose with lysine.

α_{s_1}-Casein

The results of calcium binding to α_{s_1}-casein heated at different temperatures in the absence and presence of calcium are given in Table 2. The percentage changes in binding caused by the presence of calcium during heating are also given. It can be seen that the calcium binding to this casein fraction was not affected by the various heat treatments employed in these experiments, either in the presence or absence of calcium.

From the electrophoretograms of discontinuous PAG electrophoresis in tris-glycine buffer at pH 8·3 in the absence of urea and mercaptoethanol, it is shown that α_{s_1}-casein is not appreciably affected by different heat treatments, either in the presence or absence of calcium, under the experimental conditions. Despite the heat-induced disaggregation of casein molecules, which takes place above 70°C (Kresheck *et al.*, 1964), and the physical changes the α_{s_1}-casein molecules may undergo at high temperatures, the data of this study indicate that the accessibility and/or the number of calcium binding centres of α_{s_1}-casein are not increased by heating, so that the amount of calcium bound to α_{s_1}-casein, following different heat treatments, remains unchanged. Possibly the calcium-induced conformational changes of the protein molecule (Imade *et al.*, 1977; Holt *et al.*, 1975) bring about aggregation, resulting in a tightening of the molecule (Dalgleish, 1973) and burying calcium binding centres within the aggregate, before they have been made accessible to calcium ions. Therefore the degree of calcium binding remains unchanged at the whole range of temperatures used, near to the original level. This finding is not in agreement with the results of Dalgleish and Parker (1980), where the binding of Ca^{2+} to α_{s_1}-casein is increased by increasing temperature. This discrepancy may be explained by the fact that these authors carried out their experiments at low temperatures (up to 40°C). Conformational changes in the α_{s_1}-casein molecule induced by calcium at low concentrations and heat are not observed by PAG electrophoresis, as

they are presumably reversible on cooling. Dalgleish (1973) demonstrated that the process of precipitation of α_{s_1}-casein by calcium is reversible, redissolution occurring by lowering the calcium/protein ratio.

β-Casein

In contrast to α_{s_1}-casein, heating of β-casein with calcium at temperatures 95 and 115°C increased its ability to bind calcium by 19% compared to unheated samples (Tables 2). On the other hand, when β-casein was heated at the same temperature, but without calcium, its calcium binding capacity decreased by 21·9% and 22·6% after heating at 95 and 115°C, respectively, compared to unheated samples. Table 2 shows that the increases in calcium binding by β-casein, caused by the presence of calcium *per se* during heating at 95 and 115°C were 50·0 and 51·0%, respectively, compared to samples without calcium present on heating. However, the PAG electrophoretograms, showed no apparent change in the electrophoretic mobility of β-casein, after heating at 80 or 95°C in the presence of calcium, compared to unheated samples. As for α_{s_1}-casein, the conformational changes in the molecule of β-casein induced by heat and/or calcium may be reversible on cooling. The presence of lactose during the heating of β-casein did not change its calcium binding ability, which was contrary to expectations. It was observed in these experiments that solutions of β-casein with or without lactose, when heated at 80, 95 and 115°C in the presence of calcium, aggregated as judged by the high degree of opacity developed. On cooling to room temperature the opacity quickly disappeared, indicating that the changes involved were at least partially reversible. That some of these alterations must be irreversible is indicated by the fact that the amount of calcium bound to β-casein, previously heated at 95°C or 115°C and cooled to room temperature, decreased compared with unheated samples (Table 2). It was reported that, on heating β-casein to temperatures similar to those applied in these experiments, the molecule unfolds (Kresheck *et al.*, 1964). It is reasonable, therefore, to assume that such unfolding brings out a number of binding centres, which bind calcium when it is present during heating. The unfolding of the molecule may be followed by a polymerisation or aggregation step, possibly through hydrophobic interactions, resulting in the burying of some of the calcium binding centres and so rendering them inaccessible to calcium, even after cooling to room temperature.

β-Lactoglobulin

Heating of β-lactoglobulin in the presence of calcium at 80, 95 and 115°C does not much affect the amount of calcium bound to it (Table 2). These data are in agreement with those reported by Demott (1969) and Zittle *et al.* (1957). If, however, β-lactoglobulin was heated in the absence of calcium, its

calcium binding showed two characteristics: first, an increase of 21·5% after heat treatment at 80°C and cooling, and second, a decrease of 40% after heat treatment at 95°C, compared to calcium binding of unheated samples (i.e. treated at 25°C).

The electrophoretograms of β-lg heated at different temperatures with or without calcium are presented in Fig. 1. It is shown that β-lg is completely aggregated when heated with calcium for 30 min at 80 and 90°C and for 10 min at 115°C (b, c, d). However, when it was heated under identical conditions, but without calcium, the aggregation was considerable but less compared to samples heated with calcium. When heating β-lg in the absence of calcium the aggregation is maximal at 95°C while, at 80°C and 115°C, it becomes less than that at 95°C, as judged by the density of their travelling bands in polyacrylamide gels (b′, c′, d′). Treatment of β-lg at 25°C with calcium does not bring about any aggregation (a, a′). Consideration of the calcium binding results and the electrophoretograms of these experiments may lead to the conclusion that the presence of calcium during heating promotes the speed and enhances the degree of thermodenaturation of β-lg, presumably by establishing intra- or inter-molecular linkages between calcium-binding centres.

The present calcium-binding results indicate that calcium-binding groups,

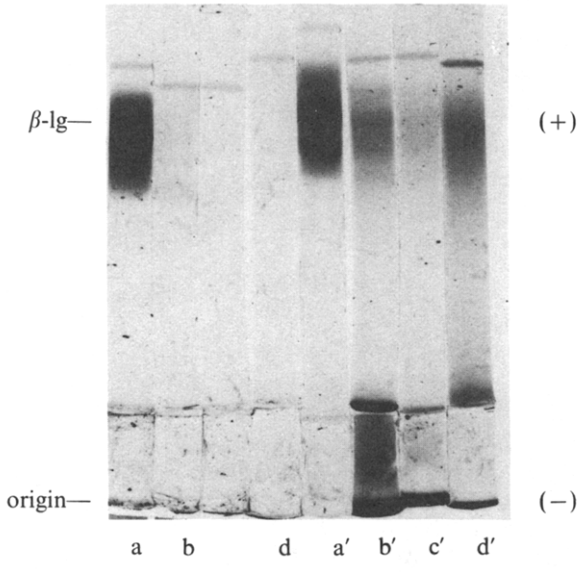

Fig. 1. The effect of heating at a range of temperatures on β-lactoglobulin (β-lg) in the presence and absence of calcium as shown by discontinuous PAG electrophoresis in tris-glycine buffer, pH 8·3. Samples heated at (a) 25°C; (b) 80°C; (c) 95°C; (d) 115°C with 3 mM calcium; and at (a′) 25°C; (b′) 80°C; (c′) 95°C; (d′) 115°C without calcium during heating but with calcium added to 3 mM (after heat treatment and cooling the samples to 25°C).

i.e. carboxyl groups, may become unscreened during the heat-induced unfolding of the molecule of β-lg. On cooling, some of the heat-induced conformational changes appear to be at least partially irreversible, so that a considerable number of the calcium-binding groups are still accessible to calcium. This might explain the increase in calcium binding observed after the heating of β-lg at 80°C, followed by cooling to room temperature. On the other hand, by heating at about 90°C, the rate of denaturation of β-lg changes (Lyster, 1970) and the irreversible denaturation step of β-lg may occur. Presumably, the number and accessibility of calcium-binding centres decrease below the level existing in the protein in the native state, as a result of their being buried in the interior of the molecule. This could explain the drop in the degree of calcium binding by β-lg after heating at 95°C without calcium and subsequent cooling. However, on heating β-lg in the presence of calcium, two opposing changes may occur simultaneously: first, a heat-induced unfolding of the protein molecule and second, conformational changes caused by the bound calcium *per se* (Klotz, 1950; Imade *et al.*, 1977). Presumably the net result of these two effects on the degree of calcium binding is to keep it almost constant throughout the range of temperatures used.

ACKNOWLEDGEMENTS

We thank Prof. H. Nursten, Dr F. Grimbleby and Prof. C. Polydoropoulos for providing facilities throughout this work and helpful discussions. The first author acknowledges the Greek State Scholarship Foundation for the financial support given to him.

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