N° 27/02.
Chimie.

References

1. L. de Jong, *Neth. Milk Dairy J.* 30 (1976) 242-253.
2. L. de Jong, *Neth. Milk Dairy J.* 31 (1977) 314-327.
3. A. Noomen & H. Mulder, *Neth. Milk Dairy J.* 30 (1976) 230-241.
4. A. Noomen, *Neth. Milk Dairy J.* 31 (1977) 75-102.
5. A. Noomen, *Neth. Milk Dairy J.* 31 (1977) 103-108.
6. L. de Jong, *Neth. Milk Dairy J.* 29 (1975) 162-168.
7. J. Stadhouders, *Milchwissenschaft* 29 (1974) 329-337.
8. A. Noomen, *Neth. Milk Dairy J.* 29 (1975) 153-161.
9. L. de Jong, *Neth. Milk Dairy J.* 32 (1978) 15-25.
10. D. G. Schmidt, P. Walstra & W. Buchheim, *Neth. Milk Dairy J.* 27 (1973) 128-142.
11. L. de Jong & Aliza E. A. de Groot-Mostert, *Neth. Milk Dairy J.* 31 (1977) 296-313.
12. F. M. W. Visser & A. E. A. de Groot-Mostert, *Neth. Milk Dairy J.* 31 (1977) 247-264.
13. P. J. de Koning, Studies on rennin and the genetic variants of casein. Thesis, Amsterdam, 1967. Veenman & Zonen, Wageningen, the Netherlands.
14. R. Djurtoft, B. Foltmann & A. Johansen, *C. r. Trav. Lab. Carlsberg* 34 (1963) 287-298.
15. T. J. Geurts, P. Walstra & H. Mulder, *Neth. Milk Dairy J.* 28 (1974) 102-129.
16. P. Walstra, *Neth. Milk Dairy J.* 23 (1969) 245-249.
17. L. de Jong, A. Noomen & H. Mulder, to be published.
18. D. T. Davies & A. J. R. Law, *J. Dairy Res.* 44 (1977) 213-221.

Neth. Milk Dairy J. 32 (1978): 15-25

Protein breakdown in soft cheese and its relation to consistency. 3. The micellar structure of Meshanger cheese

L. de Jong

Dairying Laboratory, Department of Food Science, Agricultural University, Wageningen, the Netherlands[1]

Received: 31 October 1977

Key words: cheese structure, microscopy, electron microscopy, protein breakdown, consistency

Summary

The structure of Meshanger cheese was studied by fluorescence and interference contrast microscopy as well as electron microscopy. Structural changes in the protein matrix during the ripening are described and compared with those in other soft and in hard cheeses. It is indicated that there are no visible differences in structural changes during the ripening of soft and hard cheese. The consequences concerning cheese ripening and casein micelle structure theory are discussed.

1 Introduction

Of the three major components of cheese, fat, water and protein, the last one primarily constitutes the structural matrix of the cheese. This protein matrix is formed after the proteolytic conversion by rennet of $\varkappa$ casein into para-$\varkappa$ casein (1, 2). The paracasein micelles form a gel in the presence of Ca^{2+} and the curd obtained can be transformed into cheese in many ways. The conditions during this transformation determine the properties and ripening of the cheese.

Meshanger cheese (4, 5, 6) is prepared and ripened under such conditions that its consistency changes very fast; it liquefies within 3 weeks. The softening is governed by the rate of conversion of α_{s1} casein, which constitutes about 38 % of the whole casein (3), into its primary decomposition product α_{s1}-I.

[1] Present address: Netherlands Institute for Dairy Research (NIZO), Ede, the Netherlands.

In previous papers (8, 9) we showed the close relation between the chemical change of α_{s1} casein and the softening of Meshanger cheese; the relation with changes in the micro-structure of the cheese had not yet been dealt with.

The micro-structure of cheese, soft as well as hard, has been studied by light and electron microscopy (10-19). The papers of Knoop et al. (20-23) on Camembert cheese structure and that of Mulder et al. (24) dealing with the structure of hard cheese present a good survey of our present knowledge.

This paper presents a description of the structural changes during the ripening of Meshanger cheese, by various microscopical techniques. Implications concerning casein micelle structure are discussed.

2 Methods and material

2.1 Cheese preparation

The Meshanger cheeses were taken from the normal production in the pilot plant of our laboratory. The cheese was prepared according to Noomen (6).

2.2 Microscopy

2.2.1 Fluorescence microscopy

Cubical samples with edges of a about 5 mm were taken from the centre of the cheeses. With solid carbon dioxide the unfixed cubes were rapidly frozen in a droplet of water on the sample holder of a Reichert microtome. Sections of approximately 2 μm were cut with a pre-cooled knife and stretched on a droplet of water on a microscope slide. Water was used, since no differences were observed between preparations made with water, cheese whey and salt solutions. The sections were air-dried and stained by immersion in a solution of acridine orange (0.1 %). After rinsing with water the air-dried sections were embedded in Canada balsam and studied with a Zeiss WL microscope, equipped for fluorescence with excitation from below. The excitation filter had a cut-off wavelength of about 400 nm; the barrier filter transmitted light above 530 nm. The pictures were photographically recorded.

2.2.2 Interference contrast microscopy

The slices prepared for fluorescence microscopy (see Section 2.2.1) were also studied with the interference contrast technique. We used the Zeiss microscope mentioned before, fitted with Nomarski interference contrast condensers and objectives.

2.2.3 Electron microscopy

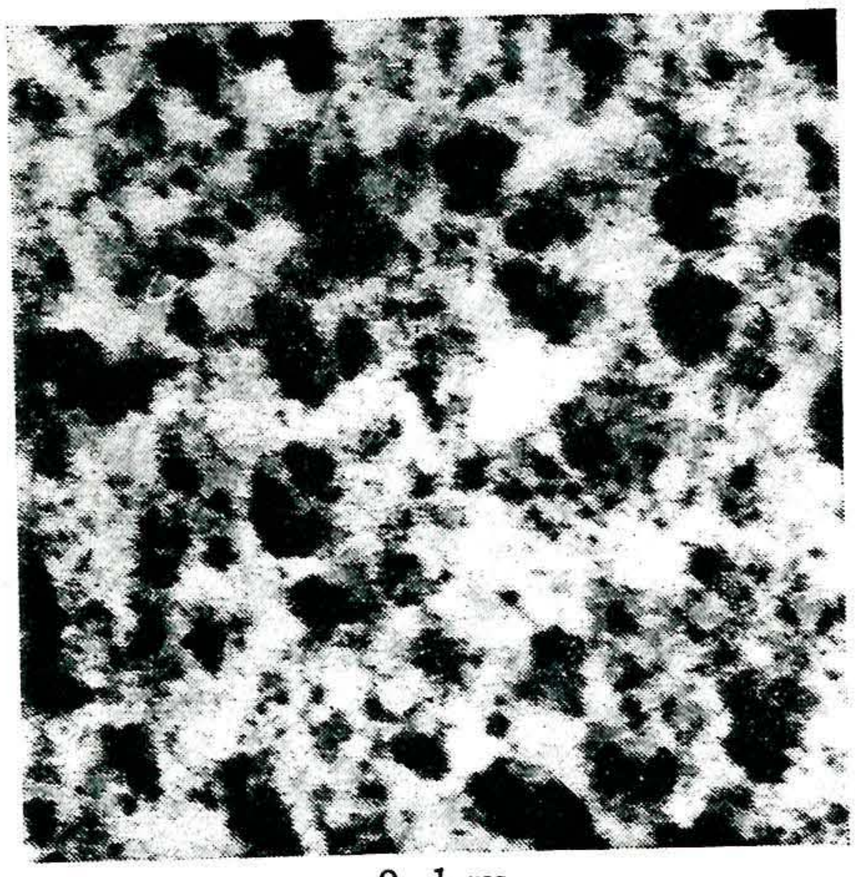
2.2.3.1 Embedding technique. From the centre of pre-cooled cheeses cubes were cut with edges of about 1 mm. After fixation in a solution of 4 % formalin with a pH equal to that of the cheese, the cubes were post-fixed in a 1 % solution of OsO_4 in a veronal buffer with a pH equal to that of the cheese. After one hour the cubes were rinsed with water and dehydrated in a series of ethanol-water mixtures with increasing ethanol concentration. After treatment with propylene oxide the cubes were embedded in Epon 812 and kept for about 12 hours at temperatures of 34, 45 and 60 °C.

Sections ~ 50 nm thick were cut with glass knives on a LKB ultra-microtome Ultrotome III and post-stained with lead citrate according to Reynolds (25). Electron micrographs were made with a Philips EM 300 at the Technical and Physical Engineering Research Service (TFDL), Wageningen, the Netherlands.

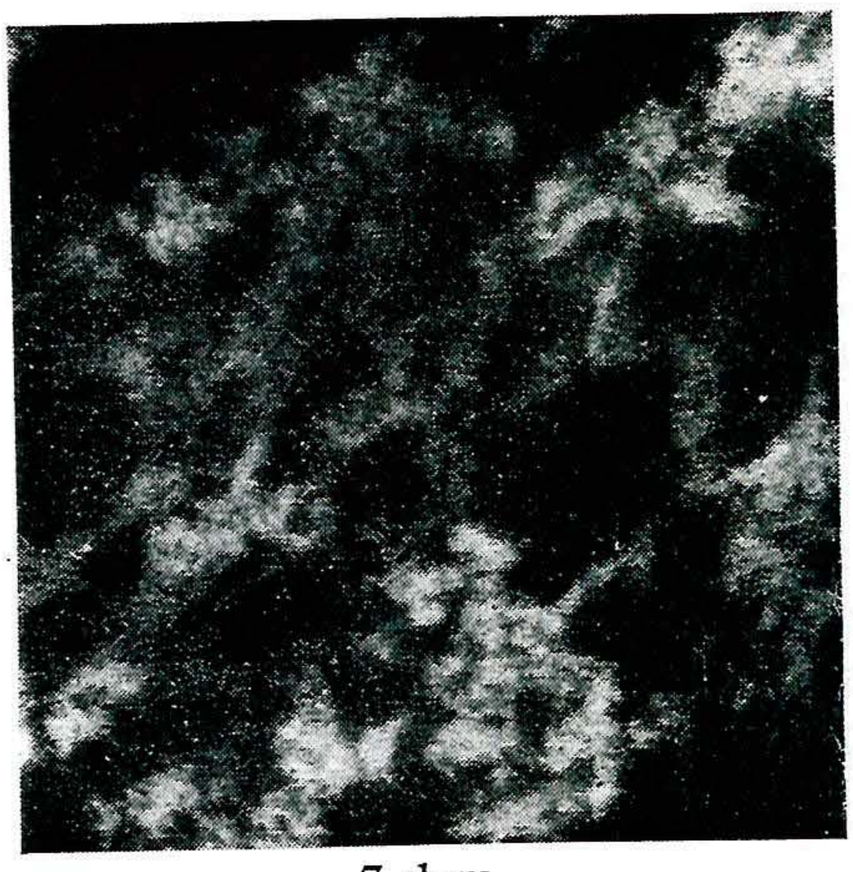
2.2.3.2 Freeze fracturing. Pieces (about 1 × 1 × 1 mm), were cut from the centre of cheeses, mounted on specimen holders, frozen in melting freon and stored in liquid nitrogen. Freeze fracturing was done in a Baltzer freeze etching unit (BAF 301). The fractured surface was coated with a thin layer of Pt and C, approximately 2 nm thick, followed by a supporting layer of C. The replicas were rinsed by immersion in HNO_3 and NH_3 solutions and mounted on uncoated 400 mesh grids. Electron micrographs were taken as described in Section 2.2.3.1.

3 Results

Micrographs of Meshanger cheese at 0, 7, 14, 21 and 29 days after preparation are shown in Fig. 1. The pictures were made by the fluorescence technique described in Section 2.2.1. Although the resolving power with the fluorescence technique is better than in normal light microscopy it is still only of the same order as the average diameter of the paracasein micelles: about 100 nm (7). The picture at 0 days shows that the protein matrix of the cheese contains individual particles sticking together and forming a network. The best impression of this network is seen where small strands of protein 'cross a black hole'. The holes have contained the milk fat globules which have disappeared during the preparation of the slices. The size of the holes, 0.3 - 5 μm, is well within the range mentioned by Mulder & Walstra (27) for milk fat globules: diameter 0.1 - 10 μm. This indicates that the preparation technique does not grossly alter the dimensions of the objects studied. Some of the holes mentioned above may have been filled with whey. The micrographs from cheeses 7 and 14 days old show that the protein part had become more dense. The holes in the matrix

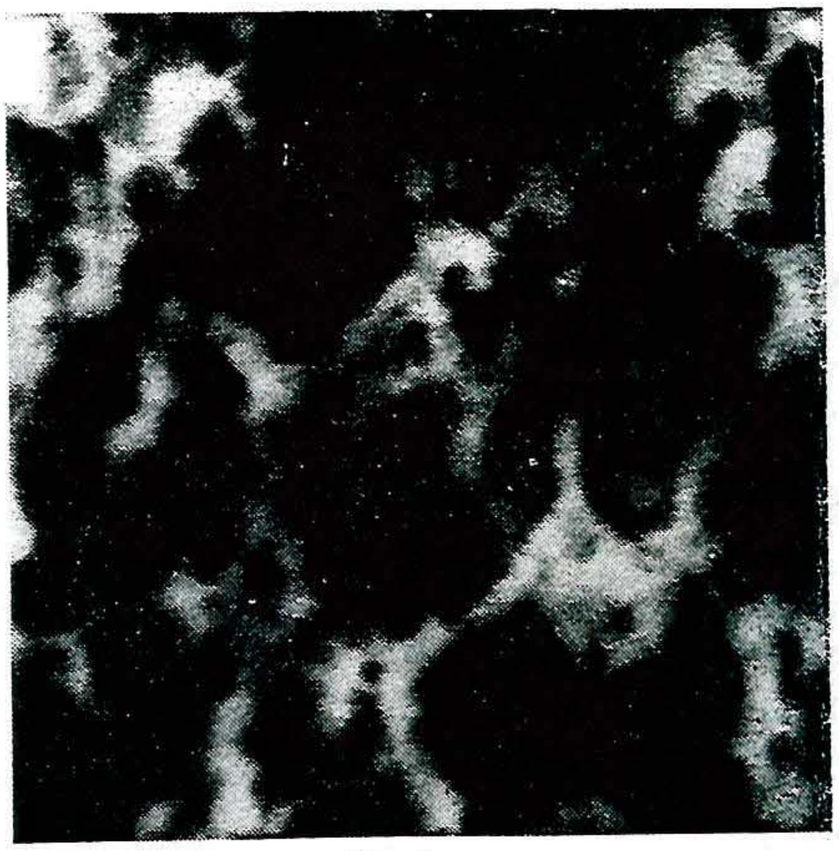


0 days



7 days

14 days



21 days

29 days

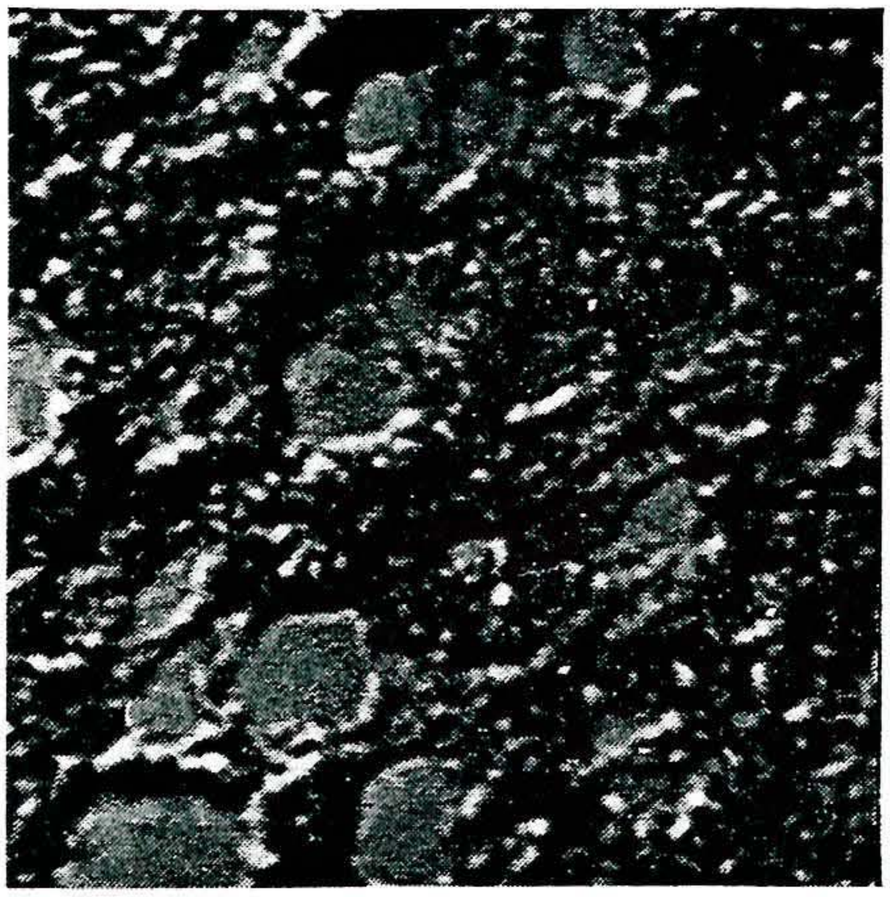
Fig. 1. Fluorescence micrographs of Meshanger cheese. Microscope objective × 63, N.A. 1.4; magnification × 2200.

had grown but the places where the fat globules were present are still recognizable. On the micrographs representing the cheese at 21 and 29 days after preparation, the protein looks like a homogenous mass without a recognizable internal structure. The distances between the protein strands had grown as compared with those in younger cheeses. It might be inferred that parts of the protein matrix had lost their contact with the continuous protein matrix during the ripening of the cheese and had disappeared during the preparation of the sections. The disappearance of the internal structure, due to the proteolysis during cheese ripening, may also be related to the change in consistency reported previously (8, 9).

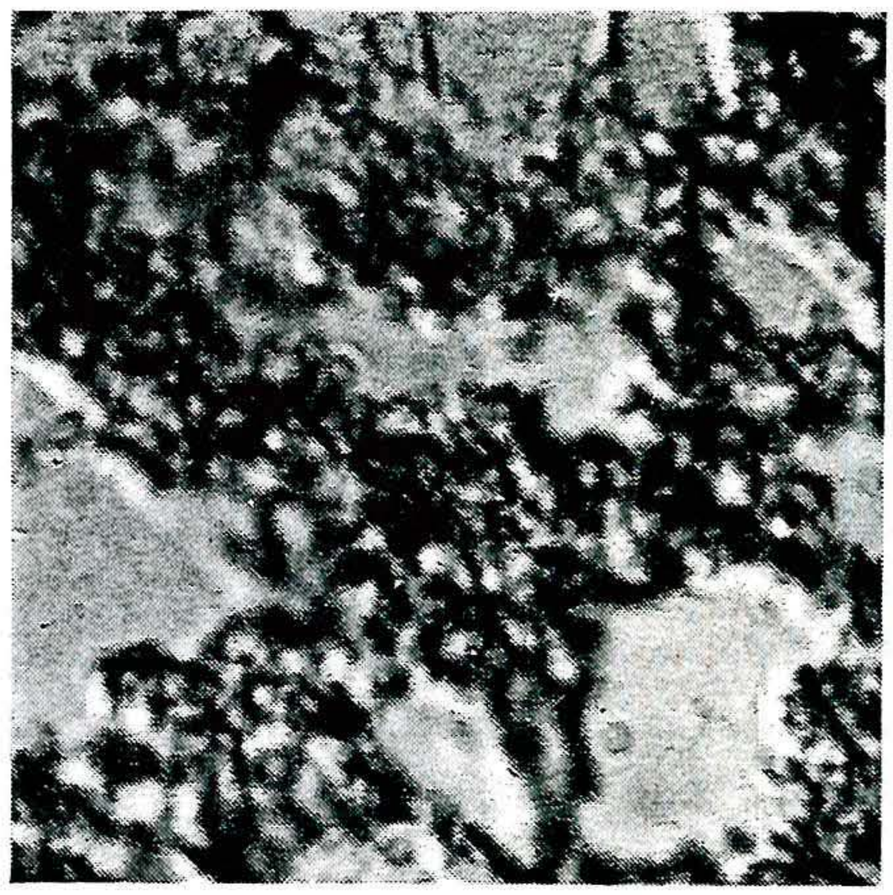
The micrographs obtained by interference contrast microscopy are shown in Fig. 2. The magnification is a little larger than in the fluorescence micrographs. The spherical particles composing the framework of the cheese are clearly detectable on the micrographs representing the curd and the immature cheese (0 and 7 days). The holes from which the fat had disappeared are also very well visible. The transition from a granular structure to a homogeneous mass, without the recognizable elements reported above, was also observed using interference contrast techniques.

Fig. 4 shows the electron micrographs of Meshanger cheese at the ages as indicated, which were obtained by the methods described in Section 2.2.3.1. The picture of the curd (0 days) shows a loose network of clearly recognizable paracasein micelles, in which the remnants of the fat globules and their membranes are to be seen. With further maturing the boundaries between the micelles disappear and the framework seems to become more compact (7 days). This compactness should not be confused with the one reported at the end of the ripening as mentioned above. At the age of 14 days the protein matrix shows a granular structure. The diameter of the spherical particles, which become still better visible at the age of 21 days, differs by about 1 order of magnitude in size from that of the paracasein micelles visible at 0 days. It seems reasonable to identify these particles as a submicellar structure, bearing in mind however that there is a great difference between these particles and the submicelles constituting the paracasein micelles at 0 days (7); an extensive breakdown of α_{s1} casein in particular by rennet has occurred (8). The consequences of this proteolysis will be discussed in the next section. The electron micrograph representing the cheese after 14 days of ripening gives us the impression that the paracasein micelles are losing contact. This in contrast to the decreasing distance observed in the first ripening period, and it may also be caused by the proteolysis already mentioned.

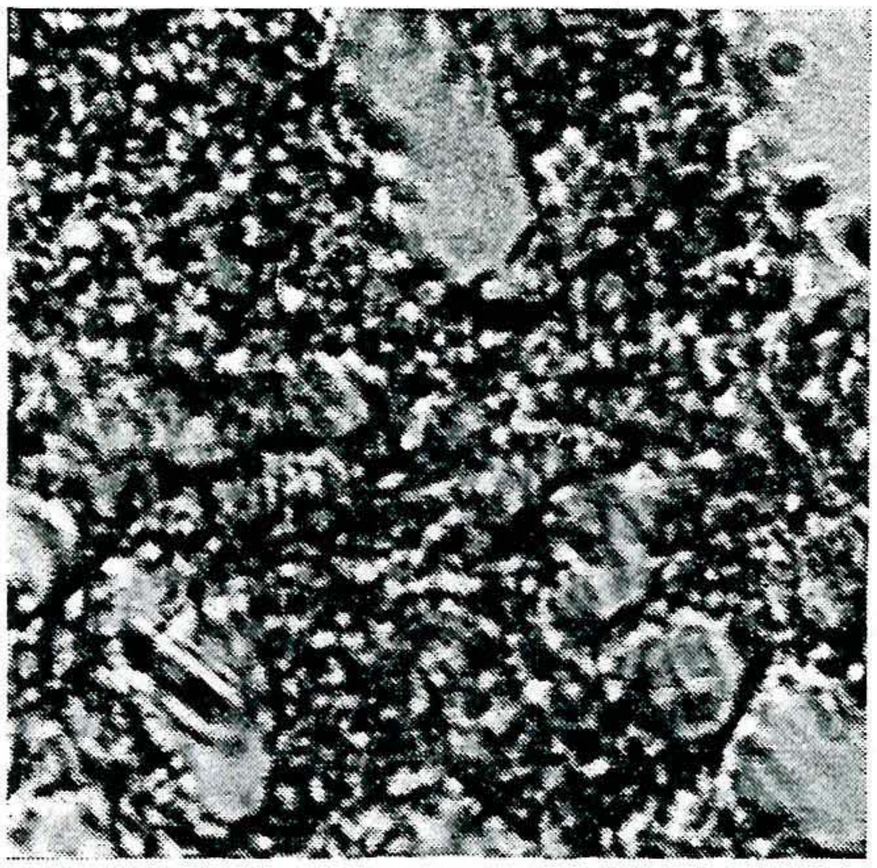
Clearly the structural changes during the ripening of Meshanger cheese appear to be similar to that of Camembert cheese as reported by Knoop &



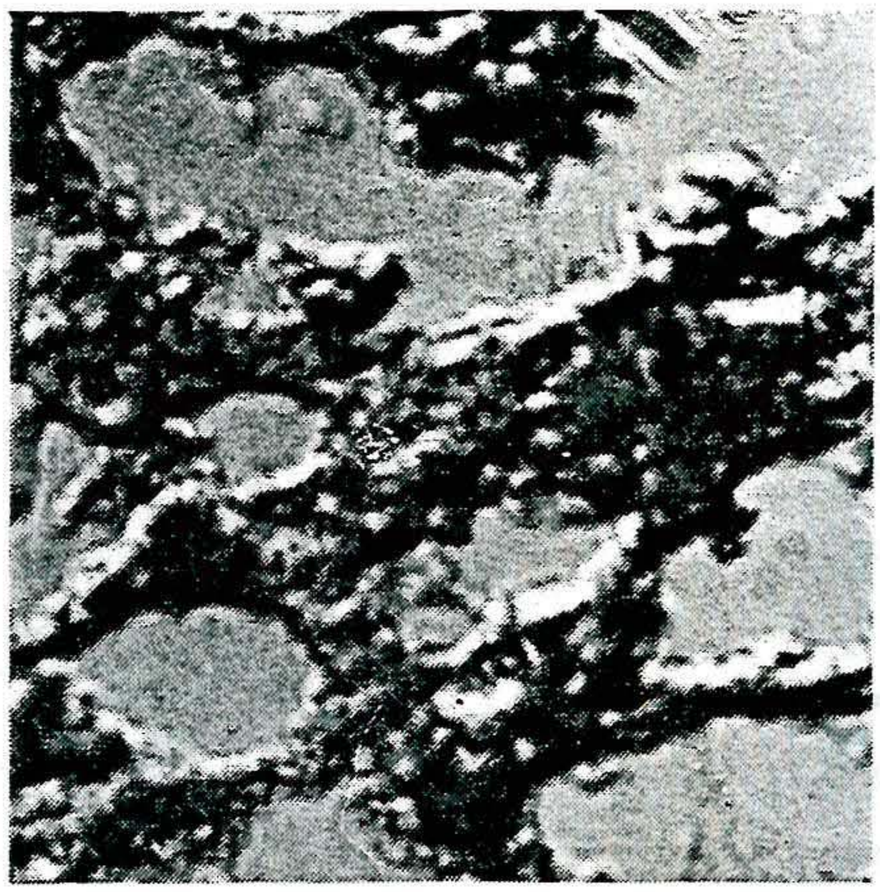
0 days



7 days



14 days



21 days

29 days

Fig. 2. Interference contrast micrographs of Meshanger cheese. Microscope objective × 100, N.A. 1.00; magnification × 3400.

Fig. 3. Meshanger cheese at 28 days of age. Electron micrograph (freeze fracturing) by TFDL. Magnification × 44 000.

Peters (22). Their explanation that the proteolytic enzymes of the surface flora would be responsible for Camembert softening is questionable, as was reported previously (9). Both the electron micrographs and the photomicrographs demonstrate that fat globules exist in the mature cheese, which indicates that contrary to for example Cheddar cheese, the fat is and remains a discontinuous phase during the ripening (14).

Fig. 3 shows an electron micrograph of a sample of Meshanger cheese at the age of 33 days prepared according to the freeze fracturing procedure (Section 2.2.3.2). The picture shows the protein mass and part of a milk fat globule. The protein seems to consist of small spherical units, whose diameter is approximately equal to that of submicelles.

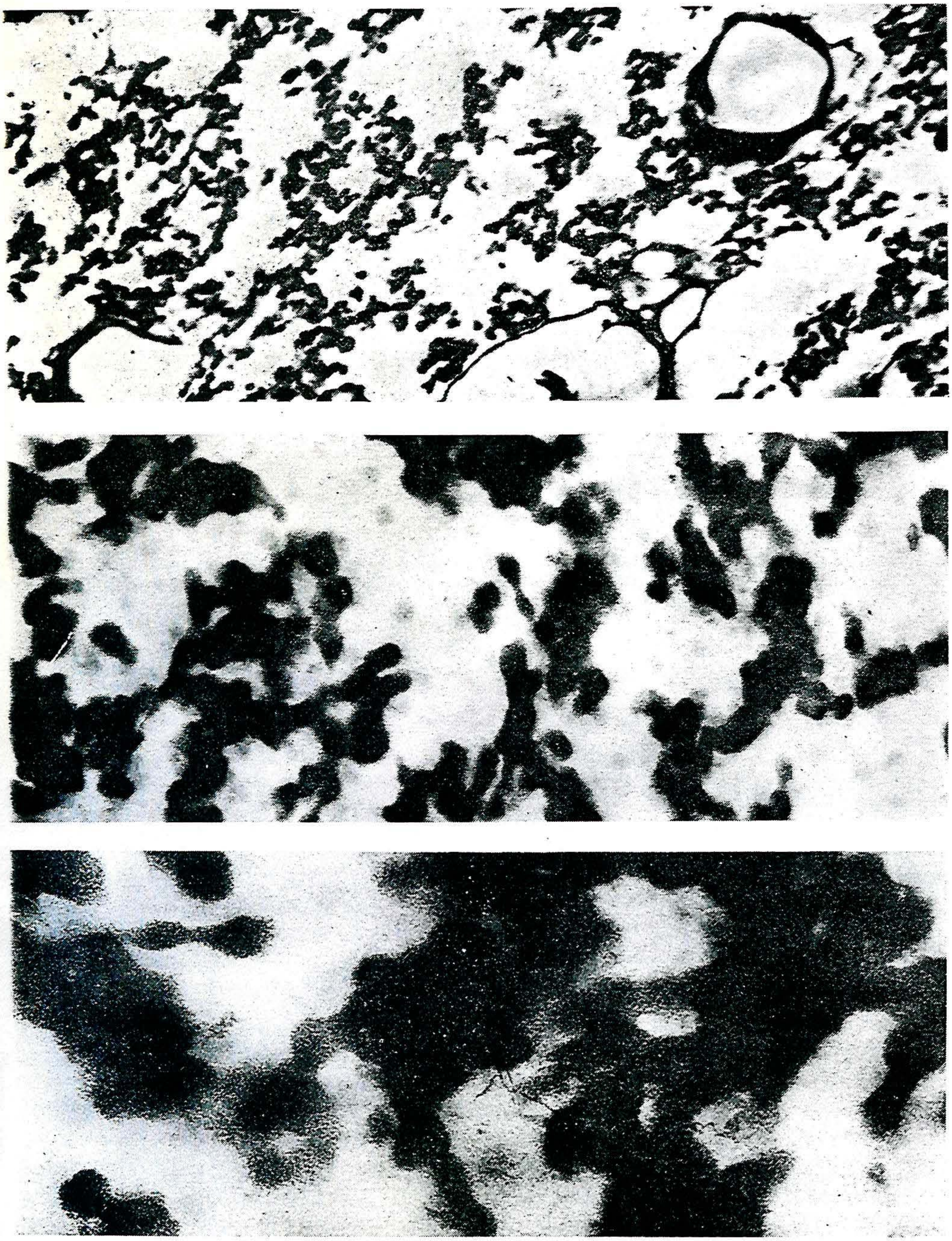
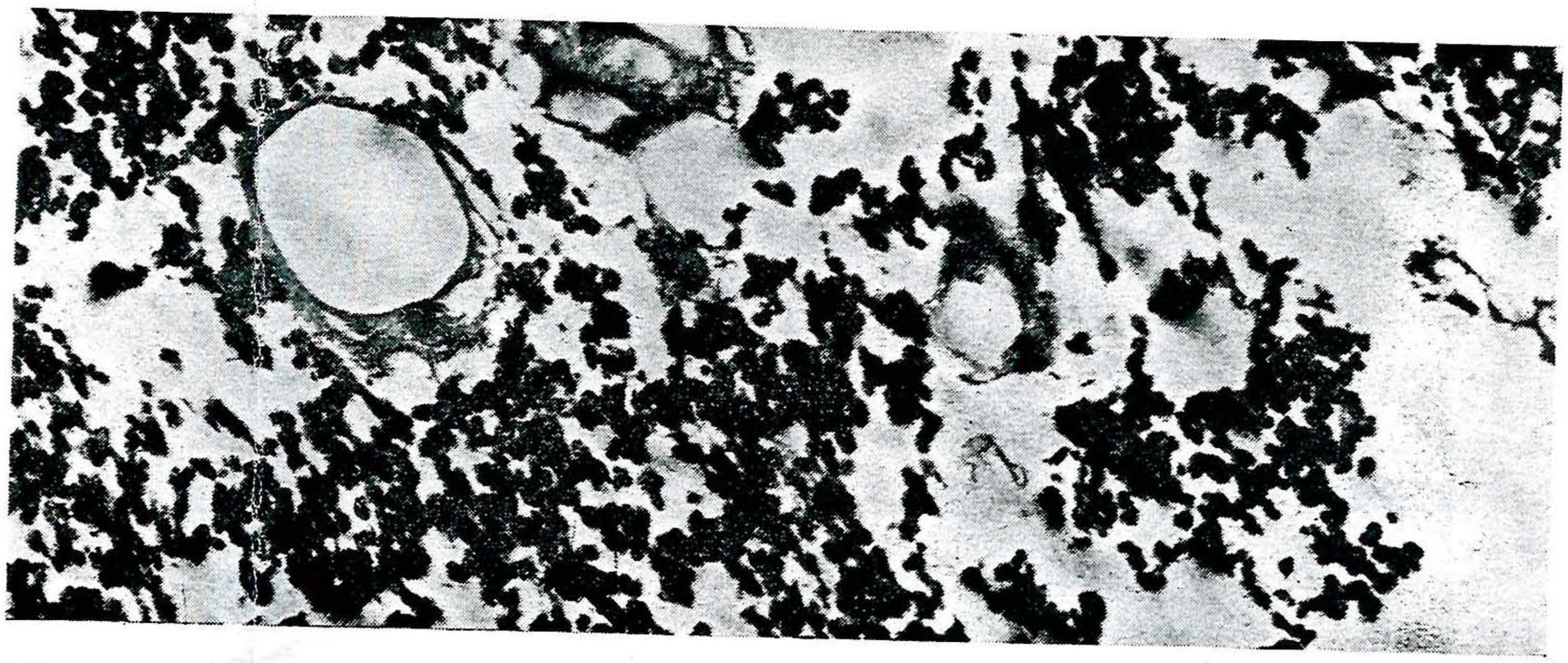
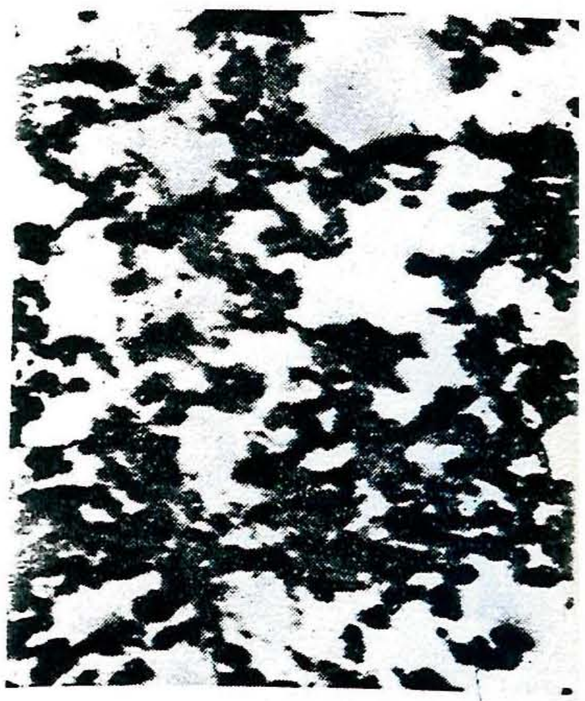
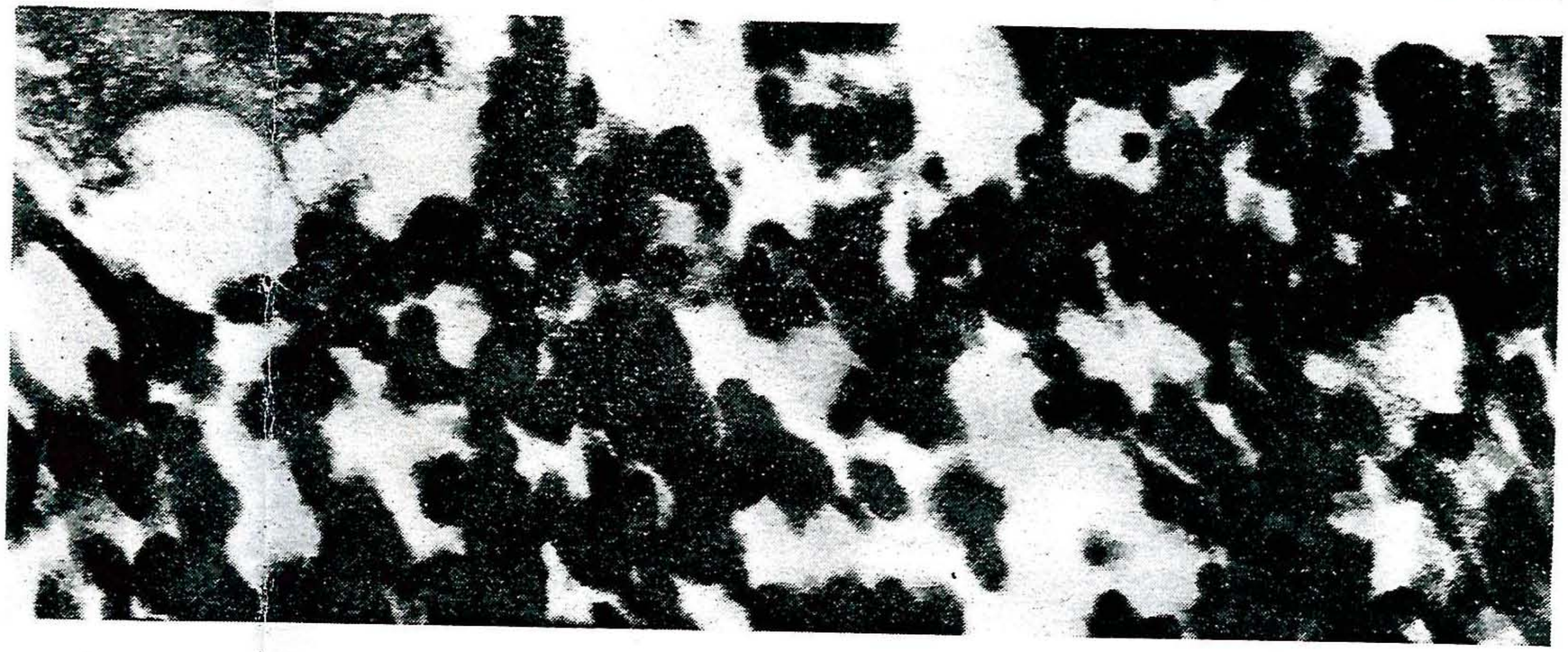
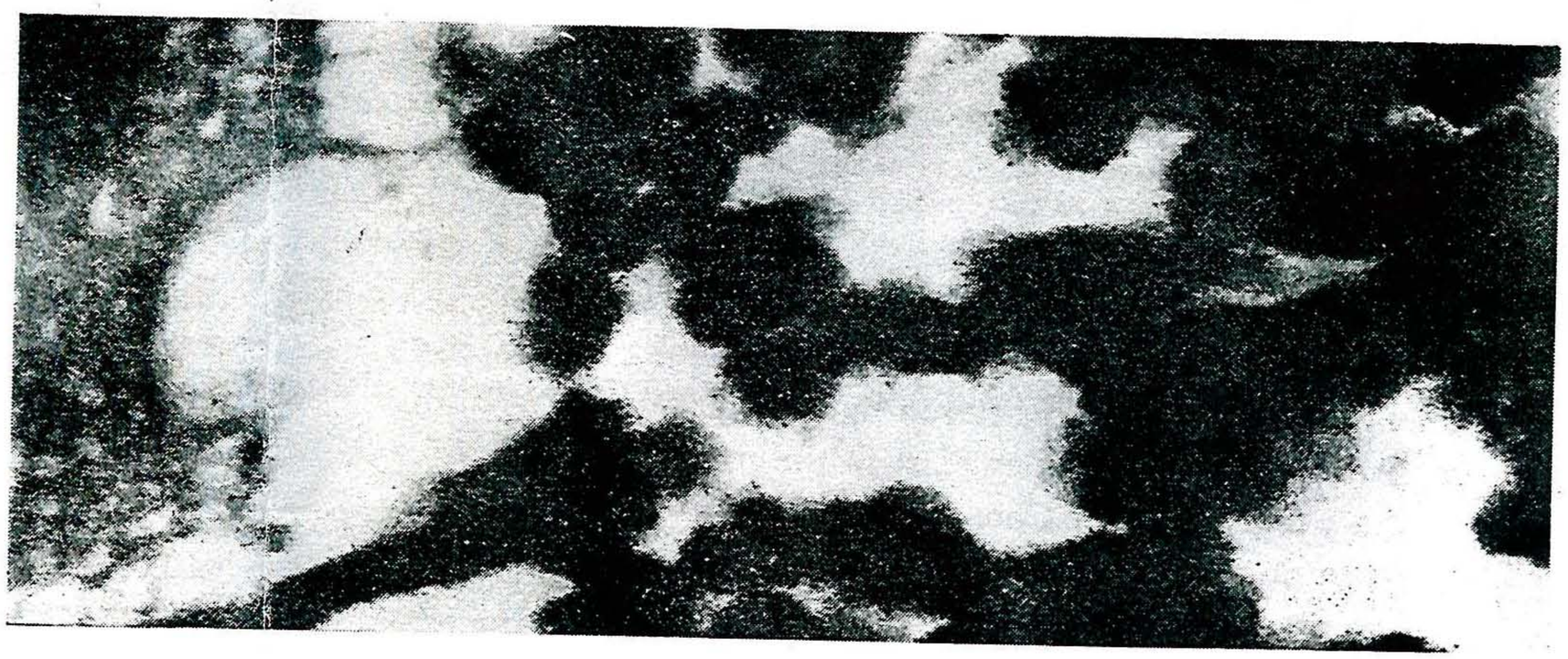


Fig. 4. Meshanger cheese at (from left to right) 0, 7, 14 and 21 days of age. Electron micrographs (embedding technique) by TFDL. Magnification × 10 500 (top), × 34 000 (middle) and × 70 000 (bottom).









4 Discussion

The photomicrographs obtained with the fluorescence and the interference contrast technique both show the same picture of structural changes during the ripening of Meshanger cheese, a soft cheese with a high moisture content. It is striking that these structural changes completely agree with those reported by Mulder et al. (24), concerning Gouda and Edam cheese. In both cases a structure composed of small units changed into a smooth structure without recognizable internal organization.

Fig. 5 shows an electron micrograph of an embedded specimen of Gouda cheese. It is published by courtesy of Dr Schmidt, NIZO, Ede, the Netherlands. Comparison between this picture and Fig. 4 does not reveal any difference between the structure of mature Meshanger and Gouda cheese. Other photos by D. G. Schmidt (to be published) show no difference between immature soft and hard cheese. Meshanger cheese, however, softens while Gouda and Edam cheese do have a change in consistency (29) but without softening.

Since the structural changes during the ripening of hard and soft cheeses seem similar and are governed by the breakdown of their protein, under comparable physico-chemical conditions in the cheese (pH, salt content and Ca ion concentration) and at a similar degree of α_{s1} casein decomposition, the

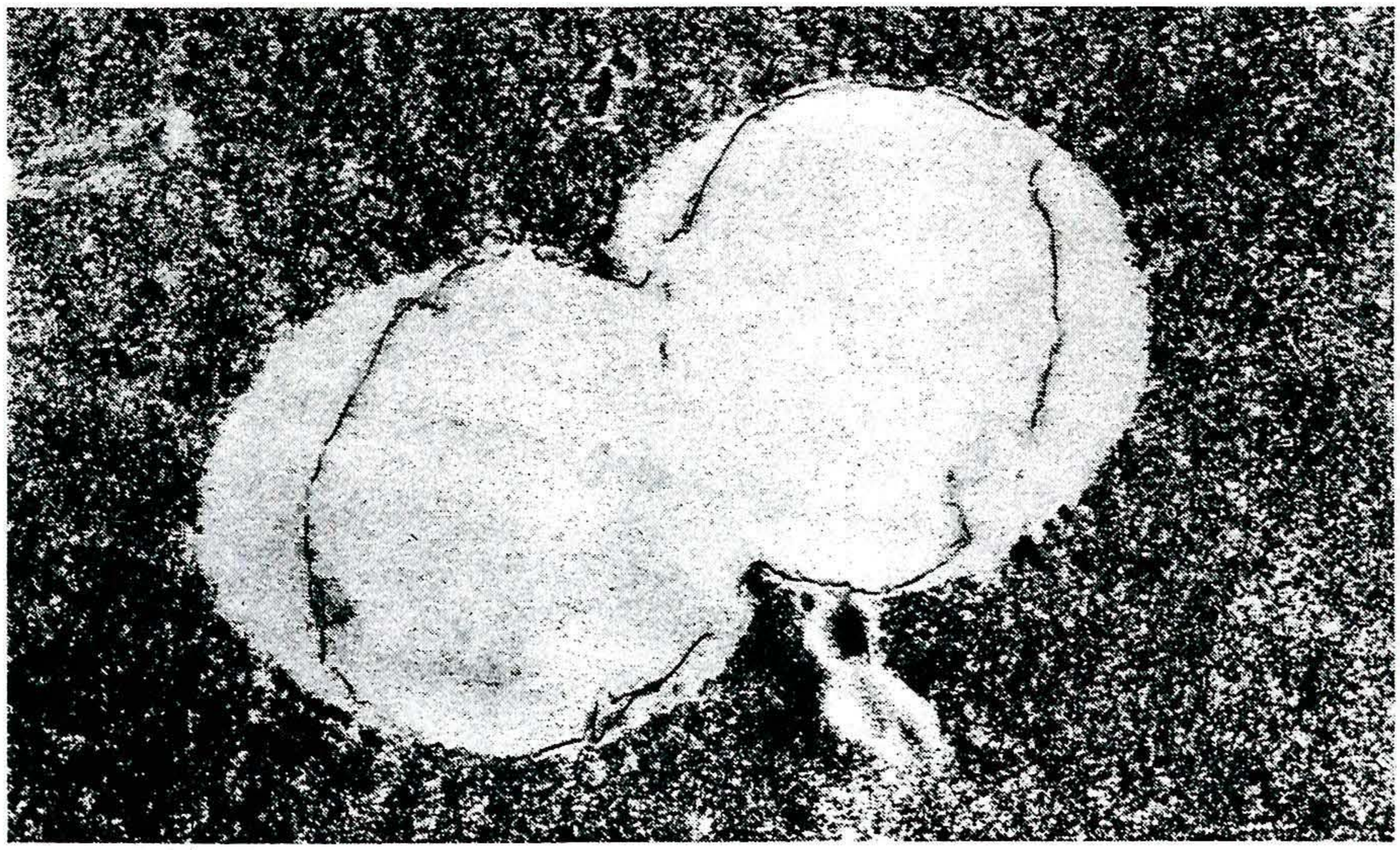


Fig. 5. Gouda cheese (12 weeks of age). Electron micrograph (embedding technique) by TFDL. Magnification × 31 000. By courtesy of Dr D. G. Schmidt (NIZO, Ede, the Netherlands).

moisture content regulates the rheological behaviour of the mass of partly broken down protein.

In a previous paper (9) we reported that Meshanger cheese without active rennet enclosed does not have any perceptible α_{s1} casein breakdown and as a consequence no softening. Unfortunately the technique for preparing 'rennet-free cheese' was not yet available when the research reported in this paper was performed. Therefore no micrographs of those cheeses can be presented at this time. It is likely that those cheeses do not show any structural change after a few days of ripening, and that the structure will remain unaltered. The observations of Knoop & Peters (20) who describe a continuing change of the coagulum in time may be explained by the protein breakdown by rennet, which they did not consider. This proteolysis is considerable (30) and even stronger at higher incubation temperature (31). More experiments with systems in which no active rennet is present should be performed to obtain results which permit definite conclusions to be drawn.

Since the building elements of the protein matrix of the freshly formed cheese are paracasein micelles, understanding the cheese structure requires knowledge of casein micelle structure. The micelle is thought to be constructed of submicelles (7). As has already been mentioned (8, 9) the degree of breakdown of α_{s1} casein, about 38 % of the protein in the micelles, determines the consistency of Meshanger cheese. A few remarks may however be made. Knoop & Peters (28) demonstrated that, in contrast to former opinions, renneted milk did coagulate at low temperatures (4 °C), although very slowly as was to be expected. The coagulum thus formed could not be electron microscopically distinguished from gels produced at higher temperatures. As β casein is said to diffuse to a great extent out of the casein micelle at low temperatures (7, 32-38), this implies on the one hand that β casein does not play an important structural role in the casein micelle and on the other that it favours the idea of α_{s1} casein as a structure former of the casein submicelle.

Schipper (39) reported on the function of phosphate in relation to calcium in the building of the calcium caseinate-calcium phosphate complex in milk. Schmidt & Buchheim (40) and Schmidt et al. (41) have reported that the casein micelle disintegrates into submicelles when the calcium is removed by dialysis. At low pH values calcium is also removed from the micelles which results in their disintegration. Monib (42) has reported that, at pH 5.2, 50-60 % of the calcium present in Edam cheese is dissolved in the cheese serum. This dissolution will cause a change in calcium bonds in the paracasein micelles. However, Meshanger cheese without active rennet enclosed (9) reveals no softening at pH 5.2 whereas normal Meshanger cheese softens rapidly when α_{s1} casein is broken down. Whatever exact mechanism may be respons-

ible for the building of the casein micelle from submicelles, it is inferred from this that apart from calcium phosphate bridges, as postulated by Schmidt & Payens (7), other binding forces between casein submicelles exist.

Acknowledgment

'Stichting J. Mesdagfonds, Kaascontrolestation Friesland' at Leeuwarden, the Netherlands financially supported this work. Electron microscopy was done in co-operation with 'Stichting Technisch Fysische Dienst voor de Landbouw' (Technical and Physical Engineering Research Service (TFDL)) at Wageningen, the Netherlands (Mr S. Henstra and Eugenie Werdmüller von Elgg).

Fig. 5 is published by courtesy of the Netherlands Institute for Dairy Research (NIZO), Ede, the Netherlands (Dr D. G. Schmidt).

Samenvatting

L. de Jong, *Het verband tussen de eiwitafbraak en de consistentie van zachte kaas. 3. De micellaire structuur van Meshanger kaas*

De structuur van Meshanger kaas werd bestudeerd met behulp van zowel de lichtmicroscoop (fluorescentie en interferentie-kontrast) als met de elektronenmicroscoop. De veranderingen in de eiwitstructuur tijdens de rijping werden beschreven en vergeleken met die in andere zachte en harde kazen. Aangegeven wordt dat er geen principiële verschillen bestaan tussen de structuurveranderingen in Meshanger en Goudse kaas. Het reologisch gedrag van deze kazen wordt bepaald door het vochtgehalte, afhankelijk van de mate van afbraak van α_{s1}-caseïne. De implicaties voor de opbouw van het caseïnemicel worden besproken.

References

1. A. G. Mackinlay & R. G. Wake, In: H. A. McKenzie (Ed.), Milk proteins, Vol 2: 175. Academic Press, New York, 1971.
2. R. G. Wake, *Aust. J. biol. Sci.* 12 (1959) 479-489.
3. D. T. Davies & A. J. R. Law, *J. Dairy Res.* 44 (1977) 213-221.
4. A. Noomen & H. Mulder, *Neth. Milk Dairy J.* 30 (1976) 230-241.
5. A. Noomen, *Neth. Milk Dairy J.* 31 (1977) 75-102.
6. A. Noomen, *Neth. Milk Dairy J.* 31 (1977) 103-108.
7. D. G. Schmidt & T. A. J. Payens, Surface and colloid science, Volume 9: 165-229, John Wiley & Sons, 1976.
8. L. de Jong, *Neth. Milk Dairy J.* 30 (1976) 242-253.
9. L. de Jong, *Neth. Milk Dairy J.* 31 (1977) 314-327.
10. E. Hekma, *Milchw. Forsch.* 3 (1926) 350-358.
11. G. Eigel, *Milchwissenschaft* 3 (1948) 46-51.
12. M. Rüegg & B. Blanc, *Schweiz. milchw. Forsch.* 1 (1972) 1-8.
13. E. Knoop, *Milchwissenschaft* 27 (1972) 364-373.
14. D. M. Hall & L. K. Creamer, *N. Z. Jl Dairy Sci. Technol.* 7 (1972) 95-102.

15. M. H. Abd El-Salam & Safinza El-Shibiny, *J. Dairy Res.* 40 (1973) 113-115.
16. M. Rüegg, R. Sieber & B. Blanc, *Schweiz. milchw. Forsch.* 3 (1974) 1-5.
17. K. H. Peters & A. M. Knoop, *Milchwissenschaft* 29 (1974) 193-200.
18. K. H. Peters & A. M. Knoop, *Milchwissenschaft* 30 (1975) 205-209.
19. D. Prokopek, A. M. Knoop & W. Buchheim, *Kieler milchw. ForschBer.* 28 (1976) 245-268.
20. A. M. Knoop & K. H. Peters, *Kieler milchw. ForschBer.* 27 (1975) 315-330.
21. A. M. Knoop, E. Knoop & A. Wiechen, *Neth. Milk Dairy J.* 27 (1973) 121-127.
22. A. M. Knoop & K. H. Peters, *Milchwissenschaft* 26 (1971) 193-198.
23. A. M. Knoop & K. H. Peters, *Milchwissenschaft* 27 (1972) 152-159.
24. H. Mulder, J. J. de Graaf & P. Walstra, *Proc. 17th int. Dairy Congr.* (1966) D2: 413-420.
25. E. S. Reynolds, *J. Cell Biol.* 17 (1963) 208.
26. A. Kimber, B. E. Brooker, D. G. Hobbs & J. H. Prentice, *J. Dairy Res.* 41 (1974) 389-396.
27. H. Mulder & P. Walstra, The milk fat globule: Emulsion science as applied to milk products and comparable foods. CAB, Farnham Royal, Bucks., England/Pudoc, Wageningen, the Netherlands, 1974.
28. A. M. Knoop & K. H. Peters, *Milchwissenschaft* 31 (1976) 338-345.
29. H. Mulder, *Versl. landbouwk. Onderzoek* 51 (1945) 467-487.
30. L. de Jong & Aliza E. A. de Groot-Mostert, *Neth. Milk Dairy J.* 31 (1977) 296-313.
31. P. F. Fox, *J. Dairy Sci.* 52 (1969) 1214-1218.
32. T. A. J. Payens & B. W. van Markwijk, *Biochim. biophys. Acta* 71 (1963) 517-530.
33. R. A. Sullivan, M. M. Fitzpatrick, E. K. Santan, R. Annino, G. Kissel & F. Palermiti, *Arch. Biochem. Biophys.* 55 (1955) 455-468.
34. C. W. Kolar, Jr. & J. R. Brunner, *J. Dairy Sci.* 50 (1967) 941.
35. D. Rose, *J. Dairy Sci.* 51 (1968) 1897-1902.
36. W. K. Downey & R. F. Murphy, *J. Dairy Res.* 37 (1970) 361-372.
37. W. K. Downey, *Neth. Milk Dairy J.* 27 (1973) 218-219.
38. L. K. Creamer, Gillian P. Berry & O. E. Mills, *N. Z. Jl Dairy Sci. Technol.* 12 (1977) 58-66.
39. C. J. Schipper, Het caseïnaatphosphaatcomplex van melk. Thesis, Wageningen, 1961.
40. D. G. Schmidt & W. Buchheim, *Milchwissenschaft* 25 (1970) 596-600.
41. D. G. Schmidt, P. Walstra & W. Buchheim, *Neth. Milk Dairy J.* 27 (1973) 128-142.
42. A. M. M. F. Monib, The calcium-paracaseinate-phosphatecomplex under conditions similar to those in cheese. Thesis, Wageningen, 1962.