291. Studies on the Primary Structure of Cow κ-Casein. – Structural Features of para-κ-Casein; N-terminal Sequence of κ-Caseinoglycopeptide Studied with a Sequencer[1])

by **Jacqueline Jollès**[a]), **Françoise Schoentgen**[a]), **Charles Alais**[b]), **Anne-Marie Fiat**[a]) and **Pierre Jollès**[a])

Laboratory of Biochemistry, University of Paris VI, 96, Bd. Raspail, F-75006 Paris[a]) and Department of Biochemistry, University of Nancy, F-54 Nancy[b])

(27. VII. 72)

Summary. Several long tryptic peptides obtained from reduced maleylated κ-casein were sequenced: they belonged to the N- and C-terminal (37 residues) moieties of para-κ-casein. Several tryptic peptides could then be joined by chymotryptic overlap peptides. 102 amino acid residues of para-κ-casein were thus placed into two long and four shorter sequences. Some structural results were established with a Sequencer which was also employed for a verification of the N-terminal sequence of the κ-caseinoglycopeptide; a previously described short glycopeptide was found again and was reinvestigated.

1. Introduction. – κ-casein plays a major role in the stabilization of the casein micelle in its natural environment and in the clotting phenomenon induced by the action of rennin (EC 3.4.4.3); it is also the only casein fraction that contains sugars [1]. During the clotting of milk by rennin, the only significant alteration occurs in κ-casein yielding a) an insoluble para-κ-casein which complexes with α_s-casein and forms the strands of the clot [2]; it contains all the aromatic, sulfur-containing and basic amino acids (except 3 lysine residues) of κ-casein [3]; b) a soluble fraction containing the 'caseinoglycopeptide' [4] when the starting product is a sugar-rich κ-casein or the 'caseinopeptide' or 'macropeptide' when the starting product is a sugar free κ-casein [5] [6]. The primary action of rennin on casein is constituted by the split of a Phe → Met linkage, as shown by *Jollès et al.* [6] [7] and the release of the (glyco)peptide from κ-casein, thus destroying the micelle-stabilizing properties of this latter. Previously (1965–1970) some structural features of cow κ-casein have been published by our group: a) the structure of the soluble tryptic peptides of para-κ-casein which constitutes the N-terminal part of κ-casein [8]; b) the structure of the tryptic peptide containing the rennin-sensitive Phe → Met linkage [9] which is constituted by the C-terminal sequence of para-κ-casein and the N-terminal sequence of the κ-caseinoglycopeptide; c) the N-terminal octadecapeptide [8] [10] and the C-terminal tetrapeptide (Ser-Thr-Ala-Val) of the κ-caseinoglycopeptide [11]; d) a heptapeptide of the κ-caseinoglycopeptide containing a sugar moiety (O-glycosidic linkage between a residue of Thr and GalNAc) [12].

κ-casein contains 2 Met residues [5]; *Jollès et al.* [1] [8] [13] localized one of these residues in the C-terminal sequence of para-κ-casein and the other as the N-terminal residue of the κ-caseinoglycopeptide. After cleavage of κ-casein with cyanogen bromide, its C-terminal moiety, namely the κ-caseinoglycopeptide devoid of its N-terminal Met

[1]) 27th communication on caseins.

residue, was recently purified by *Mercier et al.* [14], who established its primary structure. They confirmed our previous results concerning the sequences of the N-terminal octadecapeptide [8] [10] and of the C-terminal tetrapeptide [4] [11]; the C-terminal sequence of *Pujolle et al.* [15] could not be verified.

In order to establish the complete primary structure of $\varkappa$-casein, a detailed study of its N-terminal part, para-$\varkappa$-casein, was undertaken. Trypsin gave rise to an important tryptic 'core' which could not be entirely purified; thus $\varkappa$-casein was maleylated prior to enzymic digestion. The present paper deals with the establishment of the structure of some long maleylated tryptic peptides belonging to the N- and C-terminal moieties of para-$\varkappa$-casein as well as with some structural data concerning $\varkappa$-casein and obtained with a Sequencer.

2. Material and Methods. 2.1. Cow $\varkappa$-casein was prepared according to *McKenzie & Wake* [16] from pooled milk and $\varkappa_A$ casein by the same procedure from the milk of homozygous cows. Trypsin (EC 3.4.4.4), chymotrypsin (EC 3.4.4.5) and carboxypeptidases A and B (EC 3.4.2.1/2), were purchased from *Worthington*; aminopeptidase O (EC 3.4.1.–) was a gift from *Röhm & Haas* (Darmstadt). Sephadex G-25 and G-50 fine as well as DEAE-cellulose (DE 32) were obtained from *Pharmacia* and *Whatman*, respectively. All other reagents were obtained from *Merck* or *Prolabo*, except maleic anhydride (*Sigma*) and those employed for the Sequencer which were purchased from *Beckman*.

2.2. The *reduction* and aminoethylation as well as the reduction and alkylation of cow $\varkappa_A$-casein was achieved according to *Jollès et al.* [8] [17] and the maleylation according to *Butler et al.* [18].

2.3. *Tryptic hydrolysis and separation of the soluble maleylated tryptic peptides* (TM). 950 mg of reduced alkylated and maleylated $\varkappa$-casein were digested during 2 h with 10 mg trypsin at 37° in 50 ml of a 0.1M Tris-HCl buffer, pH 8.5. Trypsin was pretreated during 16 h with 0.0625N HCl at 37°. The soluble tryptic peptides were chromatographed on a 24 × 4 cm DEAE-cellulose (DE 32) column equilibrated with the above mentioned Tris-HCl buffer. Gradient elution was achieved as indicated on Fig. 2. The peptides were characterized by a ninhydrin determination after alkaline hydrolysis. The purified peptides were filtered on a Sephadex G-25 column (25 × 4 cm) with 0.01M NH_4OH as eluent. The peaks were dried, demaleylated (30% acetic acid, 24 h, 40°) and dried again.

Several peptides were pure, some others have been submitted to further purifications either by filtration on Sephadex G-50 (200 × 1.7 cm; 30% acetic acid as eluent) either by paper chromatography (*Whatman* No. 3) in solvent A: *n*-butanol/pyridine/acetic acid/water (15:10:3:12 *v/v*). The purity of the peptides was also examined by paper electrophoresis (*Whatman* No. 3) at pH 6.5 (pyridine/water/acetic acid 100:900:4 *v/v*) and at 15 V/cm.

2.4. *Chymotryptic hydrolysis and separation of the enzymic digest.* 185 mg of reduced aminoethylated $\varkappa_A$-casein were digested during 24 h with 3 mg chymotrypsin at 37° and pH 8 in 0.1M NH_4HCO_3 and dried. The chymotryptic peptides dissolved in 50% formic acid were filtered on a Sephadex G-25 column (200 × 1.8 cm) with 30% acetic acid as eluent. Eight fractions were collected and submitted to a preparative paper electrophoresis (*Whatman* No. 1) at pH 6.5 and at 50 V/cm.

2.5. *Determination of the structure of the peptides by classical methods.* The structures were established in detail following the usual methods chiefly the *Edman* procedure, dansyl method, enzymic digestions. The different procedures have recently been described [19] [20]. A special problem is constituted by the peptides with a N-terminal glutamine residue. After their purification or desalting in an acidic medium (filtration on Sephadex with 30% acetic acid as eluent), a cyclisation occurred with the formation of pyrrolidonecarboxylic acid; these peptides could be submitted to the '*Edman*-dansyl' procedure only after a pretreatment (1N NaOH; 72 h; 20°); after neutralization, the degradation must be started as soon as possible.

2.6. *Determination of the structure of peptides with a Sequencer.* Automated *Edman* degradation [21] was carried out in a *Beckman* Sequencer, Model 890B, by the quadrol method. The thiazo-

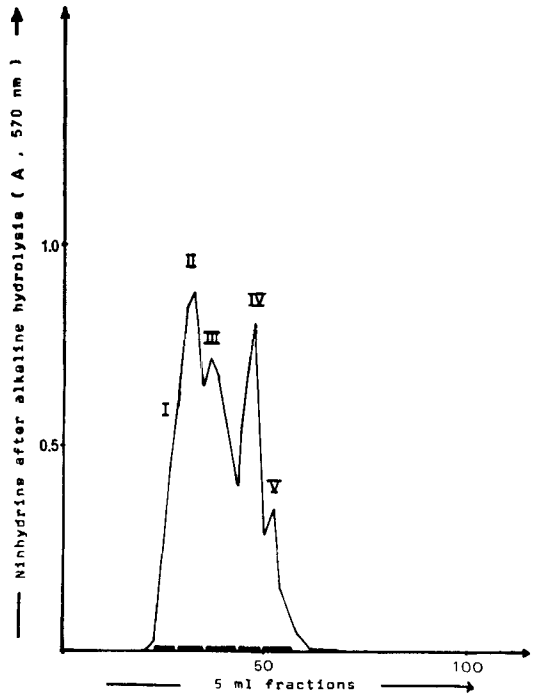


Fig. 1. *Filtration on Sephadex G-50* (150 × 1.8 cm) *of the core obtained after tryptic digestion* (6 h; 37°) *of 500 mg reduced aminoethylated* κ_A*-casein and dissolved in 2 ml 50 % formic acid.* Eluent: 30 % acetic acid

Table I. *Rf values (in solvent A), mobility* m *(at pH 6.5), yield, composition and*

Peptide	Rf (A)	m [a] (pH 6.5)	Yield (%)	Composition
TM_1	0.55	+0.50	25	Ser(0.9) Val(0.9) Leu(1.0) Arg(0.98)
TM_2	0.50	+0.05	7	SCMCys(0.78) Asp(1.0) Thr(2.8) Ser(2.7) Glu(4.95) Pro(2.75) Ala(3.4) Val(1.95) Met(0.54) Ile(0.7) Leu(1.45) Lys(0.7) Arg(1.0) Trp (*Ehrlich* +)
TM_3A	0.35	+0.60	20	Pro(1.08) Ala(2.7) Val(1.0) Tyr(0.84) Lys(0.93) Arg(0.8)
TM_3B	0.50	+0.05	9	identical with TM_2
TM_4	0.15	−0.46	25	Asp(1.08) Glu(5.87) Pro(0.95) Ile(1.0) Arg(0.85)
TM_5	0.50	+0.05	5	identical with TM_2
TM_6A			36	mainly the caseinoglycopeptide
TM_6B	0.06	−0.43	50	SCMCys(0.9) Asp(1.0) Glu(1.7) Lys(0.6) Arg(0.85)
TM_7A	0.65	+0.21	10	Asp(1.06) Ser(0.95) Glu(1.0) Pro(0.98) Ala(1.0) Ile(2.4) Tyr(1.65) Phe(1.55) Lys(1.95)

[a] $m = 0$ for Gly; $m = +1$ for Arg.
[b] *Jollès et al.*, Helv. *53*, 1918 (1970).

linones were converted into PTH-amino acids and these latter were characterized by thin-layer chromatography, by gas-liquid chromatography (*Beckman* GC 45 chromatograph) or with an amino acid Autoanalyzer after regeneration of the free amino acid.

3. Results and discussion. – 3.1. *Tryptic digestion of reduced aminoethylated $\varkappa_A$-casein: the tryptic 'core'.* By its action on $\varkappa_A$-casein, trypsin gave rise to soluble peptides which have already been studied [8] and to an important tryptic 'core'. This latter was dissolved in 50% formic acid and filtered on Sephadex G-50 (Fig. 1). Five peaks (I–V) were characterized; only one peptide could be entirely purified after rechromatography; it was contained in peak IV and its structure was studied in detail (see Table II).

3.2. *Tryptic digestion of reduced alkylated and maleylated $\varkappa_A$-casein.* In view to increase the solubility of the tryptic peptides, and also to lengthen those of them containing a C-terminal lysine residue, we decided to submit the reduced maleylated $\varkappa_A$-casein to the digestion of trypsin. Eight peaks (TM1–TM8) were characterized by chromatography on DEAE-cellulose (Fig. 2). In Table I are indicated the Rf and mobility *m* values, the yields as well as the structures of some of the maleylated tryptic peptides. The establishment of the structure of the long peptide TM2 is summarized in Table II: it contains the 'core' peptide IV which could be lengthened by two 'soluble' [8] tryptic peptides. The C-terminal sequence of peptide TM7A (Table I) was identical to 'soluble' tryptic peptide 14 [8]; digestions with carboxypeptidase A as a function of time established Gln-Tyr as C-terminal amino acids instead of Tyr-Gln; this result was also verified with the Sequencer.

chemical structure of tryptic peptides obtained from maleylated cow $\varkappa_A$-casein

Structure
Val-Leu-Ser-Arg (identical with peptide (1+2+3) F[b]))
see Table II
⟵ C4c ⟶ Tyr-Ala-Lys-Pro-Ala-Ala-Val-Arg 25%35%80% ⟵ (1+2+3)D[b]) ⟶
Gln-Gln-Gln-Asn-Glu-Glu-Glu-Pro-Ile-Arg (identical with peptide 13[b]))
SCMCys-Glu-Lys-Asp-Glu-Arg (identical with peptide 9A[b]))
Phe-Phe-Ser-Asp-Lys-Ile-Ala-Lys-Tyr-Ile-Pro-Ile-Gln-Tyr 70%100%
⟵ 10[b]) ⟶ ⟵ (1+2+3) E[b]) ⟵ 14[b]) ⟶

⟝ Amino acid characterized by carboxypeptidases A and B
⟶ Amino acid characterized by dansylation

3.3. *Chymotryptic digestion of reduced aminoethylated $\varkappa_A$-casein.* The chymotryptic digest was filtered on Sephadex G-25 and 8 fractions were characterized (Fig. 3). 12 peptides could be purified; their Rf and mobility *m* values, their yields and their structures are indicated in Table IV. Several chymotryptic peptides containing basic amino acid residues allowed to join some of the tryptic peptides.

The C-terminal sequence of para-$\varkappa_A$-casein, contained in peptide C2b, was again studied in detail; a third histidine residue was characterized as previously noted by *Mercier et al.* [14] (2.4 residues of histidine on the basis of 1 residue of leucine after 18 h hydrolysis). After tryptic digestion of C2b, a peptide with the following amino acid composition was isolated: Ser_1, Pro_2, Leu_1, Phe_1, His_3. Its N-terminal sequence, His-Pro, was determined by the *Edman* procedure. The characterization of a third residue as PTH-amino acid by thin layer and gas liquid chromatographies seemed to be

Table II. *Establishment of the*

Method	Composition: SCMCys$_1$, Asp$_1$, Thr$_3$, Ser$_3$, Glu$_5$, Pro$_3$, Ala$_4$, Val$_2$, Met$_1$, Ile$_1$ Isolated substances
Dansylation	Ser
Carboxypeptidase B (24 h) + A (1 h)	
'Partial' chymotryptic digestion (E/S = 1/1000; 1.5 h; 30°)	Ser-Pro-Ala-Gln-Ile-Leu-Gln-Trp-Gln-Val-Leu ($m = 0$) 39%61%82%
Chymotryptic digestion (E/S = 1/20; 24 h; 37°)	Ser-Pro-Ala-Gln-Ile-Leu ($m = 0$) 25%40% Gln-Trp-Gln-Val-Leu ($m = 0$) C7a C8 – C7c Ser-(Asn, Thr, Val, Pro, Ala)-Lys ($m = +0.30$) HCl 11 N; 24 h; 37°: Ser-Asp; Thr-Val-Pro-Ala-Lys
Tryptic digestion (E/S = 1/20; 48 h; 37°)	Ser-Pro-Ala-Gln-Ile-Leu-Gln-Trp-Gln-Val-Leu-Ser-Asn-Thr-Val-Pro-Ala-Lys ($m = +0.18$) Core peptide IV
Structure	Ser-Pro-Ala-Gln-Ile-Leu-Gln-Trp-Gln-Val-Leu-Ser-Asn-Thr-Val-Pro-Ala-Lys-

[a]) *Follès et al.*, Helv. *53*, 1918 (1970).
$m = 0$ for Gly; $m = +1$ for Arg.

in favour of proline. The C-terminal sequence was established by digestion with carboxypeptidase A as a function of time; after 2, 10 and 30 minutes at 37°, we characterized Ser-Phe [9], Leu-Ser-Phe [9], and His-Leu-Ser-Phe (Table III), respectively. This latter result seemed to rule out the presence of a proline residue preceding this histidine residue, as carboxypeptidase A usually does not digest a Pro-X linkage. We propose thus the following sequence: His-Pro-(Pro, His)-His-Leu-Ser-Phe. The third and fourth residue were maintained between parentheses, as this result was not entirely in accordance with the data of *Mercier et al.* [14].

3.4. *Present knowledge of cow para-$\varkappa_A$-casein.* Table III summarizes our present knowledge concerning the sequence of cow para-$\varkappa_A$-casein: the structure of two long peptides, one of them being the C-terminal sequence, as well as of four shorter peptides are indicated, with a total of 102 amino acid residues. As $\varkappa_A$-casein and the caseino-

structure of peptide TM_2

Leu_2, Lys_1, Arg_1, Trp (*Ehrlich*+)

Thr-Met-Ala-Arg
60%75%80%90%

Ser-SCMCys-Gln-Ala-Gln-Pro-Thr-Thr-Met-Ala-Arg
⟵6[a])⟶⟵ (1+2+3) A[a]) ⟶
Ser- Cys- Gln-Ala-Gln-Pro-Thr-Thr-Met-Ala-Arg

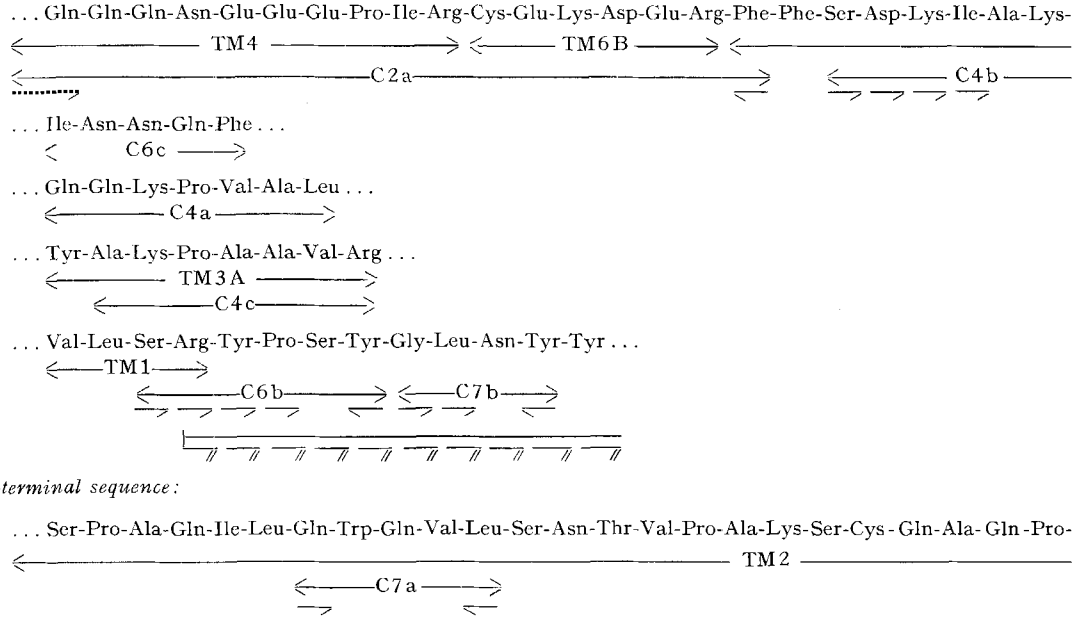
⟶ amino acid characterized by the *Edman*-dansylation procedure
⟵ amino acid characterized by digestion with carboxypeptidases A and B
⋯⟶ amino acid characterized by dansylation after pretreatment with 1N NaOH

glycopeptide contain 165 ± 5 [5] and 64 [14] amino acids, respectively, Table III might report nearly all the sequences of para-$\varkappa$-casein; however some tryptic overlap peptides have not yet been characterized.

3.5. *Preliminary results established with a Sequencer.* – 3.5.1. *$\varkappa_A$-casein and para-$\varkappa_A$-casein.* Table III reports two sequences previously established by classical methods which have been verified with the help of a Sequencer. Furthermore various techniques (*Edman* or dansyl procedures) did not allow to characterize a N-terminal amino acid for $\varkappa_A$-casein; thus the presence of a blocked N-terminal amino acid or of pyrrolidone-carboxylic acid was suspected. After an alkaline pretreatment of $\varkappa_A$-casein (see Methods), Glu was detected by *Sanger's* DNP technique with, however, several other amino acids, such as serine and threonine; a split of the polypeptide chain could not be excluded. An automatic degradation of $\varkappa_A$-casein was also tried with a Sequencer. The reduced alkylated protein without or with a pretreatment (Quadrol buffer; 20 °C; 3 days) did not give rise to a N-terminal residue.

3.5.2. *$\varkappa_A$-Caseinoglycopeptide: N-terminal sequence and study of the short glycopeptide.* Table V summarizes the N-terminal sequence (38 amino acid residues) established with a Sequencer. The first 18 amino acids were identical with those reported earlier by our group [8] [10] and the following 20 residues were in accordance with the results

Table III. *Chemical features of*



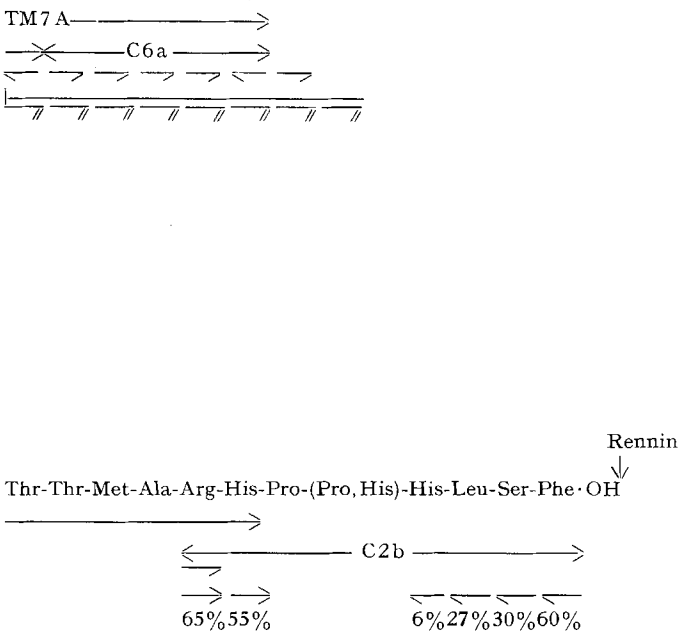
—→ Amino acid characterized by the *Edman*-dansylation procedure
·····→ Amino acid characterized by dansylation after pretreatment with 1N NaOH
—// Amino acid characterized with a Sequencer

published by *Mercier et al.* [14]. The characterization of the serine and threonine residues was quite difficult after residue No. 21.

The $\varkappa$-caseinoglycopeptide contains an O-glycosidic linkage between GalNAc and a residue of threonine [22]. The carbohydrate sequence has been studied by *Fiat et al.* [12] who isolated a short glycopeptide from $\varkappa_A$-casein; a sequence was also proposed for its peptidic part. The results presented in this paper as well as those of *Mercier et al.* [14] incited us to reinvestigate the amino acid sequence of this short glycopeptide, as a careful examination of all the published data seemed to involve the threonine residue(s) No. 26 or (and) No. 28 of the $\varkappa$-caseinoglycopeptide in the O-glycosidic linkage.

The short glycopeptide prepared according to *Fiat et al.* [12] ($m = -0.45$ at pH 6.5) was submitted to the manual *Edman* technique and the PTH-amino acids were characterized by gas-liquid chromatography. By this sensitive technique it was established that the main glycopeptide was accompanied by another, less abundant peptide. The N-terminal sequence of the main peptide was Gly-Glu-Pro-Thr; these amino acids were at each step at least ten times more abundant than the accompanying substances. As the $\varkappa$-caseinoglycopeptide contains only one glycine residue, the structure of the main glycopeptide could be thus deduced from the amino acid composition which was again established after three hydrolysis times ($Thr_{3,00}$, $Ser_{1,0}$,

cow para-$\varkappa_A$-casein



⟵ Amino acid characterized after digestion with carboxypeptidases A and B
⟶ Amino acid characterized after digestion with aminopeptidase O

$Glu_{1,10}$, $Pro_{1,90}$, $Gly_{0,70}$) as well as from previous studies [12]: Gly-Glu-Pro-Thr-Ser-Thr-Pro-Thr (residues 23 to 30, Table V). The attachment site of the polysaccharide could not be the C-terminal threonine residue, as a β-elimination process was observed [23]. Thus threonine residue No. 26 or No. 28 might be linked to the sugar part. However if Thr-X-X-Pro (and not Thr-X-Pro) [12] might be suggested as 'code sequence'

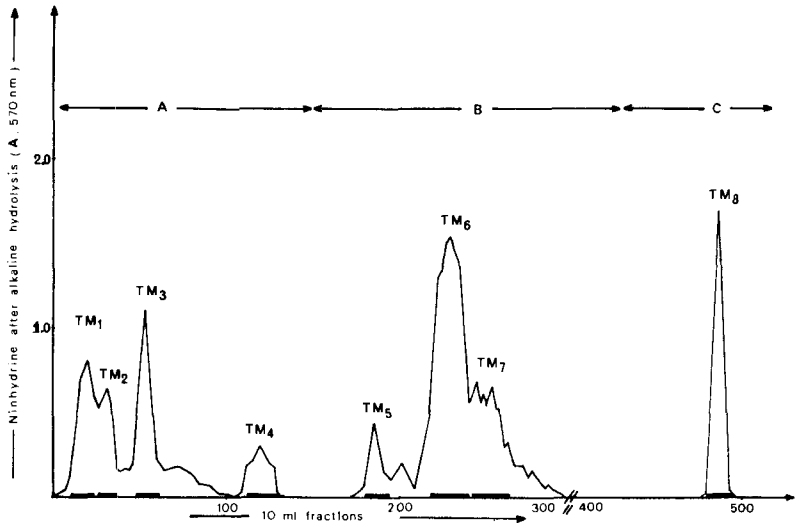


Fig. 2. *Chromatography on DEAE-cellulose* (24 × 4 cm) *of the tryptic digest* (2 h; 37°) *of 950 mg reduced, alkylated and maleylated* $\varkappa_A$*-casein.*
A: 0.1 M Tris-HCl buffer pH 8.5; B: Gradient elution; addition of 0.8 M NaCl in a mixing chamber of 2 l capacity containing buffer A; C: 0.25 N NaOH

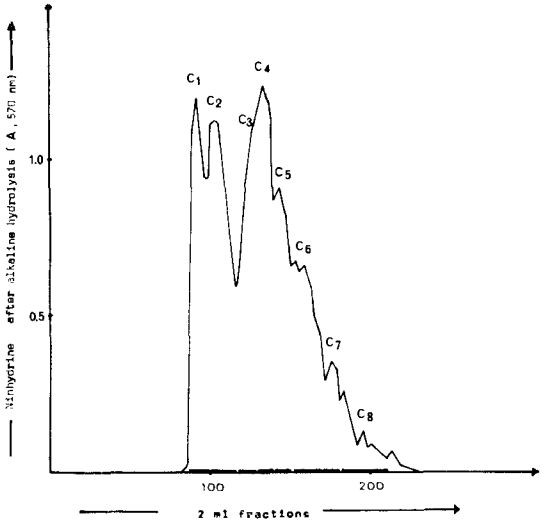


Fig. 3. *Filtration on Sephadex G-25* (200 × 1.8 cm) *of the chymotryptic digest* (24 h; 37°) *of 150 mg reduced aminoethylated* $\varkappa_A$*-casein.* Eluent: 30 % acetic acid

Table IV. *Rf values (in solvent A), mobility* m *(at pH 6.5), yield and chemical structure of chymotryptic peptides obtained in eight fractions chatacterized by filtration on Sephadex G-25 from reduced aminoethylated cow $\varkappa_A$-casein*

Peptide	Rf (A)	m [a] pH 6.5	Yield %	Structure
C_{2a}	0.03	−0.30	32	Gln-Gln-Gln-Asn-Glu-Glu-Glu-Pro-Ile-Arg-Cys-Glu-Lys-Asp-Glu-Arg-Phe
C_{2b}	0.10	+0.70	10	Ala-Arg-His-Pro-(Pro, His)-His-Leu-Ser-Phe
C_{4a}	0.53	0	37	PyrGlu-Gln-Lys-Pro-Val-Ala-Leu
C_{4b}	0.25	+0.30	36	Ser-Asp-Lys-Ile-Ala-Lys-Tyr
C_{4c}	0.22	+0.70	17	Ala-Lys-Pro-Ala-Ala-Val-Arg
C_{6a}	0.53	0	19	Ile-Pro-Ile-Gln-Tyr
C_{6b}	0.43	+0.30	21	Ser-Arg-Tyr-Pro-Ser-Tyr
C_{6c}		0	6	Ile-Asn-Asn-Gln-Phe
C_{7a}	0.43	0	6	Gln-Trp-Gln-Val-Leu
C_{7b}	0.6	0	27	Gly-Leu-Asn-Tyr
C_{7c}	0.90	0	35	Gln-Val-Leu
C_8	0.67	−0.70	40	PyrGlu [b]-Trp

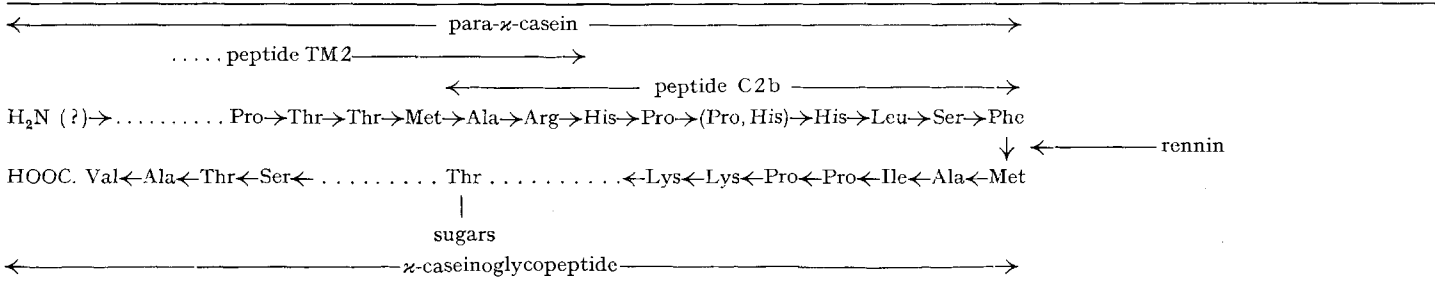
[a] $m = 0$ for Gly; $m = -1$ for Cys 0_3H; $m = +1$ for Arg.
[b] PyrGlu, pyrrolidonecarboxylic acid.

Table V. *N-terminal sequence of $\varkappa_A$-caseinoglycopeptide determined with a Sequencer*

Residue (No.)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Amino acid determined by:	Met	Ala	Ile	Pro	Pro	Lys	Lys	Asn	Gln	Asp	Lys	Thr	Glu	Ile	Pro	Thr	Ile	Asn	Thr
1. Analysis (Autoanalyzer) % recovery	44	65	49	24	16	18	21	8	12	10	6		10	6	6		4	4	
2. Thin layer chromatography	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Residue (No.)	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38
Amino acid determined by:	Ile	Ala	Ser or Thr	Gly	Glu	Pro	Ser or Thr	Ser or Thr	Ser or Thr	Pro	Ser or Thr	Ile	Glu	Ala	Val	Glu	Ser or Thr	Ser or Thr	Val
1. Analysis % recovery[a])	3	5		3	8								7		2				1
2. Thin layer chromatography	+	+	–	+	+	+	–	–	–	+	–	+	+	+	+	+	–	–	+

\+ identified with certainty.
– no amino acid could be detected: thus the presence of Ser or Thr seemed probable.
[a]) Unique amino acid characterized despite the low yield.

Table VI. *Schematic representation of the structure of cow $\varkappa_A$-casein*



for an O-glycosidic linkage between Thr and N-acetylgalactosamine and if the presence of a proline residue on the N-terminal side of the threonine residue involved in the attachment site of the polysaccharide might also be important, then threonine No. 26 seems to be the most probable residue involved in the sugar linkage.

The most probable structure of the less abundant peptide includes residues No. 22 to No. 29 (Table V).

3.6. *Conclusion.* Table VI gives a schematic representation of the structure of $\varkappa_A$-casein. In the proximity of the important rennin-sensitive linkage are situated all the histidine and methionine residues of the protein, several proline residues as well as the three threonine residues of para-$\varkappa_A$-casein.

This research was supported in part by the C.N.R.S. (ER 102), I.N.S.E.R.M. (groupe U116) and D.G.R.S.T. (contract 72.7.0470). The authors wish to express their appreciation to Mrs. M. Berger, Miss A. Zimmermann and Mr. Ly Quan Le for skilful technical assistance.

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