

## ***In vivo* milk digestion in the calf abomasum. — I. Whole-casein digestion**

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**Summary.** Peptide products insoluble in 12 % TCA and obtained in the proximal duodenum of calves at different times after ingestion of casein solutions were characterized by polyacrylamide gel electrophoresis, electrofocusing and SDS pore gradient gel electrophoresis.

With a 3 % whole-casein solution in water the disappearance of electrophoretic bands corresponding to  $\alpha_{s1}$  and  $\beta$  caseins was observed after about 1 h 30. After 3 h and up to 7 h very acidic peptides appeared. With a 3 % whole-casein solution in simulated milk ultrafiltrate, comparable patterns were obtained. Nevertheless, the acidic peptides appeared sooner, *i. e.* they were already detected in the first sample collected after meal ingestion. With the 2 diets, the importance of gastric proteolysis was demonstrated by the appearance of a great number of peptides of various sizes, charges and pHi. On the other hand, an effect of salts on casein proteolysis was detected.

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## **Introduction.**

In preruminant calves, the abomasum, the functional part of the stomach, secretes chymosin. It has been considered that, although the quantity of pepsin is very low in this stomach compartment of milk-fed calves, it has appreciable activity (Berridge *et al.*, 1943 ; Garnot *et al.*, 1977 ; Guilloteau *et al.*, 1983).

*In vitro* proteolysis of casein by chymosin or pepsins is relatively well known (Delfour, Alais and Jolles, 1966 ; Fox and Walley, 1971 ; Creamer, Mills and Richards, 1971 ; Hill, Lahav and Givol, 1974 ; Pélissier, Mercier and Ribadeau-Dumas, 1974 ; Visser and Slangen, 1977 ; Mulvihill and Fox, 1977, 1979a, b ; Addeo, Pélissier and Chianese, 1981 ; Visser, 1981).

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A limited number of publications have reported experiments on *in vivo* proteolysis of milk-proteins in the stomach. These studies deal with gastric emptying in rats (Zebrowska, 1968 ; Buraczewski *et al.*, 1971 ; Miranda and Pélissier, 1981, 1983), pigs (Braude *et al.*, 1970a, b) and calves (Toullec, Thivend and Mathieu, 1971 ; Ternouth and Roy, 1973 ; Guilloteau *et al.*, 1975, 1979 ; Pélissier *et al.*, 1983) fed with milk or casein diets. Gastric proteolysis was only partially studied in rats (Miranda and Pélissier, 1981, 1983).

In a previous work (Pélissier *et al.*, 1983), gastric emptying in preruminant calves fed with milk or whole casein solutions has been studied by the determination of N content of soluble and insoluble fractions in 12 % TCA. In the present work, we characterized peptide products which appeared in the proximal duodenum of calves at different times after the ingestion of casein solutions.

### Material and methods.

*Diets and experimental design.* — The feeding and collection procedures used for calves have been described previously (Pélissier *et al.*, 1983). The calves received an artificial milk diet containing 25 % of protein from skim-milk. This diet (125 g dry matter per kg) was distributed twice a day. In the morning of the day before sampling, sodium citrate (11 g/kg) was added to the « milk » to inhibit coagulation and increase gastric emptying (Frantzen *et al.*, 1973). In the evening, the diet was replaced by water. In these conditions, the abomasum was almost empty before the distribution of the test meal given in the morning.

Each test meal was constituted of 5 kg of the following diets : (A) 3 % whole-casein solution in water ; (B) 3 % whole-casein solution in simulated milk ultrafiltrate (Jenness and Koops, 1962). Each diet was prepared from the same milk, coming from a single cow homozygous for the 4 caseins ( $\alpha_{s1}$ ,  $\alpha_{s2}$ ,  $\beta$  and  $\chi$ ). The protein concentration used simulated milk casein concentration.

*Animals and sampling.* — Two male Frisian calves were fitted with re-entrant cannulas (Ash, 1962) positioned 10 cm below the pyloric sphincter. The test meals were given from 15 days after the operation and once a week to the same animal. The diets were given twice to one calf and once to the other.

All the digesta leaving the stomach during the first 7 postprandial hours was collected in 10-min fractions for the first hour, in 15-min fractions for the second hour and in 30-min fractions up to the end.

Each digesta was precipitated with trichloroacetic acid (TCA), 12 % final concentration, and centrifuged at 2000 x g for 20 min. The sediment was homogenized in water, extracted 10 times with an equal volume of diethyl-ether to eliminate the TCA and then lyophilized.

Sediments were analyzed by polyacrylamide gel electrophoresis (PAGE), isoelectrofocusing (IEF), SDS-pore-gradient gel electrophoresis (SDS-PGGE) and two-dimensional electrophoresis. No quantitative determination of the products was made because of the presence of unknown peptides from casein proteolysis.

PAGE was performed according to Uriel (1966) with the modifications described by Gripon *et al.* (1975). IEF, SDS-PGGE and two-dimensional electrophoresis were performed according to Trieu-Cuot and Gripon (1981).

## Results.

After about 1 h 30 min the bands, observed in PAGE with diet A (casein in water) (fig. 1) and corresponding to the major caseins ( $\alpha_{s1}$  and  $\beta$ ), disappeared completely.  $\chi$ -casein disappeared completely from the first sample collected, and a band corresponding to para- $\chi$ casein was visible for about 1 h 30 min. Before that time, we detected the transformation of  $\alpha_{s1}$  and  $\beta$ -caseins into fast migrating peptides. After 3 h and up to 7 h, bands appeared at the migration front with diet A. With diet B, (casein and minerals), a comparable pattern was observed (fig. 2). Nevertheless, the fast-moving peptides appeared sooner, *i. e.* they were already detected in the first sample collected after the meal.

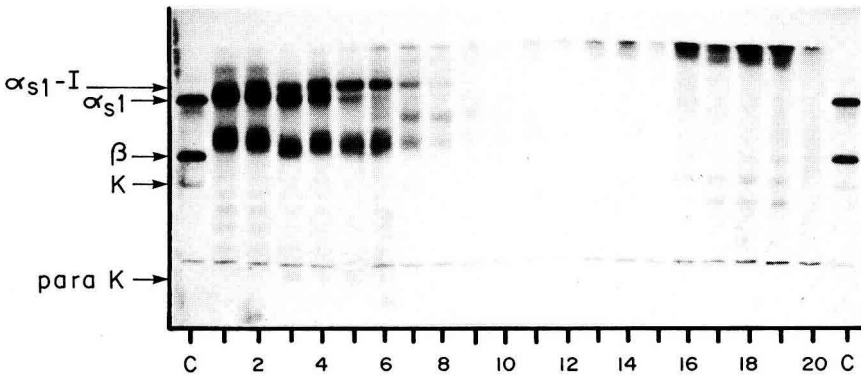


FIG. 1. — Polyacrylamide-agarose gel electrophoresis of samples obtained with diet A. C: whole casein. Times of sample collection: 1: 0-10 min; 2: 10-20 min; 3: 20-30 min; 4: 30-40 min; 5: 40-50 min; 6: 50-60 min; 7: 60-75 min; 8: 75-90 min; 9: 90-105 min; 10: 105-120 min; 11: 2 h-2 h 30; 12: 2 h 30-3 h; 13: 3 h-3 h 30; 14: 3 h 30-4 h; 15: 4 h-4 h 30; 16: 4 h 30-5 h; 17: 5 h-5 h 30; 18: 5 h 30-6 h; 19: 6 h-6 h 30; 20: 6 h 30-7 h.

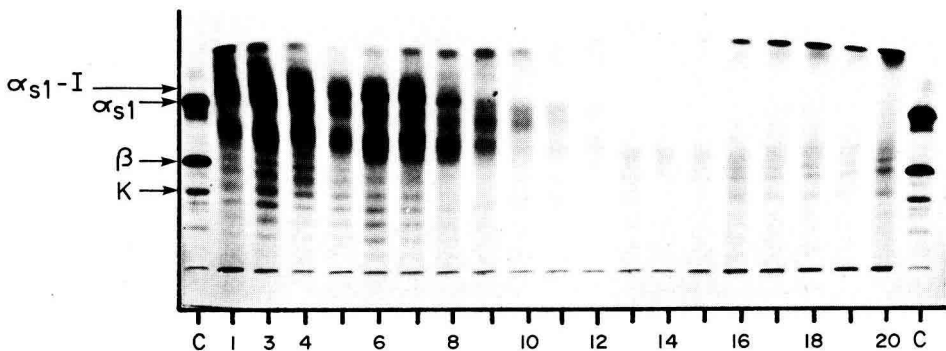


FIG. 2. — Polyacrylamide-agarose gel electrophoresis of samples obtained with diet B. See legend of figure 1.

These results were confirmed by IEF (figs. 3, 4). The pI values of a large number of peptides were distributed widely. A group of acidic peptides appeared at a rate similar to that observed for the peptides which moved with the migration front in PAGE. During the first hour, we also detected a group of basic peptides which disappeared as the caseins did. In particular, peptides migrating like para- $\chi$  casein and fragment 1-23 of  $\alpha_{s1}$ -casein were evidenced (Trieu-Cuot, 1981).

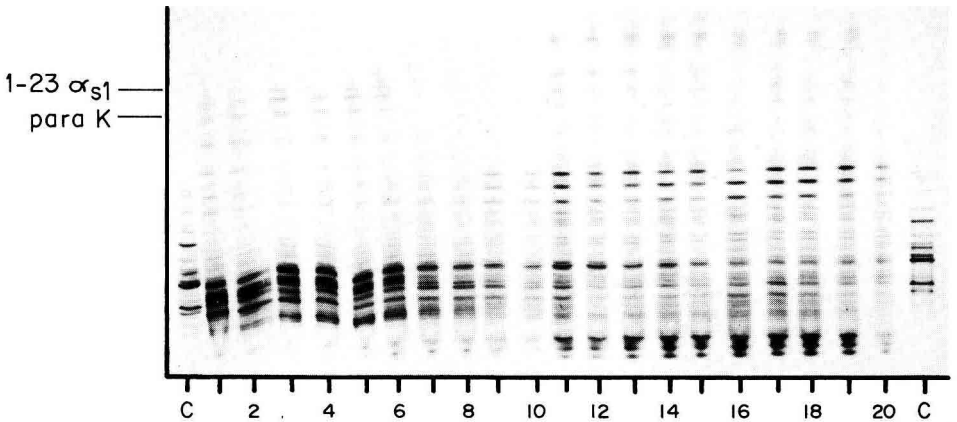


FIG. 3. — *Isoelectrofocusing of samples obtained with diet A.* See legend of figure 1.

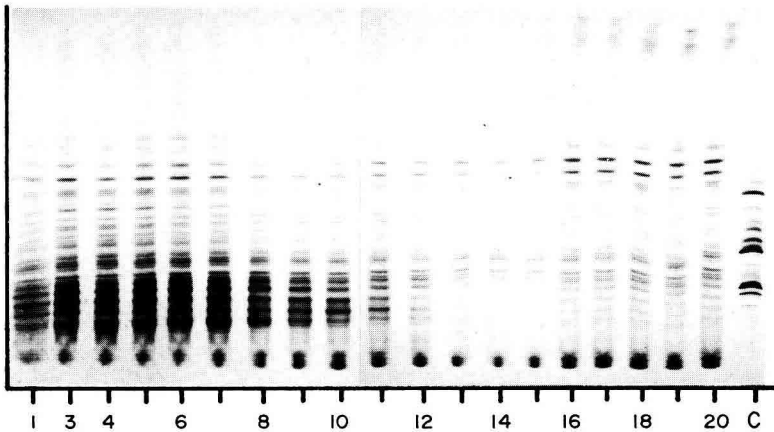


FIG. 4. — *Isoelectrofocusing of samples obtained with diet B.* See legend of figure 1.

The molecular weights (MW) of the peptides collected in the duodenum were estimated by SDS-PAGE (figs. 5, 6). For 90 min, most of the products were identified as caseins with little or no proteolysis. Peptides migrating as para kappa and as fragment 1-23 of  $\alpha_{s1}$ -casein could also be detected. On the other

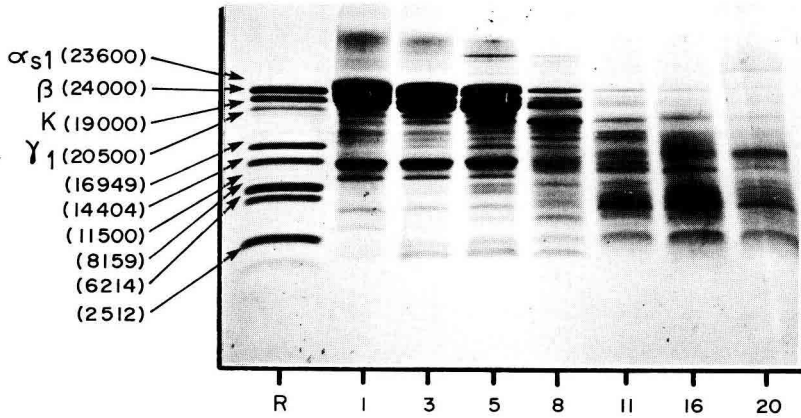


FIG. 5. — *SDS-pore-gradient gel electrophoresis of samples obtained with diet A.*  
R : molecular weight references. See figure 1 for sample collection times.

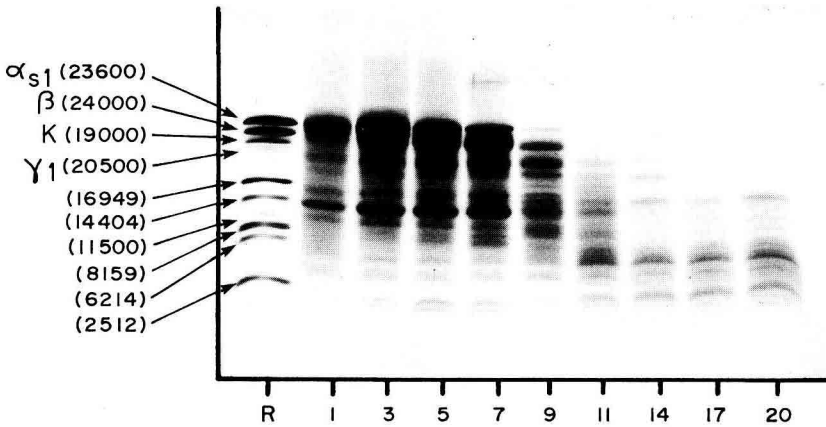


FIG. 6. — *SDS-pore-gradient gel electrophoresis of samples obtained with diet B.*  
R : molecular weight references. See figure 1 for sample collection times.

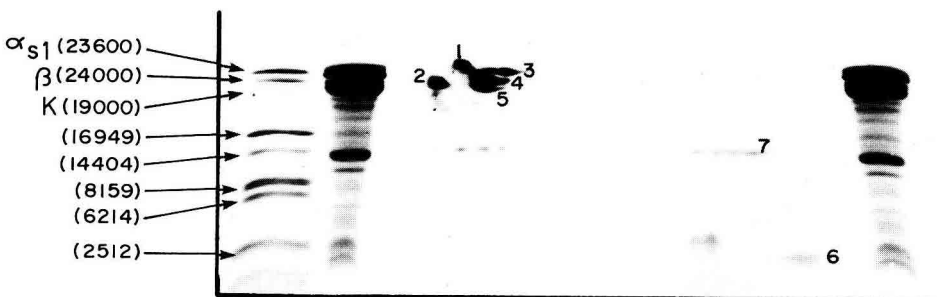


FIG. 7. — *Two-dimensional gel electrophoresis of samples obtained with diet A.*  
1 :  $\alpha_{s1}$  ; 2 :  $\alpha_{s1-l}$  ; 3 :  $\alpha_{s2}$  ; 4 :  $\beta$  ; 5 :  $\beta-l$  ; 6 : fragment 1-23 of  $\alpha_{s1}$  ; 7 : para- $\alpha$ .

hand, after 120 min the peptides which appeared during digestion had a relatively low MW but were still insoluble in 12 % TCA. These results were confirmed by two-dimensional electrophoresis (figs. 7, 8) in which these peptides migrated at the same position as  $\alpha_{s1}$ -I and  $\beta$ -I did. Digestion products from diet B appeared to be more proteolysed with this technique. In particular, acidic peptides could be detected with diet B sooner than with diet A.

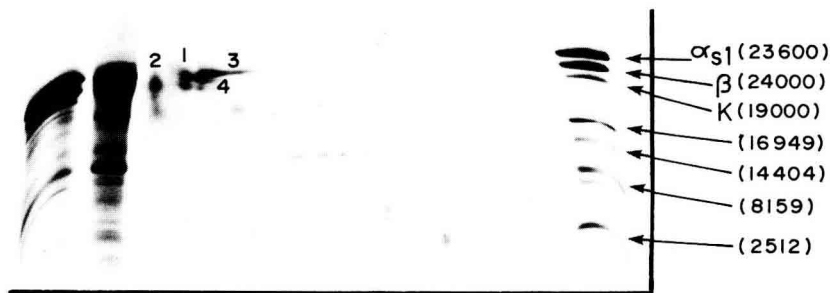


FIG. 8. — Two-dimensional gel electrophoresis of samples obtained with diet B. See legend of figure 7.

## Discussion.

With the two diets, the Phe-Met bond of  $\kappa$ -casein was split very quickly : para- $\kappa$  casein could be observed in the first collected sample. With casein in water,  $\alpha_{s1}$  and  $\beta$ -caseins were present during the first hour after the meal (times 1 to 6). During this first hour,  $\alpha_{s1}$ -casein was partially transformed into a product which migrated farther in PAGE. In IEF, a peptide which migrated as fragment 1-23 of  $\alpha_{s1}$ -casein (Trieu-Cuot, 1981) appeared at a similar rate. These results indicate that during the first hour,  $\alpha_{s1}$ -casein was partly transformed into  $\alpha_{s1}$ -I (Hill *et al.*, 1974). During the same time,  $\beta$ -casein was partly transformed into  $\beta$ -I and  $\beta$ -II (Creamer *et al.*, 1971). Nevertheless, the caseins observed in PAGE at pH 8.6, immediately after the meal, did not migrate exactly as intact caseins did. On the contrary, in PAGE at pH 2.9, their migration was exactly the same as that of intact caseins, and their MW, determined by SDS-PGGE, was similar. These « caseins » seemed to be very close to intact  $\alpha_{s1}$  and  $\beta$ -caseins but were slightly modified, probably by adsorption of endogenous products on the caseins. In particular, glycoproteins of the gastric mucosa contain sialic acid (Buddecke, 1972). The presence of such a product would explain the pattern that we obtained.

The presence of almost intact  $\alpha_{s1}$  and  $\beta$ -caseins during the first 90 min after ingestion could be explained by the absence of casein coagulation in the stomach. Their disappearance after about 1 h 30 min could be due to two mechanisms, *i. e.* a decrease of the pH, leading to casein precipitation, and the degradation of the casein into small peptides. Guilloateau *et al.* (1975) using milk-

fed calves, obtained a stomach pH of 5 after about 1 h 30 min of digestion. At such a pH, the caseins would start to precipitate.

The importance of gastric proteolysis is demonstrated by the appearance of small amounts of a great number of peptides of various sizes, charges and pI's. Around 30 % of the ingested N was emptied during the first 7 postprandial hours (Péllissier *et al.*, 1983). Nevertheless, as the casein diets did not coagulate but were precipitated at acid pH, they displayed particular gastric emptying kinetics. For about 90 min, the major part of the protein products leaving the abomasum corresponded to caseins that were proteolysed slightly or not at all. During this time, only a little more than 10 % of the ingested N arrived in the duodenum (Péllissier *et al.*, 1983). In these conditions, the largest part of the N left the abomasum in a proteolysed form.

The peptides which appeared in the duodenum were very different from intact caseins and appeared in the first sample collected. A fast appearance of peptides with mobilities lower than that of  $\beta$ -casein was observed in PAGE. Only some of these could be  $\gamma$ -caseins. The other peptides have not been identified. The rapid appearance of these products shows that casein proteolysis by gastric enzymes is different *in vivo* than *in vitro*. During *in vitro* proteolysis by gastric enzymes,  $\alpha_{s1}$ -casein is first degraded into  $\alpha_{s1}$ -I casein (Hill *et al.*, 1974);  $\beta$ -casein is successively transformed into  $\beta$ -I,  $\beta$ -II and  $\beta$ -III (Creamer *et al.*, 1971) before undergoing more important proteolysis. At this step,  $\alpha_{s1}$  and  $\beta$ -caseins were completely converted into the peptides mentioned above, prior to the significant appearance of other degradation products. On the contrary, we observed *in vivo* at the same time, little or no proteolysed  $\alpha_{s1}$  and  $\beta$ -caseins and peptides originating from a more intensive proteolysis. The kinetics of casein peptide release thus seems to be different *in vivo* than *in vitro*.

Peptides which appeared in the duodenum after about 3 h of digestion moved in PAGE with the migration front. There are two possible explanations: (1) the peptides were small but still insoluble in 12 % TCA or (2) they had a high number of negative charges. Our results using IEF and SDS-PAGE indicate that both types of peptides were present.

With diet B (casein and minerals) the electrophoretic patterns were not exactly the same. The degradation products examined by the different techniques appeared to be the same, but their kinetics of appearance in the duodenum was different. The widest differences were observed for the peptides which moved with the migration front in PAGE. These products appeared sooner *i. e.* in the first sample collected, indicating that the action of proteolytic enzymes was more significant with diet B. Indeed, the buffer capacity of diet B was certainly higher than that of diet A. On the other hand, some of the minerals present in diet B could have affected proteolysis, which would also be modified by ionic strength. According to Fox and Walley (1971), the *in vitro* proteolysis of  $\beta$ -casein by bovine rennet is strongly inhibited by 5 % NaCl and that of  $\alpha_{s1}$ -casein is strengthened. Although diet B contained only 0.8 % salts, their effect might be intensified *in vivo*, and it would be easier in these conditions for chymosin to act.

These results were obtained with fasted and cannulated calves. Since the dry matter collected from these animals during the first 7 postprandial hours

represented only about 30 % of the dry matter of the ingested meals (Pélicier *et al.*, 1983), a large part of the meal digesta was not analysed in our experiment. Nevertheless, our results seem to show that gastric proteolysis *in vivo* was very intense. On the other hand, salts had a significant effect on casein proteolysis. Hydrolysis was intensified when dietary salts were present. With the techniques used, the peptides obtained with the two diets appeared to be similar but had different appearance kinetics. These results should be confirmed by the isolation and characterization of the peptides collected.

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**Résumé.** *Digestion in vivo du lait dans la caillette du veau. I. — Digestion de la caséine entière.*

Les produits insolubles en TCA 12 % récoltés dans le duodénum proximal de veaux à différents temps après ingestion de solutions de caséine ont été caractérisés par électrophorèse en gel de polyacrylamide, électrofocalisation, électrophorèse en gel à gradient de porosité en présence de SDS.

Avec une solution à 3 % de caséine dans l'eau, la disparition des bandes électrophorétiques correspondant aux caséines  $\alpha_{s1}$  et  $\beta$  est observée après 1 h 30. Après 3 h et jusqu'à 7 h des peptides très acides apparaissent. Avec une solution à 3 % de caséine dans une solution simulant le perméat de lait ultrafiltré, des schémas comparables ont été obtenus. Cependant, les peptides acides apparaissent plus tôt. Ils sont détectés dès le premier échantillon collecté après l'ingestion des repas. Avec les 2 régimes, l'importance de la protéolyse gastrique est mise en évidence par l'apparition d'un grand nombre de peptides de différentes tailles, charges et pH I. D'un autre côté, l'effet du sel sur la protéolyse des caséines a été mise en évidence.

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