results obtained suggested that Mb suffered conformational changes at nonhelical region during freezing and thawing.

ABBREVIATIONS USED

CD, circular dichroism; ΔG_D, free energy for unfolding of a protein; $\Delta G_D^{H_2O}$, free energy for unfolding a protein in the absence of denaturant; Mb, myoglobin; metMb, metmyoglobin; Gdn·HCl, guanidine hydrochloride; $[\theta]_{222}$, mean residue ellipticity at 222 nm.

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Thermally Induced Complex Formation in an Artificial Milk System

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Artificial casein micelles were prepared from their component caseins in a milk salts buffer and mixed with whey proteins prior to heating at 95 °C for 30 s or 20 min. Trace amounts of radiolabeled κ-casein, β-lactoglobulin, α-lactalbumin, or α_{s2}-casein, in various combinations, were included in the mixtures. The heated artificial milk samples were chromatographed on Sephacryl S-300 in a dissociating buffer of 6 M guanidine hydrochloride, and the proportion of the labeled proteins that had formed high molecular weight complexes eluted at the void volume of the column. Analysis of these heat-induced complexes by gel filtration on Sephacryl S-1000 or controlled-pore glass in 6 M guanidine hydrochloride showed that β-lactoglobulin complexed most readily and that α_{s2}-casein was relatively unreactive. When a mixture of α_{s2}-casein and β-lactoglobulin was heated, complexes formed more readily, suggesting that the positioning of α_{s2}-casein within the casein micelle inhibited its reaction with the denaturing β-lactoglobulin or α-lactalbumin. The structure of α_{s2}-casein is likely to be such that its disulfide bond(s) may be less available for reaction than those of κ-casein.

Milk is heat-treated for a number of technologically important effects. This heat treatment brings about a series of protein–protein interactions in the milk. One that was identified in early studies was the formation of a complex between β-lactoglobulin, the major whey protein, and κ-casein, a cysteine-containing protein that generally lies on the surface of the casein micelle. This interaction has been studied extensively, particularly in model systems. While many studies have involved complex formation between β-lactoglobulin and κ-casein, or the loss, by thermal denaturation, of the whey proteins from the serum phase, relatively few have focused on the formation of the large protein complexes in milk. No doubt this is partly because of the complexity of the system. Creamer et al. (1978) isolated a heat-induced protein complex from heated milk that had been dispersed in citrate buffer and showed that its amino acid composition was consistent

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with being a mixture of all the whey proteins and κ-casein. Smits and van Brouwershaven (1980) used electron microscopy to show that the shape of the complexes from milk reported by Davies et al. (1978) and Creamer et al. (1978) could be obtained from the minimum system of β-lactoglobulin and κ-casein in a milk salts buffer heated for 30 min at 85 °C. Recently Creamer (1984) used column chromatography in dissociating buffers (0.1% sodium dodecyl sulfate) to show that the size of the heat-induced complex was dependent on the pH of the milk at the time of heating.

The role of α_{s2}-casein, a cysteine (or cystine) containing protein, in heat stability has been of some interest over the years, mainly on the basis that it contains thiols and is much more calcium sensitive than most other milk proteins (Hoagland et al., 1971; Toma and Nakai, 1973). Both factors would be expected to influence its involvement in heat stability. Snoeren and van der Spek (1977) found that when they isolated κ-casein and α_{s2}-casein from UHT-treated (140 °C, 4 s) milk using an Amberlite CG 50 column, β-lactoglobulin was incorporated into both of the casein fractions, suggesting that β-lactoglobulin had complexed to both of these caseins. Treatment of the heated milk sample with mercaptoethanol, 0.02 M, reversed the observed behavior and β-lactoglobulin eluted with α_{s1}- and β-casein. Kudo (1980), using purified casein fractions, found a negative correlation between α_{s2}-casein concentration and the heat stability of an artificial milk system.

In order to study the role of α_{s2}-casein in the protein complexes formed in heated milk systems, an extension of earlier work using radiolabeled tracers was considered. An excellent quantitative method of tracing proteins through complex chemical reactions is to use radiolabeled proteins. Uniform labeling of carbon or nonlabile hydrogen atom sites seemed desirable. Mills (1976) made uniformly labeled milk protein by infusing uniformly labeled amino acids into the mammary artery of a lactating cow. The specific activity of the recovered proteins was low and the method expensive. Olson et al. (1978) and Rowley et al. (1979, 1982) described the use of reductive methylation with [^{14}C]formaldehyde to give labeled β-lactoglobulin and κ-casein that behaved similarly to the precursor proteins. Koch et al. (1977) compared several methods of introducing ^{3}H into proteins, including high-energy hydrogen exchange. This specialized technique was capable of giving very high activities, but sometimes the protein had a reduced function. These authors concluded that the simplest and least intrusive method of introducing ^{3}H into a protein was by reductive methylation using formaldehyde and sodium cyanoborohydride. Consequently both ^{14}C and ^{3}H labeled proteins were prepared by the reductive methylation procedure for use in the present study.

A major difficulty in using radiolabeled α_{s2}-casein is to incorporate it into the casein micelle. If α_{s2}-casein were simply added to milk, it is likely to coat itself onto the surface of the micelle, whereas its natural position is in the micelle interior. To try to overcome this difficulty, it was necessary to make the casein micelles from proteins and minerals as described by Schmidt et al. (1977). They explored a number of artificial micelle systems and showed that many of them were very similar to the natural micelle system (Schmidt and Koops, 1977; Schmidt et al., 1977). One of these systems was chosen for use in the present study.

MATERIALS AND METHODS

Milk Proteins. A sample of freshly drawn raw milk was obtained from the bulk tank in the Dairy Barn, University of California, Davis, and skimmed by centrifugation at 4000*g* for 30 min. Addition of 1 M HCl to the skim milk at 20 °C to pH 4.6 precipitated the casein. The supernatant whey fraction was dialyzed extensively against deionized, distilled water, clarified, and freeze-dried for later use.

The casein fraction was washed in water, dissolved by addition of 1 M NaOH solution, with stirring and taking care that the pH did not exceed 7.0, reprecipitated with HCl, washed, freeze-dried, and stored at −20 °C.

κ-Casein was prepared by the method of Zittle and Custer (1963). The α_s-fraction of casein was prepared by the method of Thompson and Kiddy (1964) and was used to prepare α_{s2}-casein by ion-exchange chromatography (Mercier et al., 1968) followed by propyl alcohol fractionation as described by Kudo (1980). The purification was followed using polyacrylamide gel electrophoresis in sodium dodecyl sulfate or urea buffers (Laemmli, 1980; Thompson et al., 1964).

α_{s1}-Casein was prepared from the above α_s fraction by the method of Mercier et al. (1968).

α-Lactalbumin was purified by gel filtration chromatography of the whey protein fraction of Sephadex G-100 followed by ion-exchange chromatography (Quarfoth and Jenness, 1975).

Some samples of κ-casein and the samples of β-casein and β-lactoglobulin were purchased from Sigma. Only those samples of β-lactoglobulin that gave single peaks on gel chromatography in Sephacryl S-300 in 6 M guanidine hydrochloride were used.

Radiolabeling of the Proteins. Earlier procedures (Jentoft and Dearborn, 1979; Dottavio-Martin and Ravel, 1978) were followed for ^{14}C labeling. Typically 6 mg of protein was dissolved in 0.5 mL of a 0.02 M phosphate buffer adjusted to pH 6.58 at room temperature. It was then reacted with 500 μCi (24 μCi/μmol) of formaldehyde solution followed by 1 mL of a 6 mg/mL solution of $NaCNBH_3$ dissolved in a phosphate buffer. The solution was then dialyzed against distilled water, freeze-dried, and stored at −20 °C.

The procedure of Kraal and Hartley (1978) was followed for ^{3}H labeling of the proteins. Formaldehyde solution (30 μL of 37%) was added to a solution of protein (6 mg in 2 mL of phosphate buffer), and after thorough mixing, 1 mL of a phosphate buffer solution containing 5 mCi of [^{3}H]$NaCNBH_3$ was added to the mixture and reaction continued for 1 hr. The process was repeated with further additions of formaldehyde and [^{3}H]$NaCNBH_3$. The protein was separated from low molecular weight compounds by gel filtration chromatography on disposable 1.5 × 15 cm columns of Sephadex G-25, by collecting the void volume material and then sealing and discarding the column and contents. The protein solution was then dialyzed against distilled water, freeze-dried, and stored at −20 °C.

Artificial Milk. Artificial casein micelles were prepared in a milk salts buffer as described by Schmidt et al. (1977) with a ratio of 2.5:1.0:0.5:0.5 for α_{s1}-casein:β-casein:κ-casein:α_{s2}-casein. This ratio of 3:1 for the α_s- to β-caseins was reported to give casein micelles with structures and stabilities close to those of the natural micelles (Schmidt et al., 1977; Schmidt and Koops, 1977). The radioactive caseins were introduced as part of the casein mixture. Once the artificial casein micelles had been prepared, freeze-dried whey protein (94.5 mg) with a small addition of a labeled whey protein (e.g., 0.136 mg of β-lactoglobulin, which corresponds to 0.144% of the added whey protein) was added to the artificial casein micelle suspension (15 mL). After equilibration for at least 2 h at 37 °C, pH 6.58, three 1-mL aliquots of the milk were each heated in a thin-walled tube (made by sealing off a Pasteur pipet) in a water bath controlled at 96.5 °C, a temperature that had been found to heat the tube contents to 95 °C. Temperature was maintained at 95 ± 0.3 °C with the aid of a data logger. After the samples were heated for 30 s or 20 min, the tubes were cooled in ice water and each sample was withdrawn, mixed with 9 mg of potassium citrate, and after 1 h, mixed with sufficient solid guanidine hydrochloride to give a final concentration of 6 M. Aliquots of the dispersed milk samples were separated by gel filtration chromatography on a column of Sephacryl S-300 (Pharmacia) in guanidine hydrochloride, 0.1 M KH_2PO_4 adjusted to pH 6.58, and 4-mL fractions collected. The radioactivity of each fraction was determined on a Beckman LS7500 liquid scintillation counter and converted to dpm (disintegrations per minute) using quench correction curves and solving the simultaneous equations involved.

Table I. Percentage of Each of the Radiolabeled Proteins Found in the Heat-Induced Complex

trial	time at 95 °C	figure	protein	contribn,[a] %
1	20 min	1A	[^{14}C]-β-lactoglobulin	90–94
2	20 min	2B	[^{14}C]-α-lactalbumin	30
3	30 s	4A	[^{14}C]-β-lactoglobulin	46
3	30 s	4A	[^{3}H]-α_{s2}-casein	4.5
4	20 min	4B	[^{14}C]-β-lactoglobulin	93
4	20 min	4B	[^{3}H]-α_{s2}-casein	6
5	20 min	6B	[^{14}C]-α-lactalbumin	34
5	20 min	6B	[^{3}H]-α_{s2}-casein	5
6	20 min		[^{14}C]-α_{s2}-casein	0
7[b]	20 min		[^{14}C]-β-lactoglobulin	90
7[b]	20 min		[^{3}H]-α-lactalbumin	42

[a] Calculated as the ratio of ^{3}H or ^{14}C radioactivity in the void volume peak to the total ^{3}H or ^{14}C activity in the column effluents. [b] Neutralized whole acid casein was made into artificial micelles and mixed with whey proteins to give an artificial milk.

Analysis of the heat-induced complex was carried out with use of columns of controlled-pore glass (1.5 × 105 cm) or Sephacryl S-1000 (2.5 × 37.5 cm) as described above. The void volume of these columns was determined with a small volume of homogenized milk.

RESULTS AND DISCUSSION

The specific activities of the radiolabeled proteins were high and were typically 5 × 10^8 dpm/mg for ^{3}H-labeled and 2.5 × 10^7 dpm/mg for ^{14}C-labeled proteins. This compares with an activity of 2.5 kBq/mg (1.5 × 10^5 dpm/mg) for [^{3}H]-β-lactoglobulin produced (Smits and van Brouwershaven, 1980) by the method of Koch et al. (1977). Thus, it was possible to obtain accurate radioactivity measurements with additions of radiolabeled protein of the order of 0.01–0.2 mg of protein/15 mL of artificial milk, corresponding to less than 0.5% of the original protein.

The artificial milks were tested for the efficiency of incorporation of α_{s2}-casein into the micelles by centrifugation of the micelle suspension. There was only 4% of the radioactivity present in the supernatant. Dialysis of the supernatant showed that most of this activity was in the high molecular weight fraction. Thus, only a small proportion of the α_{s2}-casein was not in the form of normal-size micelles.

In a preliminary experiment, an artificial milk containing [^{14}C]-β-lactoglobulin and [^{3}H]-κ-casein was heated for 20 min at 95 °C, dispersed in 6 M guanidine hydrochloride, and separated by gel filtration chromatography on a column of Sephacryl S-300 to give the pattern shown in Figure 1A. Both radioactivities were eluted largely at the void volume of the column, showing that both proteins were incorporated into high molecular weight complexes (exclusion ca. 5 × 10^5 Da). The extent of incorporation of the β-lactoglobulin into the heat-induced complex that eluted at the void volume is shown in Table I. Gel filtration chromatography of the void volume fraction on a column of controlled-pore glass in 6 M guanidine hydrochloride gave the pattern shown in Figure 1B. The molar ratios of the proteins eluted from the controlled-pore glass column, Figure 1B, are shown in Figure 1C. Clearly the larger, and earlier eluting, complexes contain a greater proportion of β-lactoglobulin. These results are qualitatively similar to those obtained by Richardson and Noh (1988) with natural milk or with a system containing purified β-lactoglobulin and κ-casein as the only proteins (Noh et al., 1989).

Artificial milk systems with various paired combinations of ^{14}C- and ^{3}H-labeled κ-casein, β-lactoglobulin, α_{s2}-casein, and α-lactalbumin were prepared and heated for 30 s or

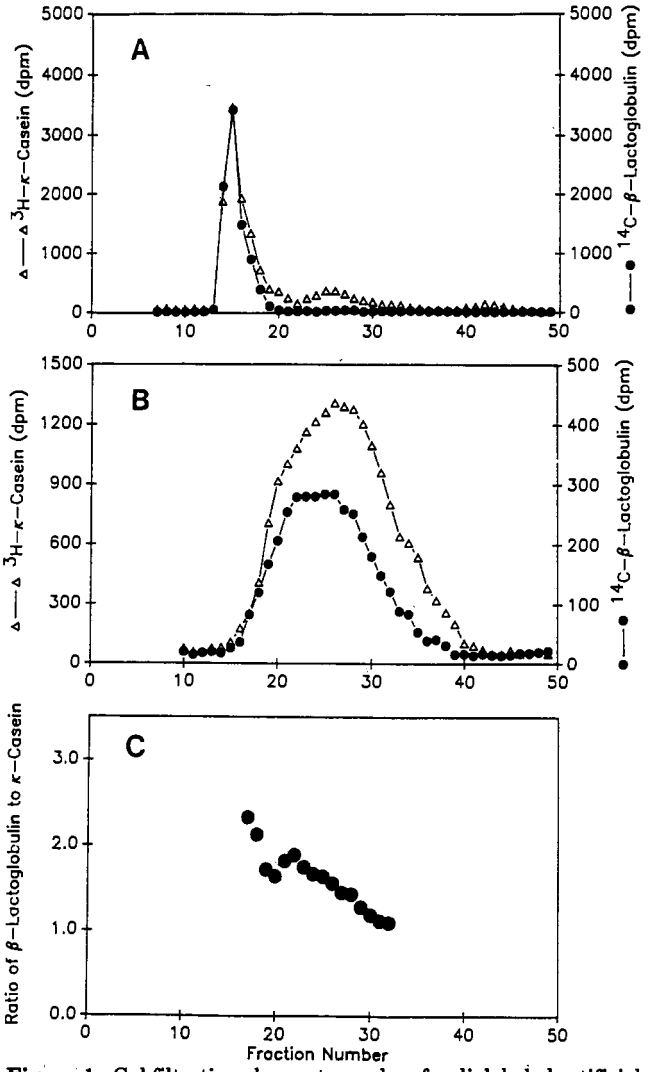

Figure 1. Gel filtration chromatography of radiolabeled artificial milk that had been heated at 95 °C for 20 min. Key: (A) Separation of heated milk on a 37.5 × 2.5 cm column of Sephacryl S-300 in a phosphate buffer (pH 6.58) in 6 M guanidine hydrochloride. The flow rate was 20 mL/h, and the fraction size was 4 mL. (B) Gel filtration chromatography of a portion of the mixture of fractions 14 and 15 of Figure 1A on a 105 × 1.5 cm column of controlled-pore glass, pore size 300 nm. The flow rate was 20 mL/h, and the fraction size was 4 mL. (C) Diagram of the molar ratio of β-lactoglobulin to κ-casein across the major peak of Figure 1B.

20 min at 95 °C and analyzed by gel filtration chromatography on Sephacryl S-300. The void volume fractions were often analyzed further on a column of Sephacryl S-1000 to determine the distribution of the proteins across the various sizes of the heat-induced complex. Figure 2 shows the results obtained when radiolabeled α-lactalbumin and κ-casein were included in the mixture. Most of the κ-casein eluted at the void volume (Figure 2), and its pattern did not change with the extent of the heat treatment (cf. Figure 2A,B). For α-lactalbumin the quantity eluting at the void volume increased with heat treatment. Figure 3 shows the results from the Sephacryl S-1000 analysis of the void volume peak from Figure 2B. In general outline it is similar to Figure 1B, with the larger aggregates containing a greater proportion of the whey protein.

Figure 4 shows the results of including radiolabeled α_{s2}-casein and β-lactoglobulin in the mixture. The quantity of β-lactoglobulin included in the void volume fraction increased with heat treatment (cf. Figure 4A,B) while the

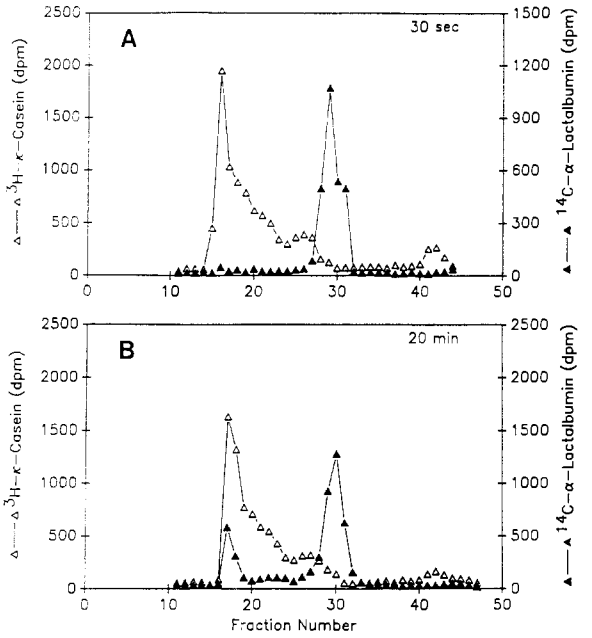

Figure 2. Gel filtration chromatography of heated milk samples containing small substitutions of [^{3}H]-κ-casein and [^{14}C]-α-lactalbumin. Conditions as in Figure 1A. Key: (A) heating at 95 °C for 30 s; (B) heating at 95 °C for 20 min.

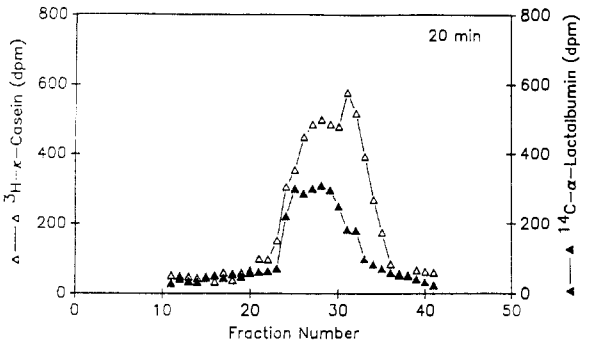

Figure 3. Gel filtration chromatography of a portion of the heat-induced complex (a mixture of fractions 17 and 18 of Figure 2B) separated from heated artificial milk containing small quantities of [^{3}H]-κ-casein and [^{14}C]-α-lactalbumin. A column (37.5 × 2.5 cm) of Sephacryl S-1000 was eluted with a 6 M guanidine hydrochloride buffer at 20 mL/h. Fractions of 4 mL were collected.

change in α_{s2}-casein was less (Table I). The further analysis of the void volume fraction is shown in Figure 5. In an attempt to force a greater degree of interaction, a system containing only α_{s2}-casein and β-lactoglobulin was heated for 20 min at 95 °C. The results (Figure 4C) show that the quantity of β-lactoglobulin eluting at the void volume was less than in the complete system (Figure 4B) but the molar ratio of α_{s2}-casein to β-lactoglobulin was greater. This shows that, in the absence of κ-casein, α_{s2}-casein will react with the denaturing β-lactoglobulin to form large complexes, as well as some smaller ones. It is likely that the placement of α_{s2}-casein in a central region of the casein micelle either in the natural milk system or in the artificial micelles inhibited its interaction with the whey proteins. In one instance (Table I, experiment 6) a sample of α_{s2}-casein did not react to form high molecular weight complexes. The low reactivity of α_{s2}-casein is of interest because, at first sight, it might be expected to have a reactivity similar to that of κ-casein. However, κ-casein is designed to form a coat of S–S-linked protein on the micelle surface, but α_{s2}-casein does not have this function so its thiols may not be so accessible for reaction. It is

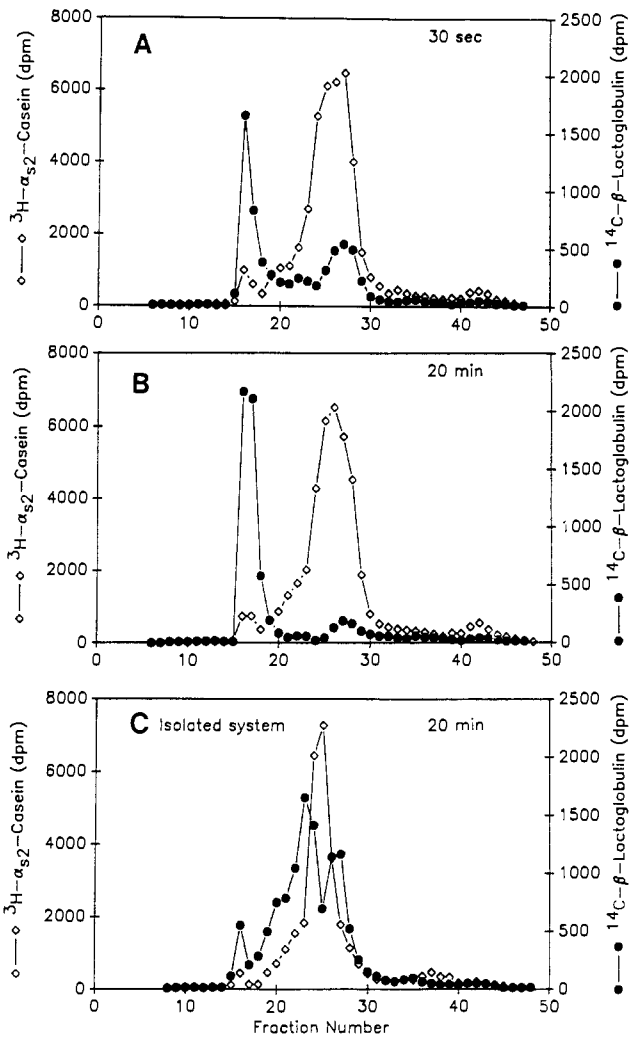

Figure 4. Gel filtration chromatography of heated artificial milk samples containing small quantities of [^{3}H]-α_{s2}-casein and [^{14}C]-β-lactoglobulin. The chromatographic conditions were similar to those described in the caption to Figure 1A. Key: (A) sample heated at 95 °C for 30 s; (B) sample heated at 95 °C for 20 min; (C) sample of α_{s2}-casein and β-lactoglobulin in artificial milk salt system heated at 95 °C for 20 min.

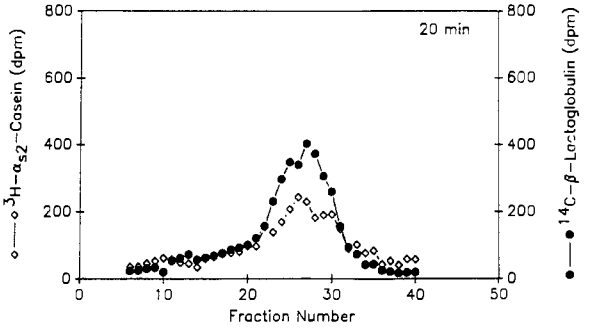

Figure 5. Gel filtration chromatography of a portion of the heat-induced complex (a mixture of fractions 14 and 15 of Figure 4B) separated from heated artificial milk containing small quantities of [^{3}H]-α_{s2}-casein and [^{14}C]-β-lactoglobulin. A column (37.5 × 2.5 cm) of Sephacryl S-1000 was eluted with a 6 M guanidine hydrochloride buffer at 20 mL/h. Fractions of 4 mL were collected.

likely to have a conformation such that the two thiol groups, that are only five residues apart, are oxidized to form an internal disulfide or to give the dimer (Toma and Nakai, 1973; Hoagland et al., 1971). The absence of κ-casein from the isolated system was likely to have been the most important factor in determining the amount of β-

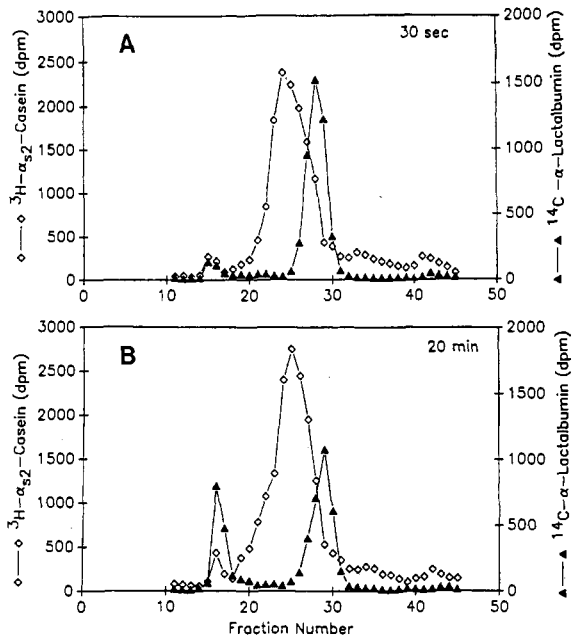

Figure 6. Gel filtration chromatography of heated artificial milk samples containing small quantities of [^{3}H]-α_{s2}-casein and [^{14}C]-α-lactalbumin. The chromatographic conditions were similar to those described in the caption to Figure 1A. Key: (A) sample heated at 95 °C for 30 s; (B) sample heated at 95 °C for 20 min.

lactoglobulin that eluted at the void volume of the Sephacryl S-300 chromatogram (Figure 4C).

The results from heated milk that included radiolabeled α-lactalbumin and α_{s2}-casein in the mixture are shown in Figure 6 and Table I. The behavior of these proteins was essentially that expected on the basis of the results obtained earlier (Figures 1, 2, and 4; Table I). The conclusion that can be drawn from the present study, that α_{s2}-casein is not very reactive and is only incorporated into the heat-induced complex to a limited extent, is not compatible with that of Snoeren and van der Spek (1977). There are two possible explanations: First, heating milk at 140 °C for 4 s, as done by Snoeren and van der Spek (1977), invokes a significantly different reaction pathway from that occurring in milk heated at 95 °C. Second, the analytical methods used may have given artifactual results. It is barely conceivable that some of a κ-casein–β-lactoglobulin complexes eluted similarly to the α_{s2}-casein in the stepwise elution of the CG 50 column. This apparent disparity could probably be resolved by experimentation.

The sensitivity of the proteins to heat appeared to be in the order β-lactoglobulin (Figures 1 and 4) > α-lactalbumin (Figures 2 and 6) > α_{s2}-casein (Figures 4 and 6) > κ-casein (Figures 1 and 2). Normal polymeric κ-casein cannot be distinguished from the heat-induced complex, because they both elute at the void volume of the Sephacryl S-300 column. Consequently, no data showing the extent of involvement of κ-casein can be presented. Clearly, β-lactoglobulin is almost totally incorporated into the heat-induced complex after 20-min heating while about 50% (Figure 4A) is converted after 30-s heating. In an earlier study Richardson and Noh (1988) showed that in skim milk about 60 and 90% of the β-lactoglobulin became associated with the casein micelles after 30-s and 20-min heating at 95 °C, respectively. These results are comparable with the present data. Smits and van Brouwershaven (1980) obtained similar results from measurements of the incorporation of [^{3}H]-β-lactoglobulin into an artificial milk, attaining about 80% incorporation after 30-min heating at 90 °C. α-Lactalbumin is less reactive, and about 35% was complexed after 20-min heating (Table I). Both Dannenberg and Kessler (1988) and Lyster (1970) found that more of each whey protein was denatured after heating at 95 °C for 20 min. One likely reason for the marked differences in the results obtained by the different groups is that some of the data refer to measurements of the quantities of undenatured β-lactoglobulin or α-lactalbumin in the heated milk systems (Lyster, 1970; Hillier and Lyster, 1979; Dannenberg and Kessler, 1988), while the present study [and that of Smits and van Brouwershaven (1980)] determined the quantities of high molecular weight complex formed in the heated milks. However, the results of the various groups are compatible if the reaction sequence in heated milk is that the proteins denature, form small complexes (not detected as undenatured proteins nor as large denatured complexes), and then become integrated into the more extensively cross-linked complexes that are centered on κ-casein polymers.

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Changes in Carbohydrate and Protein Content and Composition of Developing Almond Seeds

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Water-soluble sugars, sugar alcohols, cell wall constituents, protein, total amino acids, and free amino acids were determined in developing almond kernels (*Prunus amygdalus*) at 3-week intervals from March until harvest. A sharp drop of sugars (from 60 to 5–6% dry weight) was observed in a first stage. Reducing sugars decreased from initial high percentages to a final trace level. Changes in hemicellulose, cellulose, and lignin were also studied; the results suggest that these constituents are employed as a carbohydrate source during a period. Protein content increased steadily up to harvest, more markedly during a 20-day period. Significant variations of amino acid content were detected. The content of free amino acids is considerable in developing almonds, while ripe kernels contain insignificant amounts.

The changes in oil content and fatty acid composition of almond kernels during development to maturity were previously reported (Munshi et al., 1982; Munshi and Sukhija, 1984; Soler et al., 1988). Oil is the major fraction of this fruit (50–65% dry weight).

In this second and final paper on the evolution of the main organic constituents during active growth of the almonds, we report the protein and carbohydrate changes.

The content and composition of these fractions in ripe almond are well-known. Water-soluble sugars range from 3 to 8% dry weight; sucrose and raffinose are the main constituents (Vidal Valverde et al., 1979; Saura-Calixto et al., 1984). The content of cell wall components, hemicellulose, cellulose, and lignin, varies between 3 and 6% dry weight (Saura-Calixto et al., 1983; Lopez-Andreu et al., 1985).

On the other hand, the protein fraction (18–25% dry weight) contains a high percentage of essential amino acids, and lysine is the limiting amino acid. The free amino acid content is very low (Nassar et al., 1977; Riquelme, 1982; Saura-Calixto et al., 1982).

The study of composition changes in developing almond should provide information useful for understanding the physiological and morphological processes throughout maturation. This is the objective of the present work.

EXPERIMENTAL SECTION

Sampling. The samples used corresponded to the Pons variety, cultivated on the Spanish island of Mallorca. Samples were collected from 20 selected trees at approximately 3-week intervals, from March to September. The weight of one kernel was determined as an average of the weight of 30 kernels.

To quantitate the data, fruit set (time 0) was conventionally considered when the fruits presented the following dimensions: length 1.1 cm, width 0.9 cm, thickness 1.0 cm.

After hulls, shells, and tegument were removed, the fruits were dried and homogenized. The oil was extracted with diethyl ether on a Soxhlet extractor over 18 h. The defatted samples used for analyses are described below.

Analytical Procedures. *Soluble Sugars.* Soluble sugars were determined on extracts of 80% ethanol. Munson-Walker (Lee, 1978) and Haas (Snell and Ettre, 1973) methods were employed to quantify reducing and total sugars, respectively.

Sugar and sugar-alcohol composition was determined by gas–liquid chromatography. The solvent was removed by vacuum distillation to yield dry residues. The procedure of Sweeley et

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