

SYMPOSIUM: PRIMARY SEQUENCE OF THE CASEINS

Primary Sequence of Beta, Gamma, and Minor Caseins¹

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This report describes research of many investigators. The primary sequence of β -casein was worked out at the Institut National de la Recherche Agronomique, Jouy-en-Josas, France, by Ribadeau-Dumas, Brignon, Grosclaude, and Mercier. The isolation of γ -casein, TS-, R- and S-caseins was accomplished at the Eastern Regional Research Center by Groves et al. The hypothesis that these might be fragments of β -casein was formulated on experimental results by Gordon, Groves, Greenberg, Jones, Kalan, Peterson, and Townend, together with some information in the early publications on β -casein from Jouy.

Beta Casein: Early Research

A brief review of early important developments in the chemistry of β - and γ -caseins might begin with the observation of Osborne and Wakeman in 1918 that a small portion of acid-precipitated casein is soluble in 50% ethanol (25). The soluble protein contained only .1% P compared to .85% for unfractionated casein. These pioneers in fractionation of casein probably prepared something similar to what was later called γ -casein. That casein is a mixture of proteins was indicated by the solubility experiments of Linderström-Lang in the late 1920's (19, 20). Mellander (22) designated the major components of acid-precipitated casein in 1939 as α -, β -, and γ -caseins after his study of protein mobility in the newly developed electrophoresis apparatus of Tiselius.

At the Eastern Regional Research Center, research on casein began in the early 40's. Warner reported a breakthrough in the chemical fractionation of casein in 1944 (36). He exploited differences in solubility of α - and β -caseins in dilute aqueous solutions at 4 C and at slightly different pH values to achieve separation of the two major components. In the

early 50's Hipp et al. (15, 16) published two important methods for preparing α -, β -, and γ -caseins. Differential solubility in 50% ethanol and concentrated urea solutions were the keys to successful separations.

These early preparations of β - and γ -caseins were considered reasonably pure proteins. Both contained P: beta .6%, and gamma .1%. Amino acid analysis by our group showed their different compositions to be characteristically so because samples of each prepared by different methods gave the same analyses. Measurement of molecular weight of β -casein in the ultracentrifuge by Sullivan et al. in 1955 yielded 24,100 (34). This is almost the same as that calculated from the primary sequence; 23,982 (30). Other investigators estimated that γ -casein was a somewhat larger molecule, of approximately 30,000 daltons (24).

By the late 50's it appeared that β -casein was sufficiently pure for structural studies which were undertaken at our laboratory. In 1958 Peterson et al. described the isolation and composition of a polypeptide from a tryptic digest of β -casein (28). This consisted of 24 amino acid residues and was reported to contain all the P in β -casein, 5 atoms per molecule. Determination that the N-terminal amino acid was arginine, the same residue believed to be N-terminal in β -casein, suggested that the peptide was the N-terminal fragment of β -casein. We know now from the recent work of Manson and Annan (21) in Scotland and from the group at Jouy (29), that this tryptic phosphopeptide is the N-terminal fragment, that it has 25 amino acid residues, that it contains only 4 of the 5 P atoms, and that it has arginyl residues at both ends.

Continuing research on β -casein by Peterson was complicated by the heterogeneity of some peptides prepared from the tryptic digests. Aschaffenburg and Drewry's discovery in 1955 of genetic polymorphism in the β -lactoglobulins (2, 3), and Aschaffenburg's evidence (1) for at least three forms of β -casein (A, B, and C), initiated a new era of research in the chemistry of milk proteins. In the case of β -casein the situation was not completely clarified by Aschaffenburg's observations. Peterson and Kopfler (26) demonstrated in 1966 that

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multiple forms of β -casein A could be detected by electrophoresis in acid rather than in alkaline gels. This was an important development. We recognize now that at least five polymorphs of β -casein can be obtained from milks of Western breeds of cattle; they are known as β -caseins A¹, A², A³, B, and C. Other variants have been isolated from milks of Zebu cattle (35) but these will not be discussed here.

Compositional studies of the five Western β -caseins were undertaken at the Eastern Laboratory, at Jouy in France, and at Ede in The Netherlands. Gradually a consensus emerged regarding amino acid composition, content of P, and distinguishing amino acid substitutions. The C variant contained only four rather than five atoms of P per molecule. In all variants, the N-terminal group was arginyl followed by glutamic acid, and the C-terminal acid was valine, preceded by two isoleucyl residues. Peptide maps were similar in most respects. Thus, it was accepted that β -casein consisted of a single polypeptide chain containing 208 amino acid residues.

And then began the elegant research of Ribadeau-Dumas and his colleagues at Jouy. In 1970 the first of a series of papers described the isolation and composition of peptides from β -casein A² following treatment of the protein with trypsin, and also following specific cleavage of the polypeptide chain by cyanogen bromide at the six methionine residues (31). Fourteen tryptic peptides numbered T1 through T14, and seven cyanogen bromide peptides designated CN1 through CN7 were analyzed. Peptide T1 had virtually the same composition as the phosphopeptide isolated by Peterson et al. (28). As with Peterson's peptide, arginine was N-terminal, and T1 was considered to be the N-terminal fragment of β -casein. Similarly, peptide CN1, somewhat larger than T1, contained N-terminal arginine; it also was positioned at the N-terminus. The sum of all amino acid residues in each series of isolated peptides was 208, in agreement with previously published analyses. These results yielded a molecular weight close to 24,000 for the whole β -casein molecule (31).

Gamma Casein: Early Research

Before further discussion of the work on β -casein at Jouy, developments in the chemistry of γ -casein which occurred in the 60's will be reviewed. Groves et al. demonstrated in 1962 by starch-gel electrophoresis that γ -casein prepared by older methods was heterogeneous (13). In the same paper they described isola-

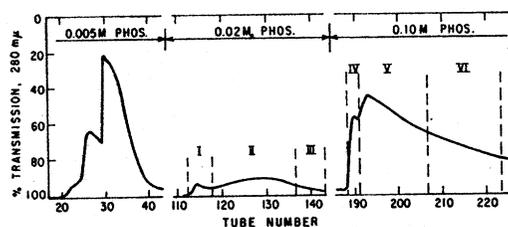


FIG. 1. Stepwise chromatography (pH 8.3) of casein (12 g dissolved in 5mM phosphate) on DEAE-cellulose column, 3.8 cm \times 50 cm, equilibrated with 5mM phosphate. The TS fraction is eluted with the 5mM phosphate starting buffer, γ -casein fractions with .02M phosphate and β -casein fractions with .10M phosphate.

tion of much purer γ -casein by column chromatography on DEAE-cellulose of an acid extract of casein. Later, it was shown that whole casein could also be used as starting material (8). In either case, a first fraction, designated "temperature-sensitive" because it is much less soluble at 25 than at 3C, is eluted in the starting buffer, 5mM phosphate, pH 8.3. A second fraction is obtained with .02M phosphate, and from this, by rechromatography, a pure γ -casein can be made. In properties and composition the pure γ -casein resembled the older, heterogeneous γ -caseins prepared by chemical fractionation; therefore, the name γ -casein was retained. Fig. 1 illustrates the chromatographic separation of the first three crude fractions obtained from casein by this method (8). In the following years TS-, R-, and S-caseins were isolated from the first (temperature-sensitive) fraction.

In the early 60's Aschaffenburg published evidence from paper electrophoresis experiments that γ -casein, like β -casein, might also be polymorphic (1). This idea received support from El-Negoumy (5), and in 1968 Groves and Kiddy confirmed two variants of γ -casein, A and B by disc gel electrophoresis at alkaline pH values (11). Subsequently, using disc gel electrophoresis at acid pH, Groves and Kiddy were able to distinguish four polymorphs of γ -casein. In milk samples from single animals the type of γ -casein was linked genetically to the type of β -casein. Thus, in milks containing β -caseins A¹, A², A³, or B, the corresponding γ -caseins were named similarly. However, in milk typed β -casein C, no γ -casein could be detected. Fig. 2 shows the different mobilities of the purified β - and γ -polymorphs in gels at pH 4.3 (12). Compositional analyses indicated that the same amino acid substitu-

TABLE 1. Possible amino acid substitutions common to γ - and β -caseins. (9, 17, 27).

Comparison	Substitution
A ¹ -A ²	His/Pro
A ¹ -A ³	His/Gln, His/Pro
A ¹ -B	Ser/Arg
A ² -A ³	His/Gln
A ² -B	Pro/His, Ser/Arg
A ³ -B	Ser/Arg, Pro/His, Gln/His

tions which differentiated the β -casein polymorphs also distinguished the corresponding γ -casein variants. The inferred substitutions are listed in Table 1. Like the β -caseins, the γ -caseins lack cystine (8, 9).

β -Casein C is not included in Table 1 because there is no γ -casein C. β -C differs from the other β -caseins in that it has an additional lysine residue substituted for glutamic acid. This is an important difference possibly associated with the lower phosphorus content of β -C. We shall return to this possibility later.

Each γ -casein contains one atom of P and one residue of tryptophan per molecule. Lysine is N-terminal and the C-terminal sequence is isoleucyl-isoleucyl-valine in each (9). This is the same C-terminal tripeptide in the β -caseins

Minor Caseins

While the work on γ -caseins at our laboratory was in progress, other proteins had been isolated in small amounts from the first temperature-sensitive fraction obtained by DEAE-chromatography of casein. From casein typed β - or γ -A², proteins named R-casein and TS-A² casein were isolated; from casein typed β - or γ -B, similar proteins called S- and TS-B caseins were separated. The names were chosen for convenience, but the TS designation indicates higher solubility in aqueous solution with decrease in temperature (8). From disc gel electrophoretic patterns of these newly isolated proteins, it became evident that their occurrence was related genetically to the type of β -, γ -casein in the whole casein. For example, R- and TS-A² caseins were never found in homozygous milks typed β -, γ -casein B; nor were S- and TS-B caseins ever found associated with β -, γ -casein A². These relationships are illustrated in Fig. 3, and gel patterns of the purified proteins are in Fig. 4 (10).

The chromatographic behavior and electrophoretic properties of these proteins led us to

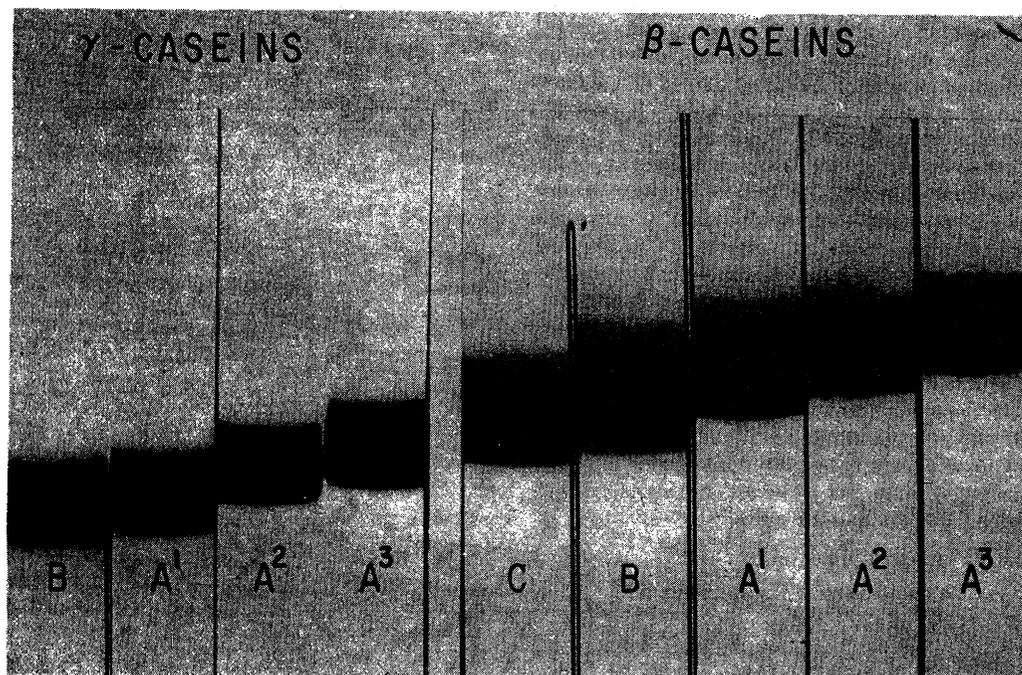


FIG. 2. Disc gel electrophoretic patterns, pH 4.3, 8M urea of γ -, β -casein polymorphs B, A¹, A², A³ and β -casein C. Migration is downward toward the cathode.

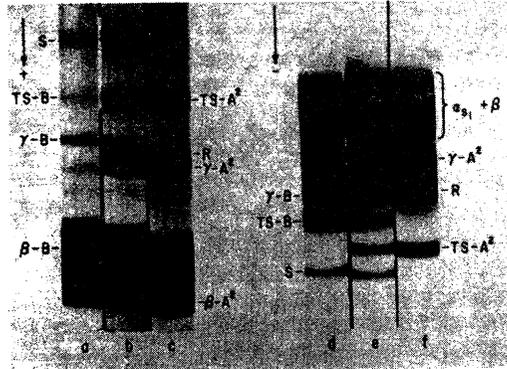


FIG. 3. Disc gel electrophoretic patterns of caseins typed γ -, β -caseins B, A² B, and A². Gels a, b, and c were run at pH 9.6, 4M urea and d, e, and f at pH 4.3, 8M urea. Gels a, d, represent the homozygous casein B; gels b, e, represent the heterozygous casein A² B; and gels c, f, represent the homozygous casein A². For the gels a, b, and c the α_1 -casein, with a mobility greater than that of β -casein, is not seen. κ -casein remains at the origin (not shown).

believe that TS-A² and TS-B made up one pair of polymorphs, and that R- and S-caseins were another pair. However, the results of compositional analyses, estimations of molecular weights, peptide mapping, and determinations of end-groups proved the pairings were wrong. Actually, TS-A² and S-caseins are polymorphic, differing only in a single amino acid substitution, arginine for serine; their N-terminal amino acid is histidine. Similarly, TS-B and R-caseins differ by the same substitution, arginine for serine, but their N-terminal amino acid is glutamic acid. All four are of about the same molecular weight, about 12,000 daltons. All have the same C-terminal tripeptide sequence, isoleucyl-isoleucyl-valine, common to the β - and γ -caseins. All have exactly the same amino acid composition except for the arginine-serine substitution and except that TS-A² and S-caseins are slightly larger molecules, containing one more histidine plus one more lysine residues than the other pair. All have one tryptophan per molecule, but none has any phosphorus or cystine (10).

TABLE 2. Comparison of amino acids in β - and γ -caseins.

	γ -A ² = β -A ² - tryptic peptides 1+9																
β -A ²	Lys ₁₁	His ₈	Arg ₁	Asp ₉	Thr ₅	Ser ₁₅	Glu ₂₉	Pro ₃₄	Gly ₅	Ala ₅	Val ₁₀	Met ₆	Ile ₁₀	Leu ₂₂	Tyr ₁	Phe ₉	Trp ₁
T1+9	Lys ₁		Arg ₂	Asp ₂	Thr ₁	Ser ₄	Glu ₇	Pro ₁	Gly ₁		Val ₂		Ile ₃	Leu ₃			
γ -A ²	Lys ₁₀	His ₈	Arg ₂	Asp ₇	Thr ₃	Ser ₁₁	Glu ₂₂	Pro ₃₃	Gly ₄	Ala ₅	Val ₁₇	Met ₆	Ile ₇	Leu ₁₉	Tyr ₁	Phe ₉	Trp ₁

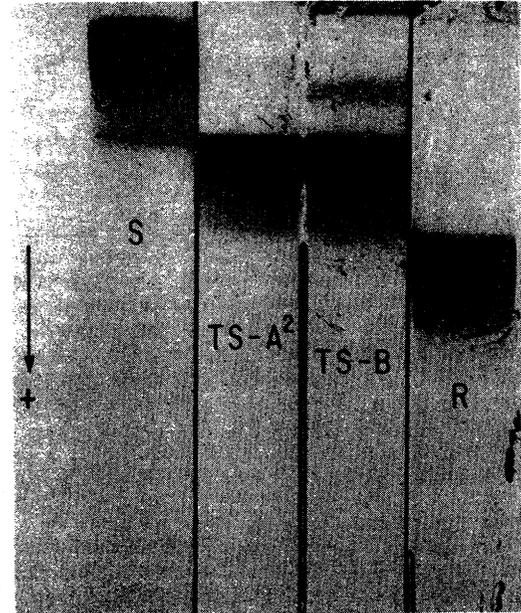


FIG. 4. Disc gel electrophoretic patterns of the purified caseins, TS-A², TS-B, R, and S caseins, at pH 9.6, 4M urea.

Molecular weights of the TS-, R-, and S-caseins were estimated by measurements of sedimentation-equilibrium in the ultracentrifuge (14) and also by gel electrophoresis in the presence of sodium dodecyl sulfate (9). Direct comparisons under identical conditions by both methods were made also of the molecular weights of β - and γ -caseins. In every comparison γ -casein was smaller than β -casein, not larger as previously believed (9).

Sequencing Caseins

This brings us back to the appearance in 1970 of the first paper on the structure of β -casein from Jouy (31). At that time we were revising our amino acid composition data for γ -casein to reflect its lower molecular weight; 20,000 rather than 25,000. Much of the data reviewed to this point generated the idea that γ -casein could be partially dephosphorylated

TABLE 3. Comparison of amino acids in β -, TS-A² and R caseins.

		R casein = β -A ² - CnBr peptides 1+5+6															
β -A ²	Lys ₁₁	His ₅	Arg ₄	Asp ₉	Thr ₅	Ser ₁₅	Glu ₃₉	Pro ₂₄	Gly ₅	Ala ₅	Val ₁₀	Met ₆	Ile ₁₀	Leu ₂₂	Tyr ₄	Phe ₃	Trp ₁
CN 1+5+6	Lys ₈	His ₂	Arg ₂	Asp ₆	Thr ₁	Ser ₃	Glu ₂₅	Pro ₁₄	Gly ₂	Ala ₃	Val ₆	Met ₃	Ile ₇	Leu ₈	Tyr ₁	Phe ₁	
R	Lys ₃	His ₃	Arg ₂	Asp ₃	Thr ₄	Ser ₁	Glu ₁₅	Pro ₂₀	Gly ₂	Ala ₂	Val ₁₀	Met ₄	Ile ₃	Leu ₁₄	Tyr ₃	Phe ₂	Trp ₁

* TS-A² = R + Lys₈ + His₁.

β -casein. Might it, in fact, be a part of β -casein? Could R-, S-, and TS-caseins be pieces of γ -casein?

The following comparisons were made. If the amino acids in the phosphopeptide portion of β -casein, that is, peptide T1 in the Ribadeau-Dumas et al. paper (31), were subtracted from the total number of amino acids in β -casein, something similar in composition to γ -casein remained. Another tryptic peptide T9, a tripeptide, was of the right composition to account for the small difference. Thus, if T1, known to be N-terminal in β -casein, were followed in the sequence by T9, the remaining portion of the molecule had exactly the composition of γ -casein including the one atom of P, the one residue of tryptophan, the same amino acid substitutions in the polymorphs, and the same C-terminal tripeptide. Table 2 shows the comparison.

Similarly, from the amino acid composition of the cyanogen bromide peptides described in the same paper, CN1 was a considerably larger N-terminal fragment which included T1 and possibly T9, and accounted for 92 amino acid residues. If the 9 amino acids in CN5 and the 7 amino acids in CN6 were added to those in CN1 and the summed amino acids were subtracted from those in β -casein, there were left about 100 residues representing almost exactly the composition of R-casein (Table 3). R-

casein is larger than the remainder by one glutamic acid and one methionine residue. This was explained when the N-terminal sequence of R-casein was found to be glutamyl-methionyl. Cleavage of the chain is expected at the carbonyl group of the methionyl residue. As already mentioned, TS-A² casein is larger than R-casein by two additional amino acid residues. Comparisons of analytical data in Tables 2 and 3 gave support to the idea that TS-A² and R-caseins might be parts of γ -casein and that γ -casein might be part of β -casein. Fig. 5 is a diagrammatic representation of how the pieces would fit in the molecule of β -casein. The total number of residues in β -casein is given as 209 rather than 208 because an extra serine residue was found later by Ribadeau-Dumas et al. in the phosphopeptide preceding γ -casein (29).

This was all guesswork at the time since there was no evidence that peptide T9 followed T1, nor that CN5 and CN6 followed CN1 in the sequence of β -casein. It was most gratifying when Ribadeau-Dumas et al. reported the positioning of the isolated peptides. The order was indeed N-terminal T1 followed by T9, and N-terminal CN1 followed by CN5, then CN6 (32). Moreover, some partial sequences were reported. A lysine residue was placed at Position 28 (later changed to 29); this could well be the N-terminus of γ -casein. A histidine residue was located at Position 105 (later 106); the right spot for the N-terminus of TS-A² casein. And a Glx residue (either

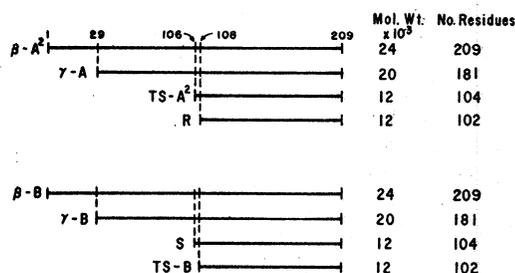


FIG. 5. Diagram of the peptide chain of β -casein A² with possible locations of γ -A², TS-A², and R caseins and a similar scheme for the β -B family.

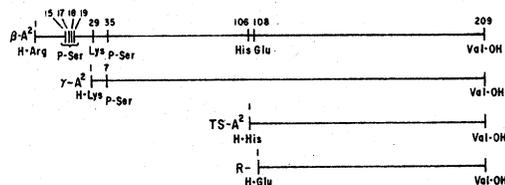


FIG. 6. Diagram of the peptide chains of β -A², γ -A², TS-A², and R caseins showing the locations of the various end-groups and the subsequent positioning of phosphoserine residues.

TABLE 4. Comparison of the N-terminal sequence of γ -, TS-A² and TS-B caseins (6) with portions of the sequence of β -casein A² as known in 1971 (32) and as finally established in 1972 (30). Residue numbers originally published (6, 32) have been increased here by one for consistency with the numbering of residues in the final sequence of β -casein (30).

Residue no. in β -casein	29	30	35	40	43
γ -casein	H • Lys • Ile • Glu • Lys • Phe • Gln • Ser [?] • Glu • Glu • Gln • Glu • Glx • Gln • Asx • Gln •				
β -casein (1971)	• Lys • Ile • Glu • Lys • Phe • Gln • Ser • Glx ₃ • Leu, Phe				
β -casein (1972)	• Lys • Ile • Glu • Lys • Phe • Gln • Ser • Glu • Glu • Gln • Gln • Thr • Glu • Asp • Glu •				
Residue no. in β -casein	106	110	115	117	143
TS-A ² casein	H • His • Lys • Glx • Met • Pro • Phe • Pro • Lys • Tyr • Pro • Val • Glu •				
TS-B casein	• His • Lys • H • Glx • Met • Pro • Phe • Pro • Lys • Tyr • Pro [?] • Val • Glx [?] •				
β -casein (1971)	• His • Lys • Lys • Glx • Met • (Pro ₂ , Phe)				
β -casein (1972)	• His • Lys • Lys • Glu • Met • Pro • Phe • Pro • Lys • Tyr • Pro • Val • Glu •				

glutamic acid or glutamine) was positioned at 107 (later 108), the inferred location for the N-terminus of R-casein. Our hypothesis was now on firmer ground. The evidence at that time could be summarized as illustrated in Fig. 6, where end groups are shown in the correct locations. However, the positions of the four phosphoserine (P-Ser) residues in the phosphopeptide and the position of the fifth phosphoserine residue were not established.

To test the hypothesis we arranged with Dr. P. W. D. Mitchell, Franklin Institute, Philadelphia, to run samples of γ -, TS-A², and TS-B caseins in the Institute's automatic sequencer. Table 4 gives the results of these runs for the first 10 to 16 residues of each protein (6) as well as the sequence of the appropriate portions of β -casein A² as known then (32) and also as finally worked out by Ribadeau-Dumas et al. (30). Question marks indicate uncertain identification of some amino acids in the table. Our results were compatible with the partial sequence and, with one major discrepancy (identification of Residue 41 as Glx instead of threonine) and several minor ones, the same as those published later in the final sequence. Our group also located phosphoserine in γ -casein as the seventh residue, that is, Residue 35 in β -casein (9 and unpublished work). The extra glutamic acid and methionine in the summed cyanogen bromide peptides referred to previously can be identified as Residues 108 and 109.

Our hypothesis based on these results was published early in 1972 (6). The investigators at Jouy thought the evidence was convincing enough for them to include γ -, TS, R-, and S-caseins in their diagram of the complete sequence of β -casein A² published later in 1972 (30) and reproduced here as Fig. 7. We have also included in the figure amino acid substitutions characteristic of the β -casein variants and their positions in the polypeptide chain as established by Grosclaude et al. (7). The arginine for serine substitution at Position 122 distinguishes the B family of these caseins from the A family; glutamine for histidine at Position 106 differentiates the A³ from the A² polymorphs; histidine for proline at Position 67 is common to the β and γ variants A¹ and B compared to A², and this substitution is also found in β -casein C compared to β -A². A second substitution which distinguishes β -C from β -A², lysine for glutamic acid at Position 37, is of particular interest because it might explain why γ -casein C was never detected. We may speculate as follows.

TABLE 5. Average hydrophobicity in calories per residue (Bigelow's parameter) of some milk proteins.

α -lactalbumin	1150
α_{s1} -casein	1170
β -lactoglobulin	1230
κ -casein	1285
β -casein	1330
γ -casein	1390
TS-B casein	1500
Galactothermin	1580

bic C-terminal half, with few charged groups.

The many proline residues distributed throughout the molecules exert a strong effect on the conformation of the molecules and preclude the presence of measurable amounts of α -helix. Another feature of the structure is a marked similarity in sequence of the phosphoserine-rich region, Residues 13 to 21 in β -casein, to a portion of the α_{s1} -casein sequence, Residues 62 to 70 (23).

The increasing hydrophobicity in the molecules of β -, γ -, and TS-casein can be assessed by calculation from their amino acid composition of Bigelow's parameter (4). Average hydrophobicities of these and other milk proteins are listed in Table 5. TS-B casein, with the highest hydrophobicity of all bovine milk proteins, is surpassed by galactothermin, a protein in human milk isolated by Schade and Reinhardt (33). Galactothermin resembles TS-casein not only in amino acid composition but in size, temperature-sensitivity, and absence of phosphorus. Is it possible that galactothermin is related to a component of human casein in the same way as TS-casein is related to β -casein?

Our hypothesis that bovine γ -casein is the same as a major fragment of β -casein and that TS-, R-, and S-caseins have the same structures as large segments of γ -casein, can be proved unequivocally only by complete sequencing of these minor caseins. All pertinent experimental evidence of which we are aware, however, supports this idea.

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