Proteolysis in soft cheese, studied on Meshanger cheese and cheese models

Promotor: dr. H. Mulder, oud-hoogleraar in de zuivelbereiding en de melkkunde.

A. Noomen

Proteolysis in soft cheese, studied on Meshanger cheese and cheese models

Proefschrift
ter verkrijging van de graad van
doctor in de landbouwwetenschappen,
op gezag van de rector magnificus,
dr. H. C. van der Plas,
hoogleraar in de organische scheikunde,
in het openbaar te verdedigen
op woensdag 17 mei 1978
des namiddags te vier uur in de aula
van de Landbouwhogeschool te Wageningen

Centre for Agricultural Publishing and Documentation

Wageningen – 1978

Abstract

Noomen, A. (1978) Proteolysis in soft cheese, studied on Meshanger cheese and cheese models. Doctoral thesis, Wageningen. (viii) + 17 p., 2 figs, 40 refs, Eng. and Dutch summaries. Agric. Res. Rep. 875, p. 3-4; Neth. Milk Dairy J. 30 (1976): 230-241; 31 (1977): 75-102; 31 (1977): 103-108; 31 (1977): 163-176; 32 (1978): 26-48; 32 (1978): 49-68; 29 (1975): 153-161.
Also: Agric. Res. Rep. (Versl. landbouwk. Onderz.) 875 p. 5-25.

Enzymes predominantly responsible for the primary degradation of protein in soft cheese and for the related changes in consistency were studied. Reconstructed Noordhollandse Meshanger cheese and preserved simulated soft cheeses of different composition were used as models in the investigation. Results for proteolysis in the simulated cheeses were comparable and also comparable to those observed with normal Meshanger cheese. Protein breakdown was studied by estimating the amount of nitrogen soluble in the moisture of cheese and by quantitative polyacrylamide gel electrophoresis. Results of the two methods were well correlated. Proteolysis, which is primarily responsible for changes in consistency of soft cheeses, was caused mainly by calf rennet enzymes. The activity of rennet at different pH and concentrations of NaCl in the moisture of cheese also revealed a major role of rennet enzymes in protein breakdown in soft cheeses with an initially very low pH, ripening under the influence of a surface flora. The role of the surface flora is merely to regulate pH and so to soften the cheese body, and to give the cheese a specific flavour.

Milk protease activity in soft cheese was studied in relation to pH, concentration of NaCl in moisture, ripening time and ripening temperature of the cheese. Its contribution to soft cheese ripening is minor, perhaps except for certain cheeses with a surface flora. Milk protease showed considerable activity in milk at favourable temperatures; proteolysis increased when cheese milk was subjected to low-temperature pasteurization.

Free descriptors; cheese varieties, Noordhollandse Meshanger cheese, soft cheese, cheese ripening, proteolysis, consistency, cheese model substrates, enzymes, rennet, milk protease, gel electrophoresis of cheese, extraction method for soluble N, effect of pasteurization of milk on milk protease activity.

This thesis will also be published as Agricultural Research Reports 875.

Stellingen

1. Stremsel speelt een hoofdrol bij de rijping van kaassoorten met een week zuivel.

Dit proefschrift.

2. De mate waarin melkprotease in koemelk proteolytisch werkzaam kan zijn, is in het algemeen sterk onderschat.

o.a. E. H. Reimerdes, H. Klostermeyer & J. Thomasow, Kieler Milchw. ForschBer. 26 (1974): 373-384.
Dit proefschrift.

3. De selectieve eigenschappen van gebruikelijke acetaatvoedingsbodems voor het tellen van lactobacillen in melk en melkprodukten kunnen belangrijk worden verbeterd door aan deze bodems 2,4-dinitrofenol toe te voegen en de telplaten te voorzien van een deklaag van hetzelfde medium.

M. Rogosa, J. A. Mitchell & R. F. Wiseman, J. dent. Res. 30(1951): 682-689.
L. A. Mabbitt & M. Zielinska, J. appl. Bact. 19(1956): 95-101.
Th. E. Galesloot & F. Hassing, Neth. Milk Dairy J. 18(1964): 177-181.

4. De vervloeiing die zich tijdens de rijping van bepaalde kaassoorten van buiten naar binnen voortzet, behoeft niet in verband te staan met de proteolytische activiteit van de oppervlakteflora.

A. M. Knoop & K. H. Peters, Milchwissenschaft 26(1971): 193-198.

5. De methode van de 'replica-plaat' zoals toegepast door Maling kan tot verkeerde resultaten leiden; bijvoorbeeld bij de bepaling van factoren die de groei van *Leuconostoc mesenteroides* remmen.

B. D. Maling, J. gen. Microbiol. 23(1960): 257-260.

6. Bezien tegen de achtergrond van de belangrijke functie van voedingsvezels in de menselijke voeding, kan de in Nederland zeer grote consumptie van appelmoes als gunstig worden gekwalificeerd.

Näringsforskning 20(1976), Supplement Nr. 14, Food and Fibre.

7. Sinds meer bekend is geworden over de thermoresistentie van melkprotease, moet men eraan twijfelen of door middel van kort-hoog verhitten (bijvoorbeeld 4 s, 140 °C) wel echt duurzame melk kan worden verkregen.

T. H. M. Snoeren & P. H. J. Evers, Zuivelzicht 70(1978): 144-145.
J. Ged & C. Alais, Lait 56(1976): 645-656.

8. De door El-Erian toegepaste methodiek ter bepaling van het gehalte aan zogenaamd oplosbare stikstof en aminozuurstikstof in kaas, is niet juist.

A. M. F. El-Erian, Meded. Landbouwhogesch. Wageningen 69-12 (1969).

9. De, overigens juiste, bewering van Rexová-Benková en Tibenský dat exo- en endopolygalacturonase van elkaar kunnen worden gescheiden op een kolom van verknoopt pectaat, wordt door de gegevens in hun tabel niet ondersteund.

L. Rexová-Benková & V. Tibenský, Biochim. biophys. Acta 268(1972): 187-193.

10. Bij een toeneming van de consumptie van UHT-melk in ons land verdient het met het oog op de voedingswaarde van deze melk aanbeveling de produktiewijze zodanig te kiezen dat de vitamines B_{12} en foliumzuur zoveel mogelijk behouden blijven.

G. Loggers, Voeding 37(1976): 454-462.
J. W. G. Porter, Milk and Dairy Foods, Oxford University Press, London, 1975.

11. Het is geenszins zeker dat de biologische functie van alle in koemelk aanwezige bactericide of bacteriostatische stoffen de directe bescherming van het kalf zou zijn.

B. Reiter, J. Dairy Res. 45(1978): 131-147.

Proefschrift van A. Noomen
Proteolysis in soft cheese, studied on Meshanger cheese and cheese models
Wageningen, 17 mei 1978

Curriculum vitae

De auteur werd geboren te Haastrecht op 17 september 1937. Hij behaalde het diploma HBS-B aan de Rijks Hogere Burgerschool te Gouda in 1954. Hierna begon hij zijn studie aan de Landbouwhogeschool te Wageningen, waarbij als studierichting de Zuivelbereiding en melkkunde werd gekozen. In 1964 werd het ingenieursdiploma behaald, met als hoofdvak de Zuivelbereiding en melkkunde (verzwaard) en als keuzevakken de Organische scheikunde en de Microbiologie. Sindsdien is hij verbonden aan de sectie Zuiveltechnologie en melkkunde van de vakgroep Levensmiddelentechnologie.

Woord van dank

Bij het gereedkomen van dit proefschrift betuig ik mijn bijzondere dank aan mijn promotor, prof. dr. H. Mulder, voor zijn adviezen en aanmoedigingen tijdens het onderzoek en voor de prettige jaren waarin ik met hem heb mogen samenwerken.

Voorts wil ik al degenen bedanken die medewerking hebben verleend bij het voorbereiden en uitvoeren van de experimenten, het gereedmaken van de manuscripten en de redactie van de artikelen en het proefschrift.

De prettige contacten met personeelsleden van de vakgroep Levensmiddelentechnologie, in het bijzonder met die van de sectie Zuiveltechnologie en melkkunde, heb ik als bijzonder stimulerend ervaren. Mijn dank gaat ook uit naar mijn gezin, familie en vrienden, die mij tot grote steun zijn geweest.

Contents

Proteolytic activity of milk protease in raw and pasteurized cow's milk
A. Noomen, Neth. Milk Dairy J. 29 (1975): 153-161.

Introduction

1 Purpose of the investigation

Proteolysis in cheese is a major process in cheese ripening. In our laboratory the crucial importance of the process initiated several studies on aspects of protein breakdown in cheese and phenomena related to that (1, 2, 3, 4).

During the ripening of cheese, the original rubbery coarse curd is converted into a smoother mass. These changes in consistency influence markedly the sensoric quality of cheese, and are most quickly perceptible in cheeses with a high moisture content. The study mainly deals with the ripening of soft cheese with particular reference to the question which enzymes are predominantly involved in the primary breakdown of protein and that may contribute to changes in the cheese consistency. Rheological properties of cheese as such were studied by de Jong (5).

2 Meshanger cheese as a model

Cheeses with a high moisture content can be classed into those which have a surface flora and where softening usually proceeds from the outside inwards (e.g. Camembert and Brie cheese), and those which may lack a surface flora and become soft throughout (e.g. Kernhem, Butterkäse, Bel Paese and St. Paulin cheese).

Before this study some work had already been done on Noordhollandse Meshanger cheese, a soft cheese formerly made on some farms in the Province of Noord-Holland. It became extinct about 1940, and knowledge of its production was almost completely lost. A surface flora, mainly of yeasts and moulds, seemed to develop spontaneously on its surface.

Unlike most other soft cheeses, softening was said to proceed from the centre outwards. The cheese had been simple to make and ripened in about two weeks, it had a mild flavour. It therefore seemed attractive as a model. The method of producing the cheese was reconstructed and then adjusted it to the needs of the investigation. Some parts of the original process were modified to suit modern methods of cheesemaking, and could be used if the cheese is ever produced industrially.

3 Estimation of protein breakdown

The study required a choice of methods for observing protein degradation in cheese. Results from the traditional methods, based on solubility of products of protein breakdown in liquids of different composition, do not necessarily reflect the total amount of nitrogenous substances soluble in cheese moisture. Since the undissolved, and hence the dissolved nitrogen compounds are associated with the consistency of cheese, a method was needed that reflected total soluble nitrogen. A simple method was developed.

During the work with model substrates, the degradation of protein was also estimated by polyacrylamide gel electrophoresis (6).

4 Proteolysis in Meshanger cheese

For proteolysis, surface flora, lactic acid bacteria, calf rennet used in making the cheese and native milk protease were all sources of proteolytic activity. The contribution of each source was tested by comparing ripening of a normal cheese with that of a cheese without surface flora and without either surface flora or bacteria. Native milk protease was present in all types. Protein breakdown and changes in consistency during ripening were largely attributable to calf rennet.

5 Rennet activity in simulated cheese

Conditions for action of enzymes may vary considerably between varieties of cheese. Even in a single variety conditions change during the ripening, for example in soft cheeses that have an initially very low pH and that ripen under the influence of a surface flora. An important question was how far proteolytic activity of calf rennet was influenced by different conditions, in particular pH and concentration of NaCl, which may be present in soft cheeses. In theory, cheese itself must be used as substrate. In our laboratory, an aseptic technique for making cheese with only rennet and milk protease as active proteolytic agents was developed (7, 8). However, the technique is laborious and it would be difficult to vary a single factor, particularly in soft cheeses. Since the study did not include sensory tests, model substrates were considered more convenient.

Results obtained with model substrates showing conditions far remote from those in cheese are difficult to interpret for the actual progress of proteolytic processes in cheese (e.g. 9, 10, 11, 12, 13, 14). Therefore, the model substrates should as closely as possible resemble conditions in cheese, at least in the essential points. With this in mind, cheese was simulated with a model that fitted as closely as possible the situation in Meshanger cheese. The simulated

cheeses were made up from the most essential components of interest in the study: calcium paracaseinate–calcium phosphate complex, water, lactic acid and NaCl. For comparison, simulated cheeses were also prepared with cheese curd. Proteolytic activity of microbial sources was prevented. The cheeses were effectively preserved by the use of thimerosal – ((carboxyphenyl) thio) ethylmercury sodium salt – at a concentration of 100 mg per litre of moisture, in combination with anaerobic storage. The preservative hardly influenced the proteolytic activity of the rennet investigated.

6 Activity of milk protease in simulated cheese and milk

Milk protease is a natural component of cow's milk (e.g. 15, 16, 17). Its activity was generally assumed to be low. The enzyme is closely associated with casein micelles and with acid-precipitated casein (18, 19, 20), and will therefore finish up in cheese. Its contribution to protein breakdown in cheese has received little attention (4, 21, 22).

Preliminary experiments revealed a considerable activity of milk protease in Meshanger – like systems of pH 6.6, preserved with thimerosal. So activity of milk protease was investigated in simulated soft cheeses at different pH and concentration of NaCl. The cheeses were prepared either with rennet-free cheese curd or with a rennet-free calcium paracaseinate–calcium phosphate complex, made by the rennet-free technique developed at our laboratory (23). The 'curd' and the complex contained the milk protease associated with the casein micelles of the milk from which they were made.

Estimation of proteolytic activity of milk protease has most frequently been based on the estimation of the amount of tyrosine liberated in certain substrates. This method is not sensitive (18, 24). The reported weak activity of milk protease in milk could thus be partly due to the low sensitivity of formerly applied methods. With modification, the method developed to measure the amount of soluble nitrogen compounds in cheese was also suitable for study of protein breakdown in milk. With this method and by electrophoresis, proteolysis was studied in preserved aseptically drawn cow's milk (7), containing less than 30 bacteria per millilitre. Protein breakdown thus could be exclusively attributed to milk protease. In former experiments a contribution to protein breakdown from proteolytic bacteria was possible.

For reasons of public health and avoidance of cheese defects, Dutch cheese manufacturers usually pasteurize cheese milk at 72 °C for about 15 s. Milk protease is considerably heat resistant and at least partially survives moderate heat treatment of milk (e.g. 16, 17,). However, there are no clear data in the literature on how far its proteolytic activity in milk, and thus possibly in cheese, is influenced by pasteurization. To gain more information, protein breakdown was studied in well preserved raw and pasteurized samples of

aseptically drawn milks. The raw milks contained less than 30 bacteria per millilitre. Any contribution to proteolysis by sources other than milk protease was thus excluded.

References

1. H. Mulder. Taste and flavour forming substances in cheese. Neth. Milk Dairy J. 6 (1952) 157-168.
2. L. A. M. Ali. The amino acid content of Edam cheese and its relation to flavour. Thesis. Agricultural University Wageningen. Meded. Landbouwhogesch. Wageningen 60, 1960.
3. G. Kleter. Methods for strictly aseptic making of cheese and effect of some bacteria on its ripening. Thesis. Agricultural University Wageningen. Agric. Res. Rep. 870 (1977) I-1 – I-24.
4. F. M. W. Visser. Contribution of enzymes from rennet, starter bacteria and milk to proteolysis and flavour development in Gouda cheese. Thesis. Agricultural University Wageningen. Agric. Res. Rep. 875 (1978) II-1 – II-32.
5. L. de Jong. Proteolysis and consistency of Meshanger cheese. Thesis. Agricultural University Wageningen. Agric. Res. Rep. 875 (1978) 27-43.
6. L. de Jong. A quantitative electrophoretic method of studying cheese ripening. Neth. Milk Dairy J. 29 (1975) 162-168.
7. G. Kleter & Tj. de Vries. Aseptic milking of cows. Neth. Milk Dairy J. 28 (1974) 212-219.
8. G. Kleter. Apparatus for making cheese under strict aseptic conditions. Neth. Milk Dairy J. 29 (1975) 295-302.
9. L. de Jong & Aliza E. A. de Groot-Mostert. The proteolytic action of rennet on different casein substrates under various conditions. Neth. Milk Dairy J. 31 (1977) 296-313.
10. L. K. Creamer. Beta-casein Hydrolysis in Cheddar cheese ripening. N.Z. J. Dairy Sci. Technol. 6 (1971) 91.
11. P. F. Fox. Influence of temperature and pH on the Proteolytic Activity of Rennet Extract. J. Dairy Sci. 52 (1969) 1214-1218.
12. P. F. Fox & B. F. Walley. Influence of sodium chloride on the proteolysis of casein by rennet and by pepsin. J. Dairy Res. 38 (1971) 165-170.
13. P. F. Fox & J. Guiney. Casein micelle structure: Susceptibility of various casein systems to proteolysis. J. Dairy Res. 40 (1973) 229-234.
14. J. A. Phelan, J. Guiney & P. F. Fox. Proteolysis of β-casein in Cheddar cheese. J. Dairy Res. 40 (1973) 105-112.
15. W. J. Harper, J. A. Robertson, Jr. & I. A. Gould. Observations on milk protease. J. Dairy Sci. 43 (1960) 1850-1851.
16. F. Kiermeier & G. Semper. Über das Vorkommen eines proteolytischen Enzyms und eines Trypsin-Inhibitors in Kuhmilch. I. Mitteilung: Zur proteolytischen Wirkung. Z. Lebensm. Unters. Forsch. 3 (1960) 282-307.
17. J. R. Dulley. Bovine milk protease. J. Dairy Res. 39 (1972) 1-9.
18. E. H. Reimerdes & H. Klostermeyer. Milchproteasen I. Micellenassoziierte Protease – Anreicherung und Hydrolyse von Caseinen. Milchwissenschaft 29 (1974) 517-522.
19. R. C. Warner & E. Polis. On the presence of a Proteolytic Enzyme in Casein. J. Amer. Chem. Soc. 67 (1945) 529-532.
20. C. A. Zittle. Purification of protease in cow's milk. J. Dairy Sci. 48 (1965) 771.
21. L. K. Creamer. β-Casein Degradation in Gouda and Cheddar cheese. J. Dairy Sci. 58 (1975) 287-292.
22. L. K. Creamer. Casein proteolysis in Mozzarella-type cheese. N.Z. J. Dairy Sci. Technol. 11 (1976) 130-131.

23. F. M. W. Visser. Method for the manufacture of rennet-free cheese. Neth. Milk Dairy J. 30 (1976) 41-54.
24. K. Yamauchi & S. Kaminogawa. Decomposition of Milk Proteins by Milk Protease. Agric. Biol. Chem. 36 (1972) 249-254.

Articles

Agric. Res. Rep. 875 p. 3-4

Cheese ripening studied in model systems. I. Introductory part

H. Mulder, A. Noomen and L. de Jong

1 Methods to study cheese ripening

The ripening of cheese is a complex of many complicated processes, in which especially protein, lactose and fat are transformed. The result is a product with the desired appearance, consistency and flavour. Together with the physical, chemical and microbial properties of the milk, the method of making and the ripening conditions determine the sort of cheese that is formed. The intricacy of all this is demonstrated by the great number of cheese varieties, so that research on cheese ripening is no simple matter. Most workers in this field study the ripening of one particular cheese; hence they are tied to the whole complex of conditions that leads to a cheese of the quality desired for the sort. Therefore every kind of cheese must be investigated separately.

Nevertheless, cheeses that are quite different still have much in common in ripening and we believe that a general, but simple model would be useful to study the main ripening processes, from which the ripening of special cheeses can be deduced. Among possible approaches to such a model are simplifying the preparation of a known cheese, resulting in a cheese with a less complicated ripening (stripped cheese), and synthesizing cheese-like systems from the necessary components (simulated cheese).

2 Stripped cheese as model

As basis we chose the soft Noordhollandse Meshanger cheese. This cheese ripens in about 2 weeks; the preparation is simple; its flavour is very mild. The stripped Meshanger cheese ripens without a surface flora. If desired it can be acidified with a lactone instead of with lactic acid bacteria; the milk is pasteurized. At first sight such a simple stripped cheese may seem ideal. However it has restrictions. Its composition depends on the properties of the milk; a possible influence for instance of whey components on ripening is not yet clear. Furthermore such stripped cheeses contain rennet enzymes, enzymes like milk protease etc.. The action of lactic acid bacteria may cause complications. A stripped cheese that contains no active rennet enzymes and no milk protease can be prepared, but its structure can differ significantly from that of normal cheese because of the severe treatment necessary to inactivate

enzymes. This treatment involves high pasteurization temperatures and manipulation with the calcium ion concentration.

Another way to prepare such a model cheese is not to work the cheese curd into cheese in the normal way, but to add ingredients like lactic acid and salt to curd (artificially composed stripped cheese). However there still are uncertainties, such as the influence of the whey enclosed in the curd.

3 Models composed from pure ingredients

The more radical way for making a model is to compose it from the individual components. The most important are calcium paracaseinate–calcium phosphate complex, lactic acid, NaCl and water. Enzymes or other substances, whose influence on ripening is to be studied, can be added. The attraction of these simulated cheeses is that their composition is exactly known. However they also have restrictions, for instance whey is left out. Using whey instead of water may reintroduce unknowns. Of course the structure of these systems differs from that of normal cheese.

One could continue, as many investigators did, and investigate the conversion of pure components of cheese, for instance by studying the proteolytic activity of enzymes in solutions of casein salts. We found however that one cannot extrapolate to cheese as, for instance, calcium has a considerable influence on casein breakdown. So the composition and properties of model systems should resemble those of cheese as closely as possible. All models, however, have some restrictions. The choice depends on the problem to be studied.

4 Motivation and approach

The motivation of our study was to gain knowledge on cheese ripening, especially on proteolysis and its consequences for cheese consistency. The factors thought to be most important were studied in the types of models mentioned. The influence of rennet (chymosin) was studied in all models whereas to study the action of milk protease the preferred system was simulated cheese. The relation between consistency and proteolysis was studied in the stripped Meshanger cheese, as was the relation between structure and consistency.

To study protein breakdown two methods were adapted. For estimation of the soluble nitrogen components, this was an extraction method and the unattacked proteins were estimated by quantitative electrophoresis.

Further details about motivation, results and conclusions of the work are reported in full in:

II – Proteolysis in soft cheese, studied on Meshanger cheese and cheese mo-

dels, reported by A. Noomen

and

III – Proteolysis and consistency of Meshanger cheese, reported by L. de Jong.

Neth. Milk Dairy J. 30 (1976): 230-241

Noordhollandse Meshanger cheese: a model for research on cheese ripening. 1. Reconstruction of the cheese

A. Noomen and H. Mulder

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

Received: 23 November 1976

Summary

The article, the first part of a series of three, deals with Noordhollandse Meshanger cheese, a soft cheese made in former times on one or two farms in the province of Noord-Holland (the Netherlands). The method of making the cheese, which was almost completely forgotten, was reconstructed not only for professional interest but mainly for the purpose of fundamental research on cheese ripening.

The making of the cheese had many points in common with that of the earlier farm-made Edam cheese. The cheese was made from cow's milk as freshly drawn as possible, no starter was used and the production conditions were aimed at a high moisture content in the cheese. The consistency of the cheese was soft to very soft. In contradiction to most known soft cheeses with a surface flora the softening of the body proceeded from the centre to the outside of the cheese. The taste was very mildly acid and somewhat yeasty.

1 Introduction

From ancient times the Netherlands enjoyed a particularly good reputation for the production of hard or semi-hard cheeses, particularly for that of Gouda and Edam cheese. These cheeses are eminently suitable for transport and trade by reason of their distinguishing characteristics, such as: a good taste which can be varied from mild to piquant in accordance with the customer's requirements, retention of their form, and a simple method of production which lends itself particularly well to mechanization and automation. For these reasons the fabrication of these cheeses has assumed such large proportions in the Netherlands that it seems to be almost forgotten that in former times cheeses

with quite different properties were known in this country, for instance cheeses with a soft body. The knowledge of the method of making these cheeses was almost completely lost. Examples of these varieties of cheese are those of the so-called meshanger type ('meshanger' means 'sticking to the knife'), such as the Noordhollandse Meshanger and the Zuidhollandse Meshanger, also called Hangmes cheese, both of them with a very weak body, and the Noordhollandse Mesklever, a cheese with a firmer but also a sticky body.

Mainly for the purpose of fundamental research on cheese ripening we have given primary attention to the reconstruction of these kinds of cheese, especially to that of Noordhollandse Meshanger cheese.

2 Historical data concerning Noordhollandse Meshanger cheese

2.1 General data

As far as can be traced the cheese was introduced by the farmer and burgomaster C. Pijper (1830 - 1907) at Hoogwoud (Province of Noord-Holland). The method of manufacture was carefully kept as a family secret, and only one outsider seemed to be aware of the manufacturing process. In this way we came into the possession of a vague description of the recipe written by a farmer Stam in the year 1921.

The cheese was made only during September and October from raw cow's milk.

For the benefit of selling the cheese every autumn small announcements were made in one or more newspapers, and that was sufficient for selling the whole production. The consumers belonged almost exclusively to the upper-ten, and the cheese was even well-known at Court. This connection would have been strongly affected by the high price of the product. In 1938 the cheese was offered for Dfl. 3 per kg, a multiple of the price of Edam cheese at that time.

The making of the cheese was stopped somewhere about 1940 (World War II), and since then the knowledge of the production of the cheese was almost completely lost. Later on C. Pijper, a grandson of the above-mentioned farmer and manager of the Experimental Dairy Farm at the Governmental Agricultural Experimental Station at Hoorn, made efforts to revive public interest in the cheese. Although from what he remembered from his youth the making of the cheese had been extraordinarily simple his efforts were not crowned with the success he desired, which he ascribed to the vastly altered conditions on the farms. Mr Pijper stimulated us to take the reconstruction of the cheese in hand, and we have benefited very much from his knowledge.

2.2 Data concerning the characteristics and the fabrication of the cheese obtained from conversations with farmers and from the literature

2.2.1 Characteristics of the cheese in matured condition, after about two weeks of ripening

– *Type (consistency).* The consistency was soft to very soft.
– *Shape.* The cheese had the shape of a small Gouda cheese. However, the cheese was made in an Edam mould and acquired its final form by sagging.
– *Dimensions and weight.* From the dimensions of an original wooden box which had been used to deliver the cheese to the consumers it could be deduced that the cheese had a diameter of about 20 cm and a height of 4 - 5 cm. By means of these data the weight of the cheese could be calculated as being somewhere between 1300 and 1700 g.
– *Surface flora.* During the ripening period micro-organisms, particularly yeasts and moulds, developed spontaneously on the cheese surface. From time to time this flora was washed away.
– *Fat content in the dry matter.* Van der Zande (1) analysed the composition of three meshanger type cheeses in 1904. The mean fat content in the dry matter was 47.6 %. The mean fat content of milk used for making these cheeses was 3 %. During later periods the fat content in the dry matter of the cheese will probably have increased as a result of the selection of cows on the basis of a high fat content in the milk. According to the actual standards (2) the cheese should be classified as 'full fat'.
– *Moisture content of the cheese.* The mean moisture content of the cheeses analysed by van der Zande (1) was 53.75 %. From his data the moisture content on a fat-free base can be calculated as being about 70 %, a value which is also mentioned by van Dam (3). Nowadays the cheese should be classified as soft cheese.
– *Aroma and taste.* The cheese had a very mild, gently acid and slightly yeasty taste.
– *Body of the cheese.* The softening of the body was said to proceed from the centre to the outside of the cheese. Thus, the cheese did not show a hard and crumbly core at the start of the ripening period. The matured cheese was soft like butter.

2.2.2 The fabrication of the cheese in early days

The cheese was made only during September and October. Production of the cheese took place in the room where Edam cheese was made, and the same equipment was used in both Meshanger and Edam cheese production. The cheese was manufactured twice a day, starting from still warm freshly drawn

milk. The recipe of fabrication was approximately as follows:
– The cheese milk was provided with a very small amount of annatto.
– At 30 °C calf-rennet was added in such a quantity that formation of the curd was obtained in about 20 min.
– The curd was cut very gently for 15 min.
– In order to maintain the temperature of the contents of the cheese vat at about 30 °C some whey was usually taken off, warmed up and added again to the vat as was done with Edam cheese.
– The curd was gently stirred for about 10 min.
– After a sedimentation time of about 10 min. the whey was taken off.
– The curd was brought into Edam moulds, turned three times, bandaged and gently pressed for two hours.
– After removal of the rind the still globular cheese was salted with a paste of sodium chloride and water and placed into a salting box used in former days for salting farm-made Edam cheese. As a result of the high moisture content the cheese sagged rapidly and finally acquired the shape of a small Gouda cheese. To control this metamorphosis the cheeses were placed against each other and turned frequently, particularly during the first hours of their stay in the salting box. Later on the cheeses were turned at each salting time. Salting of the cheese took place seven times with a frequency of three times a day.
– After salting, the cheeses were washed with luke-warm water or whey, wiped off and placed on clean ripening shelves.
– During the ripening period, which took about two weeks, the cheeses were turned daily, and were also washed clean a couple of times during this period with water or whey.

3 Considerations on the reconstruction of the cheese

As the know-how about the cheese was lost or showed large 'black holes' it was necessary to have as clear an idea as possible of the way of working and, above all, of the way of thinking at the farm in former times, since only from such a beginning is a reconstruction possible. One can easily adopt wrong methods as also happened to us (Section 3.8).

Further on it was considered that Meshanger cheese was fabricated by farmers who specialized in the production of Edam cheese. For that reason a strong resemblance of the fabrication methods of both kinds of cheese would be probable.

3.1 The milk

In spite of the lucrative character of Meshanger cheese fabrication, the production of the cheese was limited to the late summer and early autumn period. Apparently it was impossible or very difficult to produce the cheese in other periods of the year. In connection with this one could think that the composition of the milk might have been of importance. Autumn milk is relatively rich in fat, in casein and thus in calcium and phosphate, but poor in lactose (4). For that reason autumn milk was used at the outset of our efforts to reconstruct the cheese, although we were well aware that the composition of the milk nowadays is much richer than was earlier the case.

The freshly drawn raw milk was made into cheese as quickly as possible, as it had always been the common practice in the fabrication of the cheese.

3.2 Starter

As it can be seen from the recipe (Section 2.2.2) no starter was used in the fabrication of the cheese. Originally wooden milk equipment, such as pails and vats, were used on the farm. Without doubt this equipment was brushed and cleaned thoroughly with hot water and potash, but in all probability a flora of predominantly lactic acid bacteria would have lodged in joints, cracks and so on. Data from the older literature indicate a general use of slow acidifying starters in the production of the earlier farm-made Edam cheese. In this respect the use of 'long whey' has been widely known for a long period of time. It is therefore reasonable to assume that Meshanger cheese milk got inoculated spontaneously with mainly slow acidifying lactic acid bacteria.

Present-day milk will not only show a much lower bacterial count than in former times but the bacterial composition of the milk will also be quite different. For that reason we added a very small quantity of a slow acidifying starter to the milk.

3.3 Rennet and renneting of the milk

The same rennet (calf veal extract) was used for Meshanger and Edam cheese fabrication. The quantity of rennet added to the cheese milk and the renneting temperature and time were also similar in both productions.

3.4 Treatment of the curd

Because the moisture content of the cheese had to be high the curd was cut

very gently and the curd-whey mixture was handled very carefully.

Before the introduction of the Boekel-method around 1900, in which the curd was stirred to the desired dryness in the cheese vat, a rough treatment of the curd was also used in the production of Edam cheese. A considerable quantity of whey had to be removed afterwards by crumbling the curd and frequent turning. It was well known that during autumn difficulties were frequently encountered in obtaining a sufficient dryness of the curd. Many Edam cheeses lost their globular shape during that period by sagging, as a result of the too high moisture content of the cheese. This in combination with the fact that the acid formation also did not proceed satisfactorily resulted in a cheese of unsatisfactory quality, showing a sticky rind with a red surface flora. This product was known as Mesklever cheese. The cheese was not made on purpose but some cheese traders had interest in it, probably mostly in connection with the flavour but also with the low price of the product. An old merchant defined Mesklever cheese as a cheese made by farmers who did not know how to make a good Edam cheese in autumn. Kernhem cheese, a well known cheese variety in the Netherlands at the present time, can be considered as a modern version. However, this cheese is totally different from Noordhollandse Meshanger cheese. It has a red surface flora and its moisture content is lower.

3.5 Moulding

The cheese was formed in Edam moulds, bandaged and lightly pressed for a couple of hours. To our modern way of thinking this method looks inefficient and illogical. However, in connection with the ripening of the cheese we stuck to the old method (Section 3.9).

3.6 Salting

After pressing, the cheese had a closed rind but was still very, very soft. The cheese was salted by rubbing the cheese surface with a paste of sodium chloride and water. During salting the cheeses stayed in a closed box, the 'zoutkist' (salting box), to prevent cracking of the rind by draught and cooling.

The salting of the harder Edam cheese happened in an almost identical way. The only difference was that Edam cheeses were kept in special moulds, the 'zetters', during the salting period so that they maintained a globular Edam shape.

3.7 Ripening conditions of the cheese and treatment of the cheese surface

In former times a part of the barn was frequently separated specially for the

purpose of using as a ripening room. When no cows were present the barn would have been dry.

The farmer's wife was accustomed to take care of Edam cheeses which needed a clean and smooth surface. As frequently as it was thought to be necessary these cheeses were thoroughly cleaned with water or whey and oiled with linseed oil. A good appearance of the cheeses was a matter of pride for the farmer's wife.

Certainly during the first days after fabrication Meshanger cheese would have shown a wet surface as a result of the syneresis of an excess quantity of moisture. These conditions allowed a flora of micro-organisms to develop on the cheese surface and, according to our information, the farmer liked to see that happen, especially the development of moulds. He concluded from it that the ripening conditions were good. However, the farmer's wife did not like it and, as was done with Edam cheese, Meshanger cheese was cleaned from time to time.

3.8 The ripening of experimental 'meshanger-like' cheeses with a surface flora of Brevibacterium linens

In respect of the reconstruction of the cheese an essential question was whether the surface flora was of crucial importance to the ripening. Meshanger cheese had a high moisture content and no washing was applied to the curd during fabrication. Thus, in spite of the relatively low lactose content of the milk, the freshly prepared cheese would have contained a considerable amount of lactose. One could expect that lactic acid fermentation in such a cheese would lead to an acid and crumbly cheese. In such a situation a surface flora could be capable of converting a part of the milk sugar and especially of the lactic acid resulting in a decrease of the acidity. Enzymes produced by the flora could also contribute to the protein ripening of the cheese, in short, a way of ripening such as is equally observed in other soft cheeses with a surface flora.

The role of a surface flora would then be of extreme importance and one is therefore readily inclined to stimulate the development of such a flora. We started with adopting this view, in which we were encouraged very soon by the experimental results obtained.

Several times cheese was made from autumn milk to which a small quantity of a slow acidifying starter had been added. As the manner in which the cheese acquired its shape was considered to be of little importance the cheeses were made in Gouda vats of 2 kg. The cheeses were salted by keeping them for a few hours in brine until the desired quantity of salt had been taken up. The

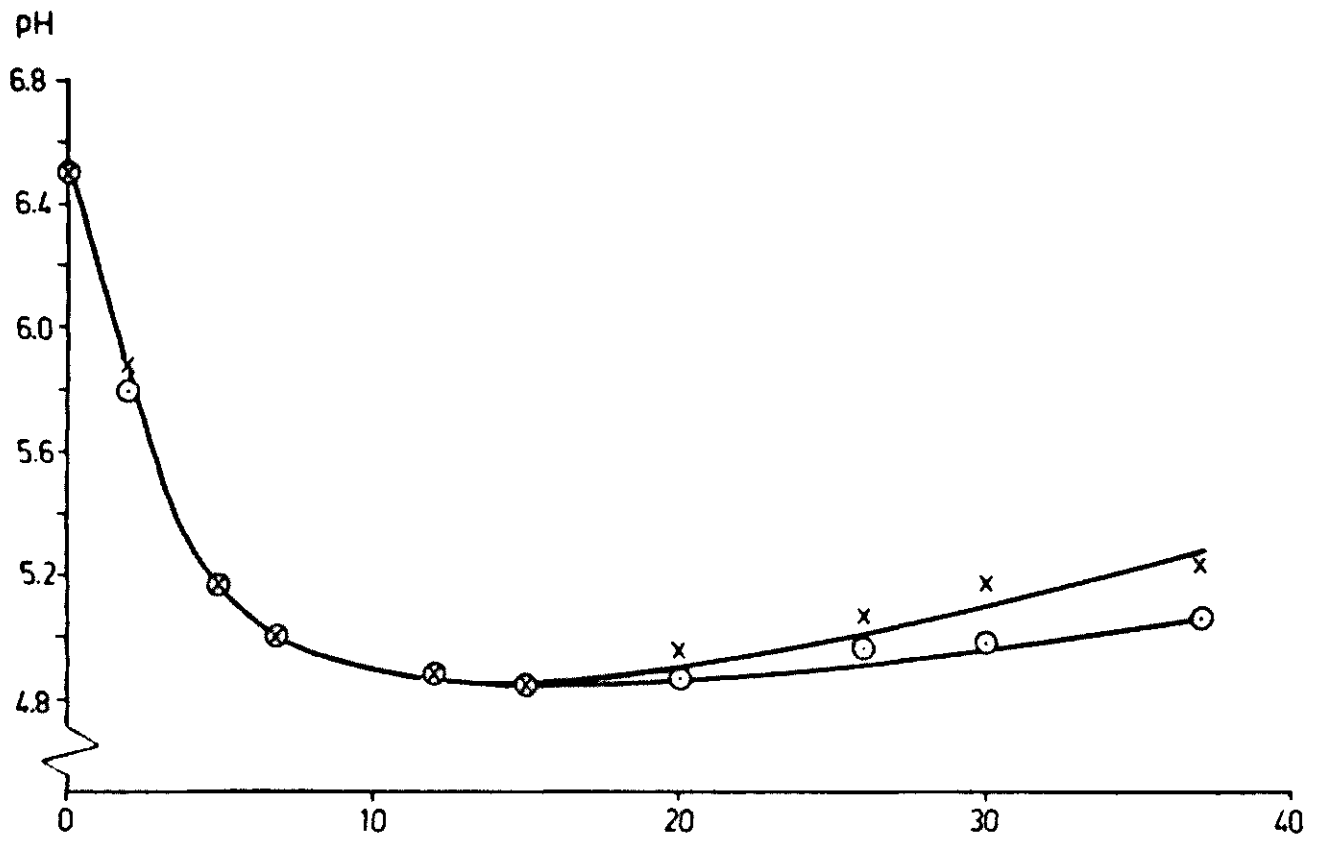

Fig. 1. Experimental 'meshanger-like' cheese with a surface flora of *Brevibacterium linens*. Course of the pH during the ripening period. x = outer layer (5 mm) of the cheese without the rind; ⊙ = centre of the cheese.

surface of the cheese was inoculated with a culture of *Brevibacterium linens* ('ferments du rouge'). The development of the surface flora was favoured by the storage conditions (temperature 13 °C, RH > 95 %) and by rubbing the cheese every day with water or a dilute salt solution. The course of the pH

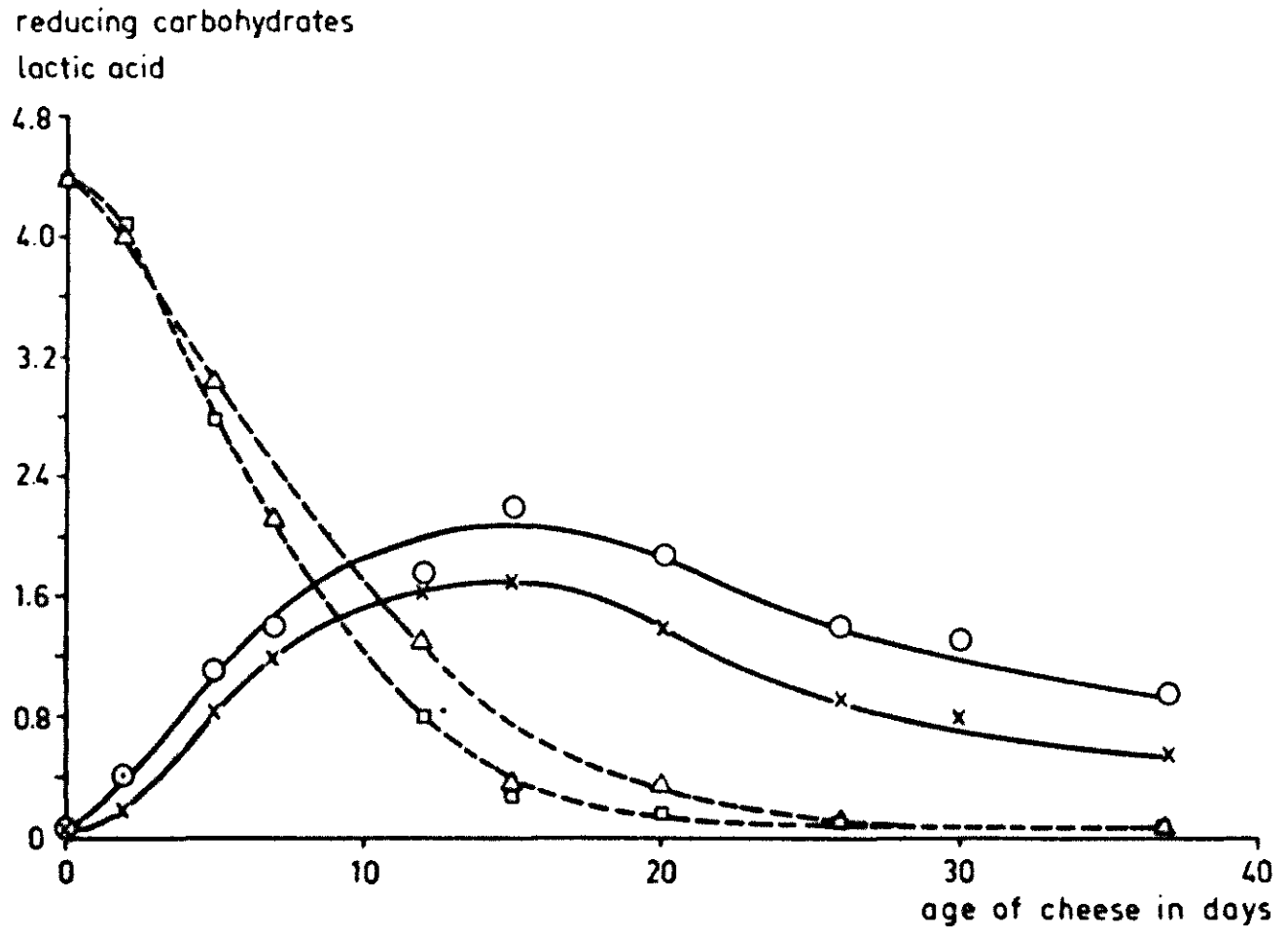

Fig. 2. Experimental 'meshanger-like' cheese with a surface flora of *Brevibacterium linens*. Contents of lactic acid and of reducing carbohydrates (%) in the cheese moisture on different days of the ripening period in the outer layer (5 mm) of the cheese without the rind and in the centre of the cheese. Lactic acid: outer layer (x), centre (○). Reducing carbohydrates: outer layer (△), centre (□).

and of the contents of reducing carbohydrates and lactic acid of such a cheese are shown in Fig. 1 and 2. The moisture content of these experimental cheeses was about 54 %, and the salt content in the cheese moisture about 3 %. The cheese got too acid and crumbly in the first days of ripening but the acidity was reduced by the activity of the surface flora. The cheese ripened in 4 to 5 weeks, the ripening proceeded from the outside to the inside of the cheese and the taste suggested that of a 'fromage à croûte lavée'. The product was very attractive in taste and stuck to the knife, but was said to be quite different from Noordhollandse Meshanger cheese. Nevertheless the results of our experiments at first made us believe that the development of a surface flora had to be stimulated.

3.9 The final reconstruction of the cheese

From the foregoing section it can be seen that we at first believed the surface flora was of crucial importance to Meshanger cheese.

Other considerations and observations, however, gave us a strong impression that we had lost our way completely by following the above-mentioned line of thought:

– Meshanger cheese should have a mild, gently acid and slightly yeasty taste. Apparently the cheese had not the distinctive flavour of a red surface flora. This could indicate that the flora was not of primary importance to the flavour of the cheese.

– The low relative humidity of the air in the barn will not have favoured the development of a surface flora, particularly not of 'ferments du rouge'. More likely, yeasts might have developed on the cheese surface during salting and afterwards, and later on also moulds. The yeasty taste of the cheese could perhaps be explained by that. Especially with regard to the yeasts the salting box might have acted as a source of contamination.

– The cheese surface was regularly cleaned well by the farmer's wife during the ripening period by which the flora would have been largely removed.

– Mr Pijper positively remembered that the ripening of the cheese proceeded from the inside to the outside. He even stated that the ripening had to be considered as a failure if the core of the cheese had become acid and crumbly because of the fact that this situation would persist. This would be in complete contradiction to the ripening of known soft cheeses with a surface flora, such as Camembert and Brie cheese, and equally to our experimental cheese with a flora of 'ferments du rouge'.

– If the statements of Mr Pijper were correct it would be very improbable that, with regard to the pH regulation in the cheese, only the activity of a

surface flora would have prevented the cheese from becoming too acid and crumbly even at the core. Apart from an extreme slow acidification of the cheese and a very high speed of transport of lactose and of lactic acid from the interior to the surface of the cheese such a situation would have required a very large activity of the surface flora. For reasons mentioned above, such an activity was not reasonable. Moreover, it takes several days for a surface flora to become active. During the first days after fabrication the flora will have shown hardly any activity, while the lactic acid bacteria in the cheese will have developed at an increasing rate during that period of time.

– Preliminary experiments on the inward migration of enzymes into soft cheese showed this to proceed very slowly. This did not suggest a significant contribution of proteolytic enzymes of the surface flora to protein breakdown in the cheese.

– A simple experiment strengthened the idea that the surface flora was not of crucial importance to the protein breakdown and to the flavour of the cheese. Experimental cheeses were stored in such a way that growth of micro-organisms on the cheese surface did not occur. Too low a pH of the cheese, particularly of its outer parts, was prevented by covering the surface with calcium carbonate or by keeping the cheese in a paste of chalk and water. Under these conditions those parts of the cheese with moderate pH values showed a softening of the body. Moreover they were judged as Meshanger cheese of good quality by some of the original Meshanger cheese connoisseurs.

All this led us to believe that the activity of a surface flora had been at most of minor importance and that some other mechanism had been of primary importance to pH regulation and to protein breakdown in Meshanger cheese. With respect to the regulation of the pH, this mechanism should then have necessarily implied that the conversion of lactose into lactic acid was far from complete.

We found the key to a solution in the salting process. The method of fabrication of the cheese and the deduced assumption that the cheese milk became inoculated spontaneously with slow acidifying lactic acid bacteria made it very probable that the cheese was salted at a time at which the lactic acid bacteria had hardly started to grow. One could imagine that the growth of these bacteria was retarded and finally almost inhibited by the increasing salt concentration in the cheese moisture as a result of the diffusion of salt into the cheese.

In this conception, the acidifying properties of the lactic acid bacteria as well as the salting process, particularly the speed of penetration of the salt into the cheese, must have been of primary importance. As this speed is strongly influenced by the moisture content of the cheese, this content had to be sufficiently high.

Further efforts to reconstruct the cheese were based upon the supposition mentioned above. Taking account of the altered bacterial composition of the milk, cheeses were made from aseptically drawn autumn milk to which a very small quantity of a very slow acidifying starter had been added. To allow the penetration of the salt into the cheese to proceed rapidly a very high moisture content of the cheese after pressing, much higher than in the experimental cheeses mentioned in Section 3.8, was created by a very slight treatment of the curd. As we now supposed that, with regard to the growth of the lactic acid bacteria in the cheese, the conditions could have been influenced by the sagging of the cheese from the Edam to the Gouda shape and by the way of salting the cheese, we returned to the original way of making the cheese in an Edam mould and to salting the cheese by rubbing the surface with a paste of sodium chloride and water. With the exception of small and unimportant modifications the cheeses were otherwise made according to the description given in Section 2.2.2. The cheeses were stored at 13 °C and about 95 % RH. The development of a surface flora was largely prevented.

After we had achieved some experience with this way of working we succeeded in manufacturing a cheese which satisfied the description of Noordhollandse Meshanger cheese. The cheese ripened in about two weeks. The weakening of the body proceeded from the inside to the outside. The taste was mild, gently acid and slightly yeasty. Indeed, the fabrication of the cheese was very simple and was merely a question of know-how.

Samenvatting

A. Noomen, *Noordhollandse Meshanger kaas: een model ter bestudering van rijping van kaas. 1. Reconstructie van de kaas*

Het artikel, het eerste van een serie van drie, handelt over Noordhollandse Meshanger kaas, een weke kaas die tot omstreeks 1940 uitsluitend in de maanden september en oktober op enkele boerderijen in Noord-Holland werd bereid. De bereidingswijze van de kaas was nagenoeg geheel verloren gegaan. Vooral ten behoeve van fundamenteel onderzoek over kaasrijping werd de bereidingsmethode gereconstrueerd.

Dit eerste artikel bevat een beschrijving van de kaas op grond van historische gegevens en van de overwegingen welke hebben geleid tot de reconstructie van de kaas. De bereiding vertoonde veel overeenkomst met die van de vroegere boerenedammer. Er werd echter steeds van zo vers mogelijk gewonnen melk uitgegaan, er werd geen zuursel gebruikt en de kaas werd op een hoog vochtgehalte afgewerkt. Waarschijnlijk werd de kaasmelk spontaan geënt met langzaamzurende melkzuurbacteriën, afkomstig uit het houten melkgereedschap. De kaas werd gemaakt in een Edammer-vorm, doch zakte uit tot een kleine Goudse kaas door het hoge vochtgehalte. Op het kaasoppervlak kwam spontaan een flora, vooral bestaande uit gisten en schimmels, tot ontwikkeling. Het week worden van de kaas verliep van binnen naar buiten, hetgeen volledig tegengesteld was aan dat wat wordt waargenomen bij bekende zachte kaassoorten met een oppervlakte-

flora. De kaas rijpte in ongeveer twee weken en had dan een milde, zachtzure en iets gistige smaak.

Omdat de wrongel niet werd gewassen bevatte de pas bereide kaas veel lactose. Een bepaald mechanisme zal dus hebben moeten voorkomen dat de kaas zuur en kort werd. Er wordt beredeneerd dat de oppervlakteflora slechts van ondergeschikte betekenis kan zijn geweest voor de pH-regeling in de kaas en eveneens voor de eiwitafbraak en de smaak.

Een kaas die aan de beschrijving van Noordhollandse Meshanger voldeed, bleek te kunnen worden bereid door uit te gaan van de veronderstelling dat de groei van langzaamzurende melkzuurbacteriën in de kaas werd vertraagd en tenslotte vrijwel werd stilgezet door het ten gevolge van de diffusie van zout in de kaas toenemende zoutgehalte in het kaasvocht.

References

1. K. H. M. van der Zande, *Versl. landbouwk. Onderz. RijkslandbProefstns* 2 (1907) 52-91.
2. Joint FAO/WHO Food Standards Programme, Report of the Seventeenth Session, Rome (1975) p. 32.
3. W. van Dam, Opstellen over moderne zuivelchemie, 2nd ed., 1922, p. 111.
4. H. Mulder, A. J. van der Have & C. J. Schipper, The composition of Netherlands milk. (Report obtainable on request.)

Neth. Milk Dairy J. 31 (1977): 75-102

Noordhollandse Meshanger cheese: a model for research on cheese ripening. 2. The ripening of the cheese

A. Noomen

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

Received: 5 January 1977

Summary

After the method of making the cheese had been reconstructed several important properties of the manufacturing process were studied. From these investigations it appeared that: 1) the moisture content of the cheese was of decisive importance; 2) the surface flora was not of crucial importance to pH regulation or to protein breakdown in the cheese; 3) the pH of the cheese was regulated by the growth of very slow acid-producing lactic acid bacteria, whose activity depended on their salt resistance and the storage temperature of the cheese, the speed of penetration of salt into the cheese which was influenced by the moisture content of the cheese, and the final salt concentration in the cheese moisture; 4) protein breakdown in the cheese could be attributed to the calf rennet used; 5) the weakening of the cheese body was very closely related to the protein breakdown under the existing conditions in the cheese. The crucial importance of the proteolytic activity of calf rennet in cheese to the consistency of the cheese is discussed.

1 Introduction

During the reconstruction of the cheese (1) strong indications were obtained that the surface flora could not have been of primary importance either to the pH regulation or to the protein breakdown and the flavour formation in the cheese. The reconstruction of the cheese became possible when we were guided by the assumption that the development of the pH was essential and that the pH regulation was mainly determined by the relation between the salt content in the cheese moisture and the growth of slow acid-producing lactic acid bacteria used in the production of the cheese. Following this line of thought, the following factors were of essential importance: the activity of the lactic acid bacteria depending on their salt resistance, the moisture content of the cheese, the speed of penetration of the salt into the cheese and the final salt concentration in the cheese moisture.

Our experiments (1) established that the development of the pH determined

the way in which the softening of the cheese was proceeding, from the centre to the outside or vice versa, at least when in the latter case the excessive acidity of the cheese was reduced by a surface flora. Therefore the research on the ripening of the cheese was directed to the following problems: the mechanism of the pH regulation in the cheese, the factors responsible for protein breakdown in the cheese, and the relation between protein breakdown and changes in the consistency of the cheese. With respect to the last problem, the surface flora, the lactic acid bacteria, the calf rennet and native milk protease could be considered as potential sources of proteolytic activity.

2 Materials and methods

2.1 Cheese

2.1.1 Normal cheese

In most experiments cheeses were produced and ripened as described in Part 3 of this study (2). Cheeses were made in Edam moulds.

After pressing, cheeses can show significant differences in weight. Because of the fact that, among other things, a surface flora and the salting process can contribute to the ripening of cheese the dimensions of the cheese can be of importance. For this reason, immediately after pressing, cheeses were selected from each batch with a weight as nearly equal as possible (for example 1540 ± 35 g).

2.1.2 Cheese without a surface flora

Cheeses, produced as indicated in Section 2.1.1, were kept under anaerobic conditions at 13 °C, this being the normal ripening temperature of Meshanger cheese. Anaerobic conditions were created by placing the cheeses into anaerobic jars after salting. Because jars suitable for our purpose could not be obtained commercially, we were obliged to construct them ourselves (Fig. 1). Cheeses were held free from the bottom of the jar on a desiccator plate. After removal of the largest possible amount of air from the jar by flushing with nitrogen gas, complete anaerobic conditions were obtained by the use of the BBL Gas Pak System, with two Gas Pak envelopes.

2.1.3 'Bacteria-free' cheese (GDL cheese)

The cheeses were made as indicated in Section 2.1.1, with the following modifications. The pH of the milk was adjusted to 6.23 with lactic acid solution (50 %). Subsequently thimerosal as a preservative (100 mg/litre), gluconic acid – δ – lactone (18 g/litre) and a normal quantity of rennet

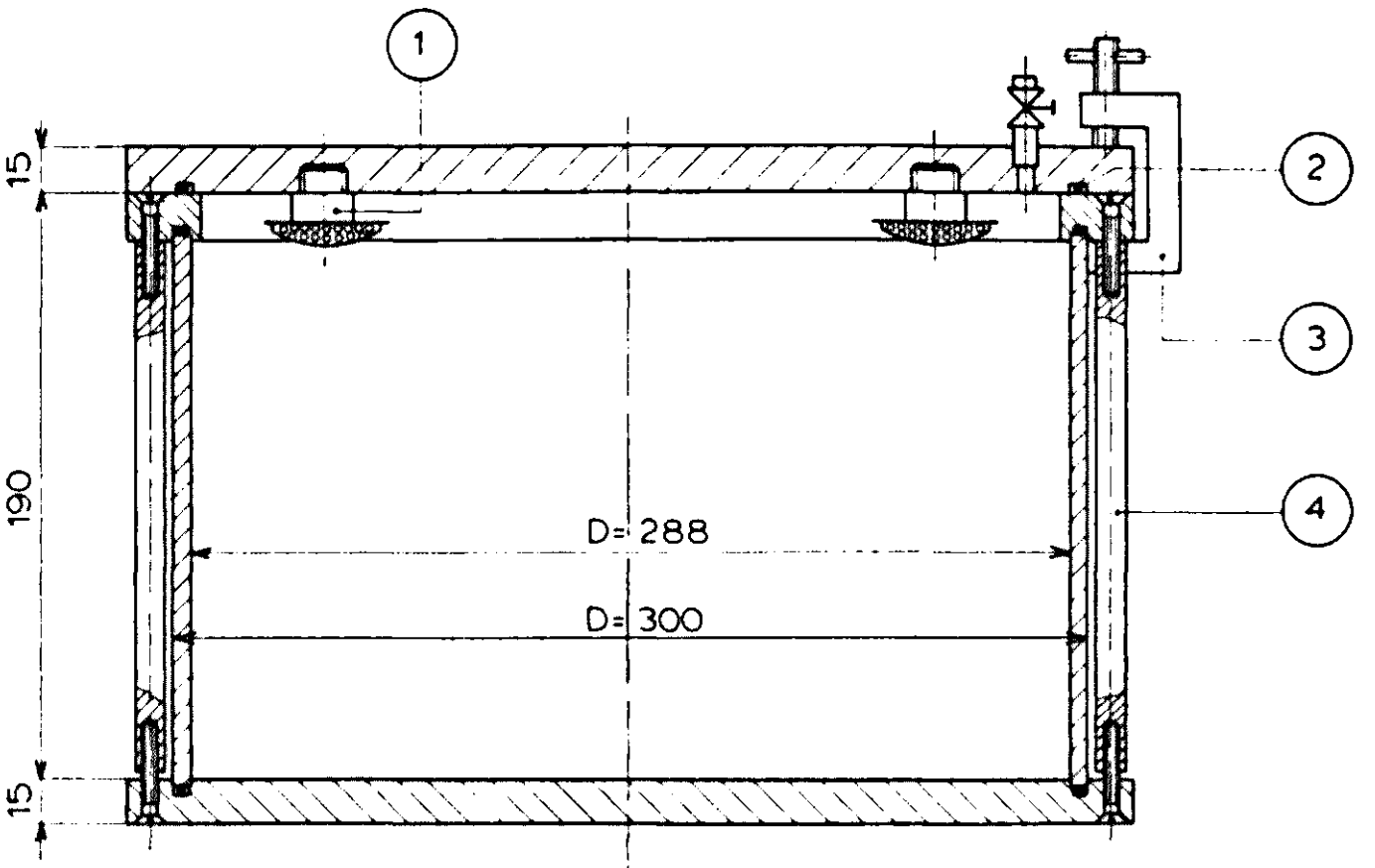

Fig. 1. Illustration of the jar used for the storage of Meshanger cheese under anaerobic conditions. 1 holder for catalyst; 2 gasket; 3 clamp (for closing the jar six clamps are placed along the circumference of the cover); 4 tie-rod. Cover, bottom and wall of the jar are made of transparent acrylic plastic. Dimensions are given in millimetres.

(40 ml/100 litres) were added to the milk. After renneting for 4 min at 30 °C the curd was cut for 4 min and stirred for 3 min. Cheeses were made in Gouda moulds (2 kg). After pressing for 30 min and salting, the cheeses were stored anaerobically as described in Section 2.1.2. The final pH of the cheese was 5.23.

It appeared to be necessary to add the lactic acid and the lactone directly to the milk. On the one hand the addition of these substances to the curd-whey mixture after taking off a large amount of whey will easily result in a cheese with too low a moisture content due to the more intensive treatment of the curd. On the other hand, probably as a result of a slow uptake of lactone by the large curd particles, there is considerable danger of too high a pH.

2.2 Sampling of the cheese

The cheese was found to show large variations in composition throughout its mass; from place to place the composition of the cheese can show large differences both horizontally and vertically. The cheese was therefore sampled in such a way that the composition, at least in the horizontal layers, was as uniform as possible. Preliminary experiments showed that a satisfactory sample could be obtained if a cylinder of cheese with a diameter of 9 cm was taken vertically through the centre of the cheese with the aid of a very sharp borer of stainless steel.

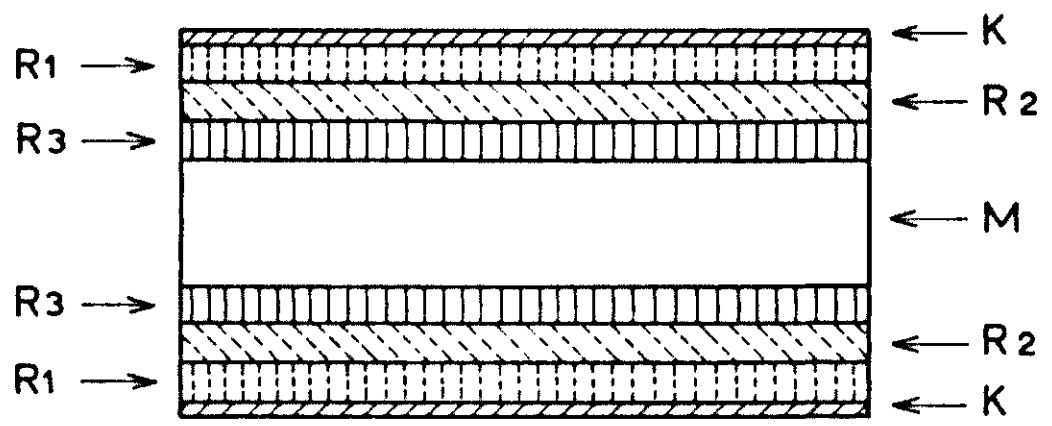

Fig. 2. Sampling of Meshanger cheese. Corresponding slices of the cylinder of cheese were combined.

With a wire the cylinder of cheese was divided into nine slices. From these five samples were prepared: a sample of the surface area consisting of the outer 2 mm of the cheese (K), three samples of the rind consisting of successive layers each of 5 mm of cheese (R1, R2 and R3, respectively) and a sample representing the centre of the cheese (M). The method of sampling of the cheese is illustrated in Fig. 2. Samples were homogenized in a mortar.

During the ripening period the cheese always becomes flatter by further sagging, particularly when it is made in Edam moulds, and also by the loss of moisture as a result of syneresis. Moreover, during the first week after production, the cheese shows the presence of much free moisture so that the loss of some moisture during sampling cannot be avoided. When assessing the analytical results obtained on these samples the possible effect of these factors must be borne in mind.

2.3 Analytical methods

2.3.1 Moisture content

The moisture content was determined according to the method used by Geurts, Walstra & Mulder (3). Portions of 2.5 - 3 g of cheese were dried on aluminium foil for 4 hours at 105 °C. The dry matter was used for the estimation of the salt content.

2.3.2 Salt content

The salt content was estimated according to Standard FIL - IDF 17: 1961.

2.3.3 pH of the cheese

The pH was determined in a homogeneous mixture of 4 g of cheese and 2 ml of distilled water by the use of a combined glass electrode.

2.3.4 Content of reducing carbohydrates in the cheese moisture

Ten grammes of cheese were homogenized with 190 ml of distilled water at

0 - 5 °C, using an Ultra Turrax. (For samples K half of the above-mentioned amounts were taken.) The mixture was centrifuged at 2 °C for 10 min at 23 000 *g*. The supernatant was stored at – 20 °C until it was analysed.

The contents of reducing carbohydrates were estimated according to the method of Barnett & Tawab (4). Estimations were performed in quadruplicate with 2 ml quantities of a dilution of the defrosted supernatant containing 10 - 60 μg carbohydrates per 2 ml. As reagents 1 ml of a 7.5 % (w/w) phenol solution and 7 ml concentrated sulphuric acid (sp.gr. 1.84) were used. Extinction was measured at 500 nm with a Coleman Junior II Spectrophotometer.

2.3.5 Content of lactic acid in the cheese moisture

Lactic acid was determined according to the method of Davidson (5). Estimations were performed in quadruplicate with 1-ml quantities of a dilution of the defrosted supernatant (see Section 2.3.4) containing 1-10 μg of lactic acid per ml. Extinction measurements were made at 570 nm with a Coleman Junior II Spectrophotometer.

2.3.6 N determinations

Nitrogen was determined by the micro-Kjeldahl method, using one tenth of the amounts of reagents used in the macro-Kjeldahl method according to NEN 3198.

2.3.6.1 Total nitrogen (TN) in cheese samples was estimated according to the method described in an earlier report (6), using a weighed amount of about 1 g of cheese.

2.3.6.2 Nitrogen soluble in the cheese moisture (SN). To a weighed amount of cheese equivalent to 10 g of cheese dry matter, $CaCl_2$ solution (0.037 *M*) was added in such a quantity that the total amount of moisture present amounted to 200 ml. The mixture was homogenized with an Ultra Turrax for 5 min at 30 °C. The pH of the suspension was adjusted to a final value of about 7.5 by the addition of 2.5 *N* NaOH. The mixture was centrifuged for 10 min at 40 000 *g* (25-30 °C). The supernatant was filtered and nitrogen was estimated in the filtrate. Soluble nitrogen was calculated as a percentage of the total nitrogen (% SN/TN).

A more detailed description of the method will be given in a subsequent paper.

2.3.6.3 Amino acid nitrogen (AN) was determined by the method described by Sirks (7). In a volumetric flask containing 25 ml of extract (Section 2.3.6.2), 15 ml of 9.2 *N* H_2SO_4 and 6 ml of a 50 % phosphotungstic acid solution were added. Next morning the volume was made up to 50 ml with distilled water.

Nitrogen was estimated in the filtrate. AN was calculated as a percentage of SN (% AN/SN) and of TN (% AN/TN).

2.3.7 Consistency of the cheese
The consistency of the cheese and the changes occurring in it were judged by sensory perception.

3 Experiments and results

3.1 Technical characteristics of the cheese during production and ripening

Cheeses were analysed at an age of 1, 2, 4, 7, 10, 14 and 21 days. After the fourteenth day no further sample R3 was taken for the reason that insufficient cheese would have remained for sample M as a result of the continued flattening of the cheese.

3.1.1 Appearance of the cheese surface
The first days after production the surface shows a very wet appearance as a result of the continuing syneresis of cheese moisture. After 3 - 4 days the surface becomes slimy, starting from the centre of the flat sides, mainly by the development of yeasts as was established microscopically. After about seven days the entire surface has become slimy, a situation which persists until about the tenth day. Subsequently the surface starts to dry up, beginning from the centre, and gradually moulds (*Penicillium*) start to populate the surface and would finally overgrow it if the surface were not washed clean from time to time.

3.1.2 Consistency and taste of the cheese
During the first week of the ripening period the consistency shows no visible change and remains curdy. Between the seventh and the tenth day, however, large differences become visible. Starting at the centre and proceeding to the outer layers of the cheese a weakening of the cheese body is observed. This process proceeds so rapidly that after 14 days almost the entire cheese has become soft and smooth. Only the outer parts K and R1 remain more firm, a situation which is hardly changed after a prolonged ripening of the cheese. At an age of about two weeks the cheese is ready to be consumed and then has a mildly acid and somewhat yeasty taste. A bitter taste is seldom observed in Meshanger cheese. On continued storage the cheese becomes over-ripe which, among other things, is reflected in the 'running' of the cheese when it is cut through.

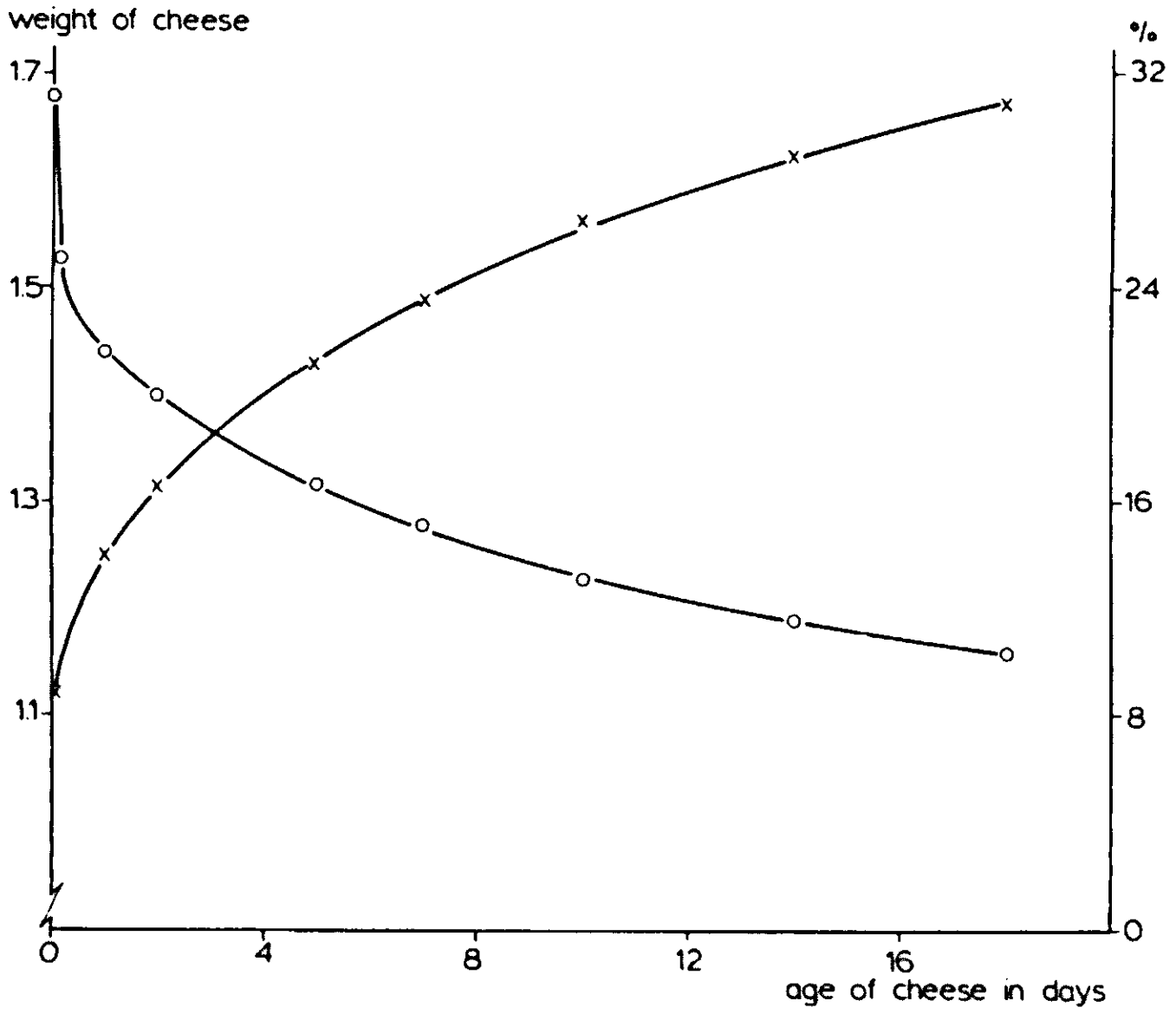

Fig. 3. Loss of weight of Meshanger cheese during the ripening period. ○——○: weight of the cheese in kg; ×——×: loss of weight calculated as a percentage of the weight after the pressing of the cheese.

3.1.3 Loss of weight of the cheese during the ripening period

During ripening the cheeses show a considerable loss of weight. A representative example of the course of the cheese weight and of the percentage loss of weight calculated on the weight after pressing is shown in Fig. 3. The data represent the mean value for 8 cheeses, which had a weight after pressing of 1685 ± 55 g.

The loss of weight is at a maximum during the first hours after pressing, and subsequently decreases gradually. Two and a half hours after pressing the cheese had lost almost 9 % of its weight. This loss had increased to 14 % after 24 hours, and to 29.1 % after 14 days, when the cheese is ready to be eaten. The mean weight of the cheese had decreased from 1.68 to 1.19 kg during this period.

After pressing the cheese loses a lot of enclosed whey and moisture being released by syneresis. The uptake of salt and the decrease of the pH of the cheese further affect the discharge of moisture. Because the influence of the above-mentioned factors decreases as the ripening period proceeds, the course of the curves is understandable. The evaporation of water from the surface may also contribute to the loss of weight.

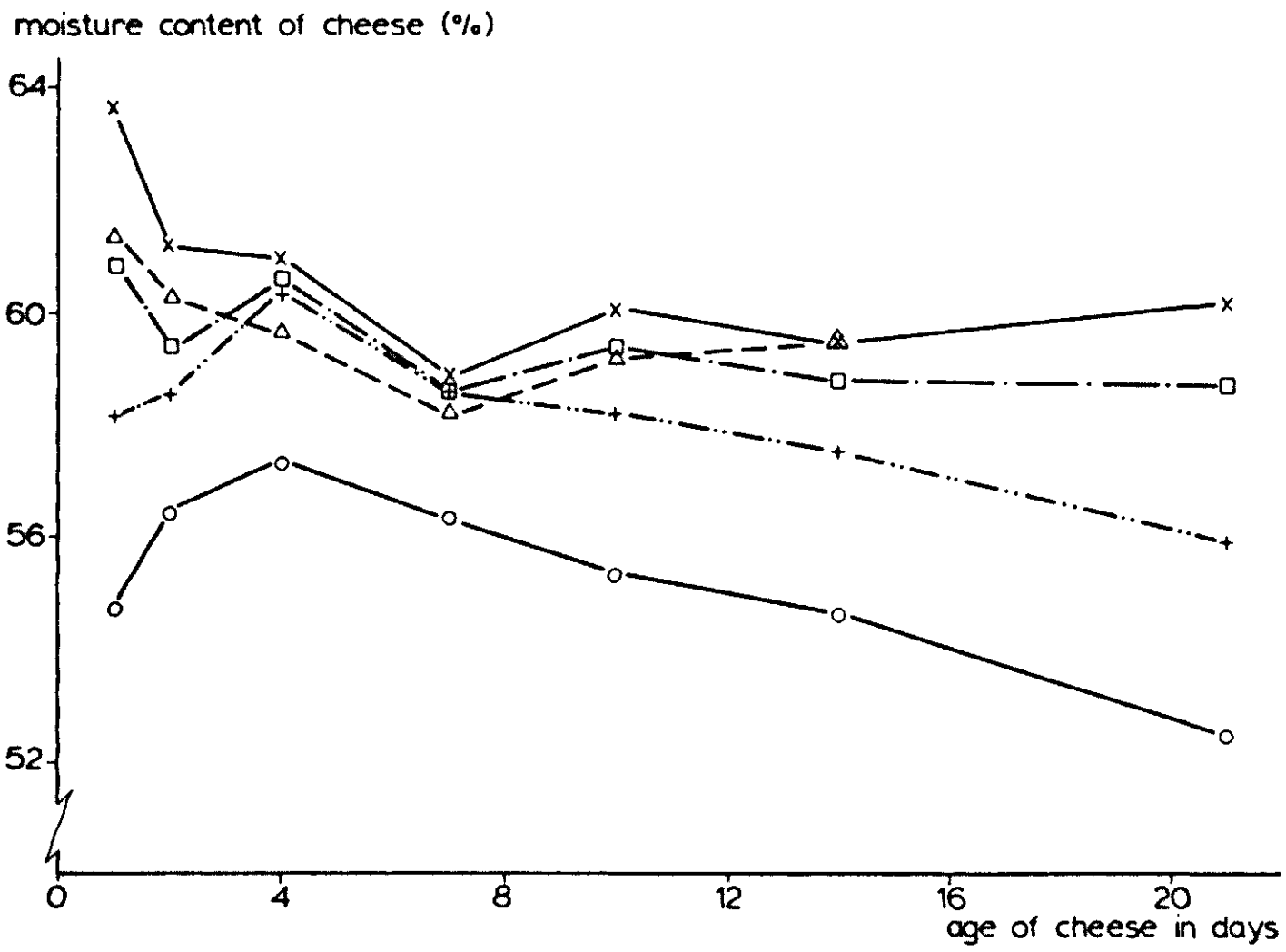

Fig. 4. Moisture contents of samples K (○——○), R1 (+ — . . — +), R2 (□— . —□), R3 (△ - - - △ and M (× —— ×) on different days of the ripening period of Meshanger cheese. Moisture content of the cheese after pressing was 66.18 %.

3.1.4 The course of the moisture content

The moisture contents of the cheese samples on different days of the ripening period after the salting of the cheese are shown in Fig. 4. In accordance with the rapid decrease of the cheese weight during the first 24 hours after pressing (Section 3.1.3), a pronounced decrease of the moisture content, which was 66.18 % after pressing, is observed during this period of time. This decrease is most marked at the surface and diminishes towards the centre of the cheese. This situation, which is still apparent after two days, will be caused mainly by the salting of the cheese. There is a relation between the inward migration of salt into the cheese and the accompanying transport of water to the outside of the cheese. Processes involved in the salting of cheese have been described recently by Geurts et al. (3).

After the first day, when the salting of the cheese has been finished, moisture from the inner layers wanders to the outer layers of the cheese resulting in a more uniform distribution of the moisture. After the eighth day the moisture contents of the portions R2, R3 and M remained almost constant at a value of about 59 %, a normal value for Meshanger cheese. As expected, the moisture content of part K remained lower than that of the other parts of the cheese and, moreover, continued to decrease after the fourth day, probably as a result of evaporation. A similar situation was observed for part R1 after about one week.

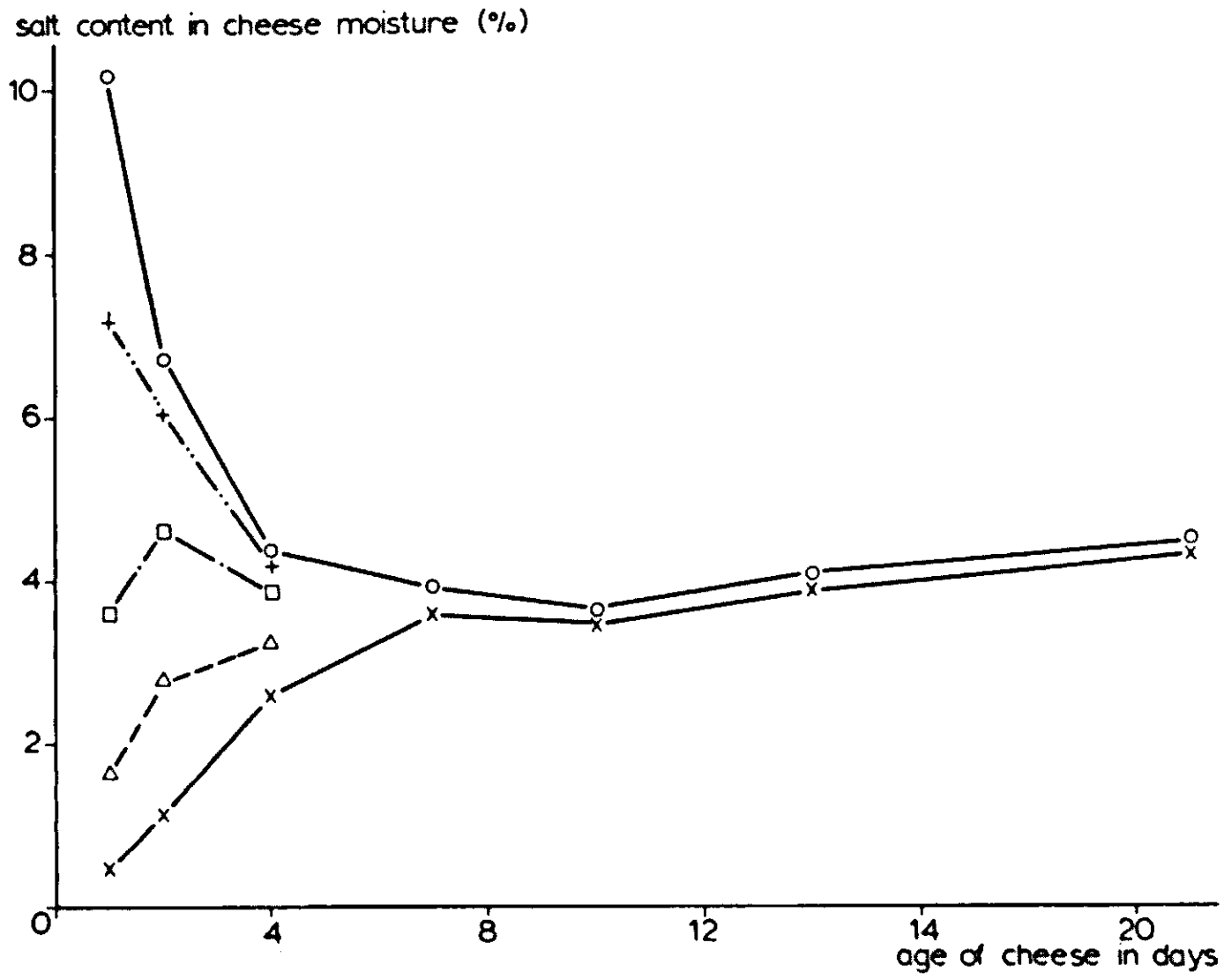

Fig. 5. Salt contents in the moisture of samples K (○——○), R1 (+ —..— +), R2 (□—.—□), R3 (△ - - - △) and M (× —— ×) on different days of the ripening period of Meshanger cheese. Values of R_1, R_2 and R_3 after 7 days and more were between or similar to those of K and M; for clearness of the figure these values are not reproduced.

3.1.5 The course of the salt content in the cheese moisture

The salt contents in the moisture of the cheese samples on the different days of the ripening period after the salting of the cheese are shown in Fig. 5. The cheese is salted by rubbing it immediately after pressing with a paste of salt and water, a treatment which is repeated after 3, 7 and 20 hours. At the beginning of the ripening period large differences are observed between the salt contents of the different parts of the cheese, but subsequently these contents approach each other rapidly, which might be expected by reason of the very high moisture content of the cheese and the accompanying high diffusion speed of the salt (3).

After about seven days the salt has become almost uniformly distributed throughout the cheese moisture, and thereafter the salt content in the moisture of the analysed cheeses was around 4 %, this being a normal value for Meshanger cheese in a matured condition.

3.1.6 The course of the pH

pH values of the different parts of the cheese on the successive days of sampling are shown in Fig. 6.

During the first days after production the pH of the surface portion K

A. NOOMEN

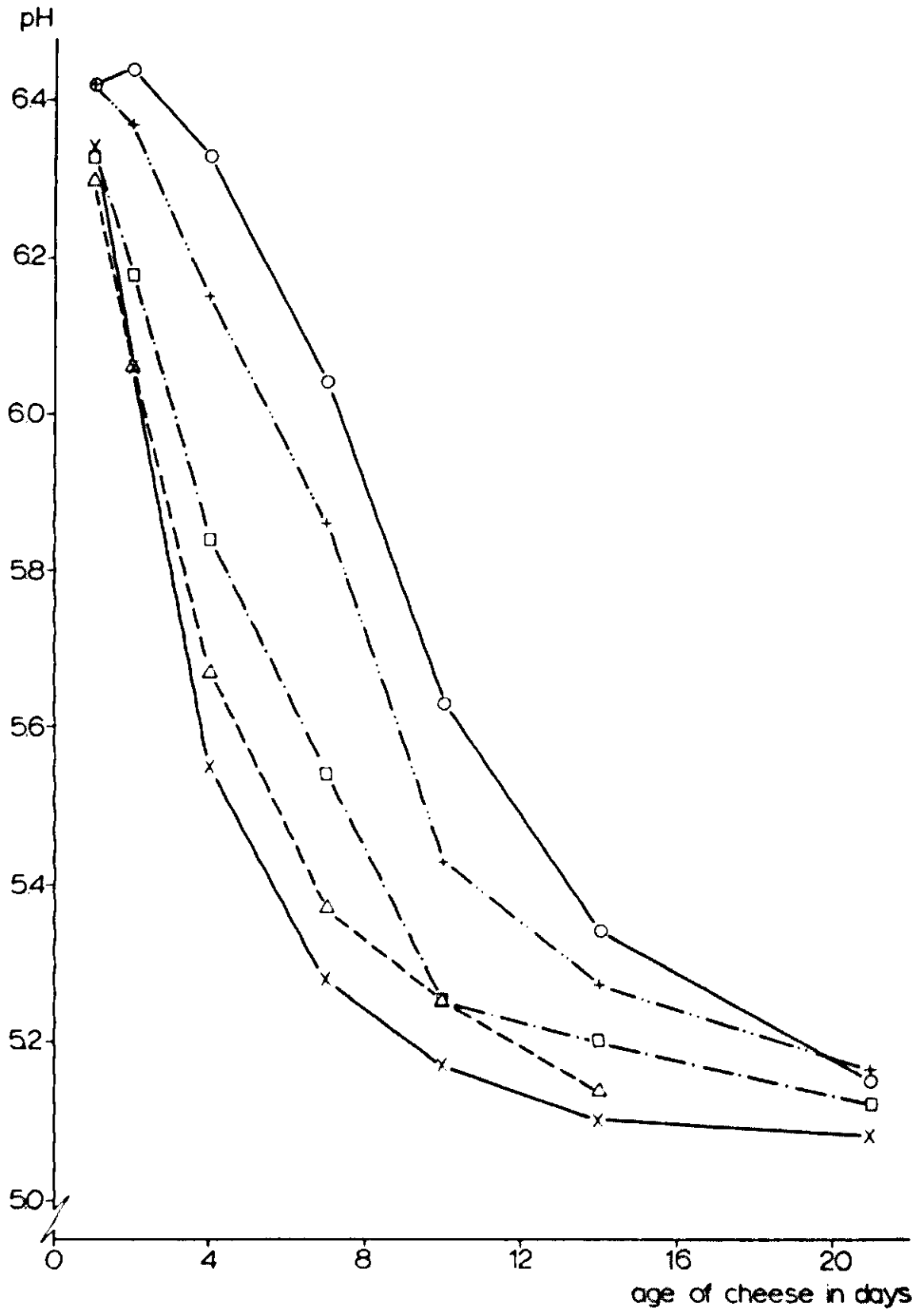

Fig. 6. pH values of samples K (○——○), R1 (+ — . . — +), R2 (□— . —□), R3 (△- - -△) and M (×——×) on different days of the ripening period of Meshanger cheese. The pH of the cheese after pressing was 6.48.

hardly changes. Subsequently a regular decrease of the pH of this portion is observed up to about the tenth day, after which the speed of this decrease is increasingly retarded.

In the central part of the cheese (M) the pH decreases more rapidly than in part K. Moreover, this decrease starts directly after pressing and continues regularly up to about the fourth day after which the speed of the decrease also is increasingly retarded.

Portions R1, R2 and R3 have pH values which lie between those of K and M.

In the last stage of the ripening period the pH values of all portions of the cheese approach each other. At an age of fourteen days, when the cheese is ready for eating, the pH of the cheese is between 5.1 and 5.2 with the exception of that of parts K and R1. This is a desirable value for Meshanger cheese in a ripened condition. If the course of the pH were not influenced by

micro-organisms on the cheese surface, on continued storage the pH of all parts of the cheese would gradually decrease further to a certain minimum value.

3.2 Conversion of lactose and of protein during the ripening of the cheese

3.2.1 The course of the contents of reducing carbohydrates and of lactic acid in the cheese moisture

The reducing carbohydrates and the lactic acid in the moisture of the cheese samples on the different sampling days are shown in Fig. 7.

In agreement with the development of the pH (Section 3.1.6) up to about the tenth day of the ripening period the contents of lactic acid are the lowest in part K and increasingly higher in the other cheese layers according as they are situated more closely to the centre of the cheese. Apart from some irregularities, as might be expected, the reserve is the case with the contents of reducing carbohydrates.

After the tenth day this situation seems to be changed. After 14 and 21 days the highest contents of lactic acid were found in part K and the lowest in part M. To explain this, one could presume that growth-stimulating substances for the lactic acid bacteria, formed by micro-organisms which have developed on the cheese surface (e.g. yeasts), enter the cheese by diffusion.

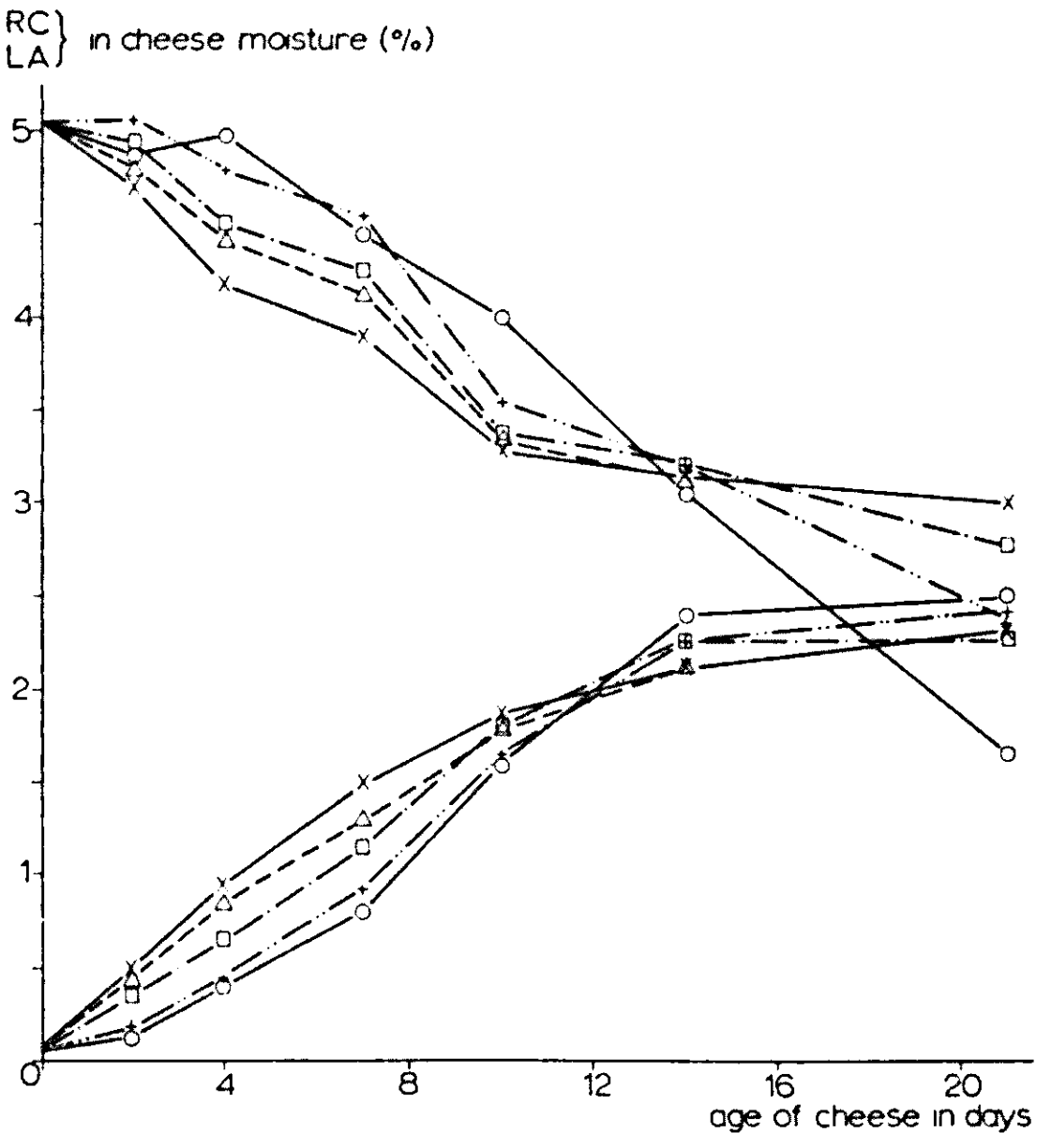

Fig. 7. Contents of reducing carbohydrates (RC; descending lines) and of lactic acid (LA; ascending lines) in the moisture of samples K (○——○), R1 (+——+), R2 (□——□), R3 (△- - -△) and M (×——×) on different days of the ripening period of Meshanger cheese.

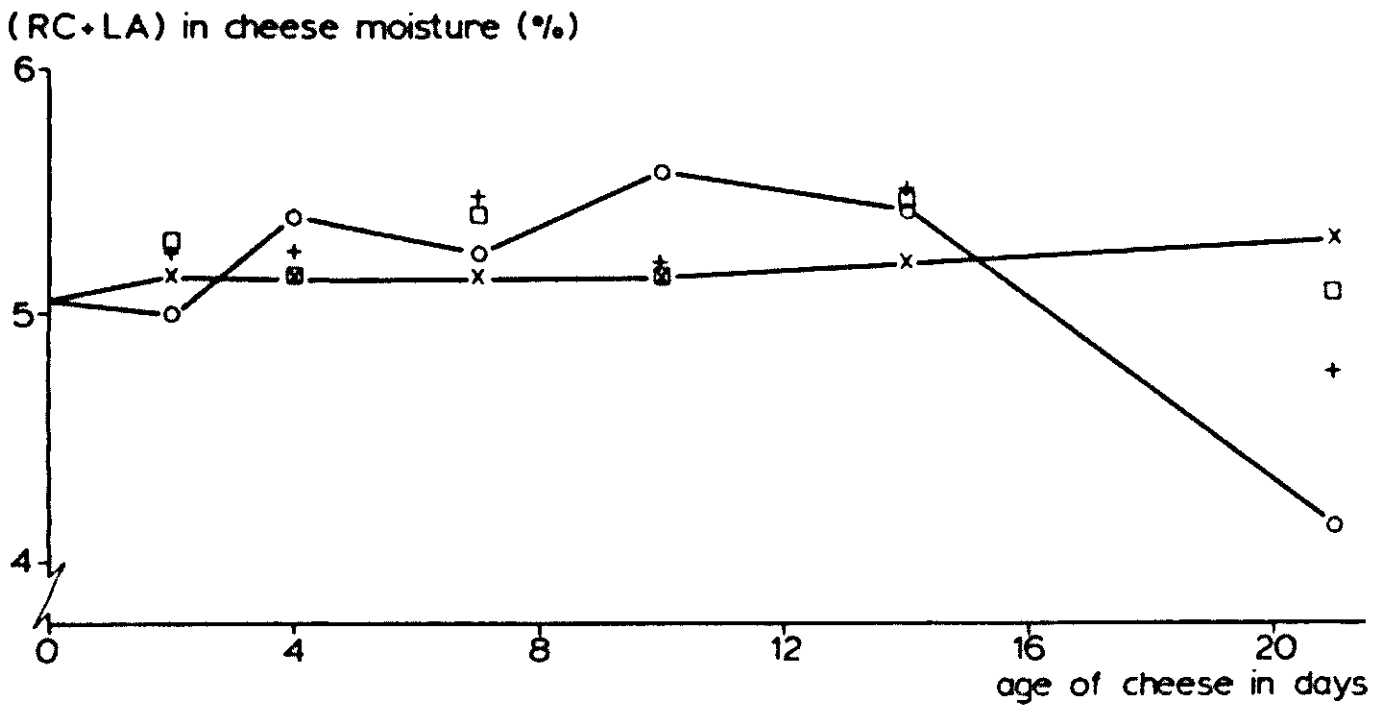

Fig. 8. Sum of contents of reducing carbohydrates and lactic acid (RC + LA) in the moisture of samples K (○——○), R1 (+), R2 (□) and M (×——×) on different days of the ripening period of Meshanger cheese. Values of R3 were almost equal to those of R1 and are not reproduced.

This reversal of the relative positions for lactic acid is not reflected in the pH of the corresponding parts of the cheese.

During homofermentative lactic acid fermentation, from a weighed amount of monosaccharide an equal amount of lactic acid is formed, while in the hydrolysis of lactose into glucose and galactose the weight of the carbohydrates is only increased by about 5 %. If the ratio between the contents of lactic acid and of reducing carbohydrates in the cheese moisture is not changed otherwise than as a result of lactic acid fermentation, the sum of the contents of carbohydrate and lactic acid must show an almost constant value during the ripening period. As shown in Fig. 8 this appears to be so for the centre M of the cheese. The values in the other parts of the cheese are less regular, probably mainly due to the too small specificity and accuracy of the analytical methods applied. However, in our opinion it is reasonable to conclude that up to about the fourteenth day the summed contents show only small changes. After the fourteenth day a strong decrease of this content is observed in part K and, to a less extent, in portions R1 and R2. This must be attributed to the consumption of sugar and of lactic acid by micro-organisms growing on the cheese surface (see also Fig. 7). Moulds in particular are increasingly populating the surface during this period (Section 3.1.1).

At an age of 14 days the moisture in the centre M of the cheese contained 3.17 % reducing carbohydrates and 2.13 % lactic acid. Owing to the high moisture content the cheese thus contained a considerable quantity of sugar at the stage where it is ripe for consumption.

3.2.2 The mechanism of the pH regulation in the cheese

In the production of the cheese a very small quantity (0.01 %) of a culture of a very slow acid-producing *Streptococcus lactis* strain was used as a starter. The activity of this starter in low-pasteurized skim milk according to the test of Stadhouders & Hassing (8) was 23 °N. Owing to this, acid formation in the cheese starts extremely slowly and also progresses at a low speed. In connection with this the temperature also acts as a growth-retarding factor. Directly after pressing the cheese is stored at 13 °C, the ripening temperature of the cheese, the result being that the growth of the lactic acid bacteria takes place almost completely at this temperature.

The cheese is salted directly after pressing, i.e. at a time at which the lactic acid bacteria have scarcely started to grow. As a result of the diffusion of salt into the cheese the development of the lactic acid bacteria thus has to proceed under the influence of continuously changing and locally very different salt concentrations in the cheese moisture, particularly in the beginning of the ripening period.

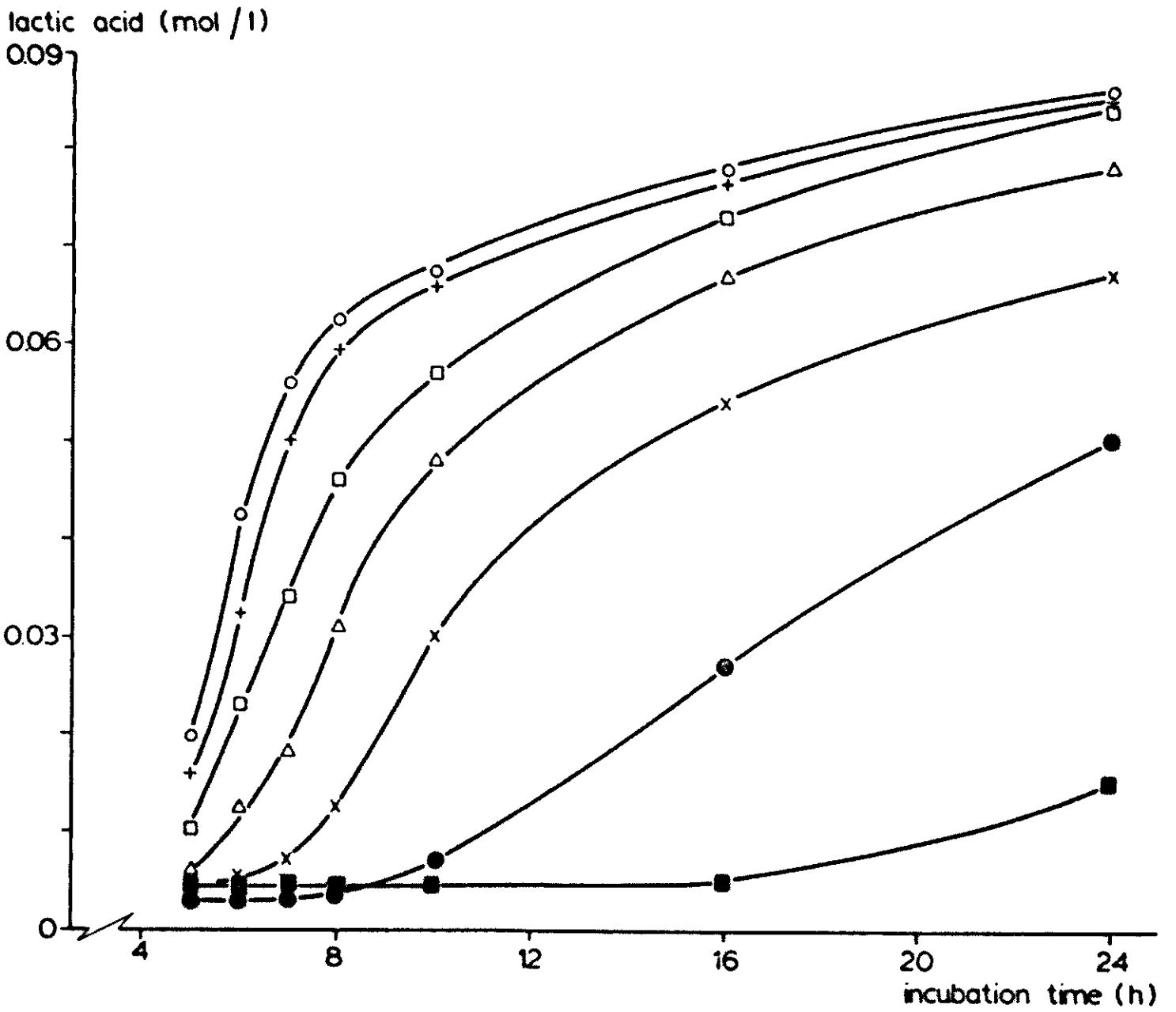

Fig. 9. Growth of *S. lactis* starter strain in MRS broth at 30 °C without salt (○——○) and with NaCl concentrations of 1 % (+——+), 2% (□——□), 3 % (△——△) 4 %, (×——×), 5% (●——●) and 6% (■——■).

The way in which the growth of the starter bacteria is influenced by salt is shown in Fig. 9. Growth is expressed as mol lactic acid formed per litre of culture medium. The bacteria was cultivated in a series of MRS broths (9) at 30 °C with increasing NaCl concentrations. After sterilization the pH of the broth was 6.6. It is clearly shown that acid formation decreases at higher salt concentrations, and that at NaCl concentrations of 4 % and higher growth is starting very slowly.

The course of the pH in the different parts of the cheese (Fig. 6) can now be explained. Before salting, the conditions for growth of the starter streptococci are almost the same throughout the mass of the cheese. However, this situation is changed drastically by the salting of the cheese. During the first days of the ripening period salt contents in the moisture of part K are higher than 6 % (Fig. 5). At these salt concentrations the growth of the starter bacteria will be very strongly retarded (Fig. 9), which is reflected in a very slight decrease of the pH. As a result of the rapid inward migration of the salt, the salt content soon reaches values at which a faster growth of the starter bacteria again becomes possible. From about the fourth day, on which the salt content in the cheese moisture has almost reached the final concentration, to about the tenth day a regular decrease of the pH in part K is observed. Subsequently the growth of the starter bacteria is increasingly retarded by the decreasing pH resulting in a more and more retarded decrease of the pH.

In contradiction to the course of the pH of part K, a direct and regular decrease of the pH of part M is observed in the beginning of the ripening period. This is understandable because of the fact that the starter bacteria are initially not at all and later on are only gradually confronted with increasing growth-retarding salt concentrations in the cheese moisture. After the fourth day the rate of acid formation is decreasing because the growth of the starter bacteria becomes increasingly retarded by the decreasing pH and also by the salt concentration in the cheese moisture which increases up to about the seventh day.

The course of the pH in the other parts of the cheese, situated between K and M, can be explained analogously from the influence of the salt content in the cheese moisture and of the pH on the growth of the starter bacteria.

The characteristic course of the pH in the cheese is thus primarily brought about by the following factors:

– by the slow rate of acid formation in the cheese. Apart from the slow acid-producing properties of the starter bacteria and their salt resistance, this speed depends on the amount of starter used in the production and on the ripening temperature of the cheese. Too fast an acid formation results in too acid a cheese and consequently in a failed product;

– by the fact that the cheese is salted directly after pressing;
– by the high moisture content of the cheese, which makes the transport of salt into the cheese so quick that even in the centre of the cheese a salt concentration in the moisture which is growth-retarding to the starter bacteria is obtained after a short period of time;
– by the final salt concentration in the cheese moisture. At too low a concentration the growth of the starter bacteria will be retarded insufficiently so that a cheese with too low a pH will be obtained. At too high a concentration the pH will decrease insufficiently. In both cases a failed product with an unsatisfactory taste and consistency will be obtained.

3.2.2.1 The uniformity of the dimensions of the cheese. From Section 3.2.2 it appears that the salting process is of very great importance to the pH regulation in the cheese. Since considerable significance is attached to a uniform manner of salting, one has to consider the uniformity of the dimensions of the cheese because the amount of salt taken up by the cheese, the final salt concentration in the cheese moisture and the speed at which a uniform distribution of the salt in the cheese moisture is obtained, are dependent on these dimensions.

3.2.3 Protein breakdown in the cheese

The contents of N soluble in the cheese moisture and of amino acid-N of the

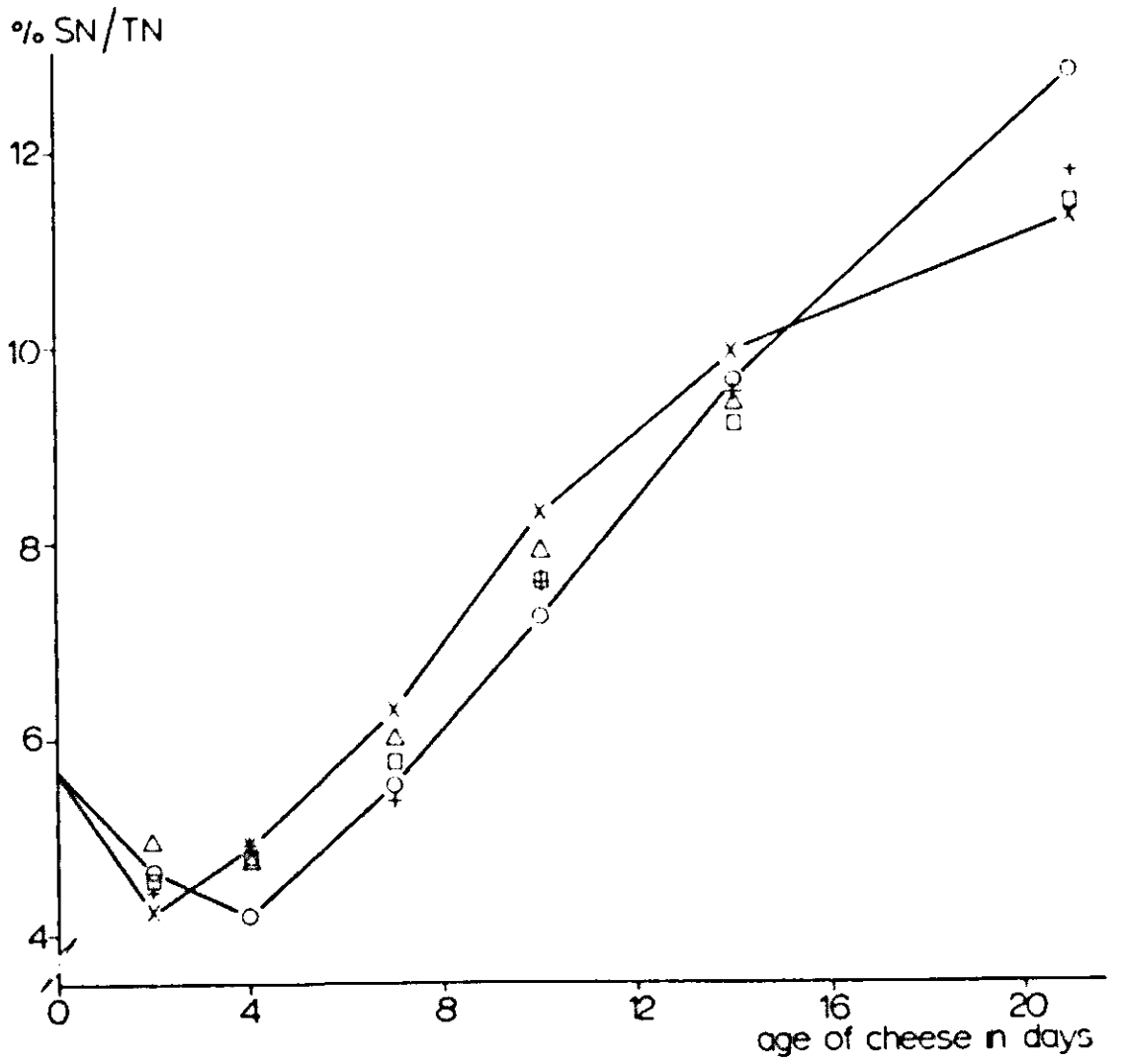

Fig. 10. Protein breakdown in Meshanger cheese. Contents of soluble N in a percentage of total N of samples K (○——○), R1 (+), R2 (□), R3 (△) and M (×——×) on different days of the ripening period of the cheese.

Table 1. Protein breakdown in Meshanger cheese. Contents of total N, soluble N and amino acid-N of samples K, R1, R2, R3 and M on different days of the ripening period.

Sample	Age of cheese in days					
	2	4	7	10	14	21
A. TN %						
K	2.96	2.82	2.98	2.98	3.14	3.32
R1	2.65	2.57	2.71	2.79	2.92	2.87
R2	2.63	2.61	2.70	2.73	2.80	2.76
R3	2.57	2.68	2.75	2.71	2.74	na
M	2.72	2.65	2.78	2.64	2.72	2.63
After pressing of the cheese, TN % was 2.31.						
B. % SN/TN						
K	4.66	4.22	5.54	7.25	9.59	12.86
R1	4.41	4.94	5.39	7.60	9.55	11.74
R2	4.56	4.79	5.74	7.58	9.18	11.49
R3	4.98	4.81	6.00	7.89	9.45	na
M	4.26	4.91	6.33	8.30	9.96	11.33
After pressing, SN % was 0.133 and % SN/TN 5.76.						
C. % AN/TN						
K	0.57	0.60	0.70	0.74	0.64	0.69
R1	0.72	0.70	0.81	0.57	0.75	0.94
R2	0.80	0.77	0.89	0.92	0.86	0.91
R3	0.86	0.78	0.91	0.89	0.91	na
M	0.77	0.79	1.22	1.02	1.03	1.03
After pressing, % AN/TN was 1.08.						
D. % AN/SN						
K	12.32	14.29	12.64	19.19	6.64	5.39
R1	16.24	14.17	15.03	7.55	7.89	8.01
R2	17.50	15.99	15.51	12.08	9.34	7.89
R3	17.19	16.30	15.17	11.21	9.65	na
M	18.10	16.13	19.27	12.33	10.33	9.06
After pressing, % AN/SN was 18.80.						

na = not analysed.

different parts of the cheese on the successive sampling days are given in Fig. 10 and Table 1.

3.2.3.1 Formation of soluble N. Initially a decrease of soluble N is observed. If the formation of soluble products of protein degradation by proteolytic enzymes present in the cheese is left out of consideration for the moment, this decrease will necessarily be seen to occur. With the large amount of moisture lost by the cheese during the first days after production, soluble N substances in the milk serum, which form a part of the total soluble N, will leave the

cheese. The following example of a calculation may serve as an illustration.

After pressing, the cheese had the following composition per 100 g: moisture 66.18 g, dry matter 33.82 g, TN 2.31 g of which 2.177 g was insoluble and 0.133 g was soluble N. Let us assume that during the first two days 15 g of moisture left the 100 g of cheese and that the salt content in the cheese moisture after these two days amounted to 6 %. If it is assumed further that with this quantity of moisture a proportional amount of the dry matter dissolved in the moisture left the cheese and that the dry matter content of this moisture was 6 % (a reasonable value for cheese whey), then $15 \times 0.06 = 0.90$ g of dry matter left the cheese. By the uptake of salt the dry matter increased by $(66.18 - 15) \times 0.06 = 3.07$ g. Thus, the initial 100 g of cheese after two days had changed into 87.17 g of cheese, consisting of 51.18 g moisture and 35.99 g dry matter. In this quantity of cheese 2.177 g insoluble N was present (2.50 %). With the 15 g of moisture $(15 : 66.18) \times 0.133 = 0.03$ g soluble N had left the cheese. Thus, the cheese contained 0.103 g soluble N (0.12 %). From these data a TN % of 2.62 and a % SN/TN of 4.58 can be calculated. In comparison to the situation directly after pressing, % SN/TN thus has decreased from 5.76 to 4.58.

In this way it can also be calculated that % SN/TN must show lower values according as the cheese has lost more moisture and has taken up more salt in the moisture. From the moisture contents and the salt contents in the cheese moisture of the different parts of the cheese (Fig. 4 and 5) than it can be deduced that two days after production % SN/TN in these parts should decrease in the sequence M>R3>R2>R1>K. Apart from a strongly deviating value for M and, to a less extent, for K this tendency seems to be present (Table 1).

A decrease of % SN/TN during the first days after production would not be observed only if % SN/TN should increase sufficiently as a result of protein degradation in the cheese. However, it may be assumed that proteolysis will have been very limited during this period of time because of the fact that lactic acid bacteria are just starting to grow, the unfavourable high pH for the proteolytic activity of rennet, the low temperature and the very high salt concentrations in the moisture of the outer layers of the cheese.

The decrease of % SN/TN during the first days is followed by an increase during the rest of the ripening period. Up to the tenth day it is observed that % SN/TN is the highest in the central part of the cheese and shows smaller values according as the parts of the cheese are situated more closely to the surface. Because of the unhomogeneity of the cheese and the small differences between the values of % SN/TN, there is no justification for drawing conclusions from these data about the degree in which the degradation of protein

had taken place in the different parts of the cheese. In model experiments (details to be published) concerning the degradation of the Ca-paracaseinate-phosphate complex by proteolytic enzymes involved in the ripening of Meshanger cheese, it was however established that this degradation must have been greatest in the central part of the cheese.

Particularly after the tenth day of the ripening period, a stronger increase of % SN/TN is observed in the portions K and R1, and somewhat later also in R2, than in the centre of the cheese. As a result of this, after 21 days % SN/TN shows the highest value in K with lower values as the samples approach more closely the centre of the cheese. This reversed situation in comparison with that in the beginning of the ripening period must be attributed to the activity of proteolytic enzymes, formed by micro-organisms which in the meantime have developed on the cheese surface.

3.2.3.2 Formation of amino acid-N. From Table 1C it can be learned that during the entire ripening period % AN/TN in the different parts of the cheese shows a reasonably constant and low level with values between 0.6 and 1 %. Apart from some exceptions in all parts a decrease of % AN/SN is observed as the ripening period proceeds (Table 1D). Particularly after the seventh day, when the chemical composition of the cheese (moisture and salt content) has almost stabilized, the experimentally found decreases of % AN/SN in the different parts of the cheese between two successive sampling days appear to be reasonably in accordance with the values which could be calculated if these decreases could have been caused exclusively by the increases of % SN/TN. From this it can be concluded that the formation of amino acids in the cheese, if present at all, will be extraordinarily limited and of no importance to the taste of the cheese.

3.2.3.3 The relation between protein breakdown and the consistency of the cheese. As mentioned in Section 3.1.2, the weakening of the cheese body starts about one week after production, beginning in the central portion of the cheese. At that time protein breakdown is most advanced in that portion of the cheese. Also during the remaining days of the ripening period an increasing protein breakdown in any given portion of the cheese is accompanied by an increasing softening of the cheese. This could indicate a strong relation between the consistency of the cheese and the degree of proteolysis, for a particular composition of the cheese. This last addition is necessary because, particularly during the period that the softening of the cheese body becomes visible, the different parts of the cheese still show considerable differences in composition, especially in their pH.

It is worth mentioning that a ripened cheese with a soft consistency became

firmer again in conditions which allowed acidification to go too far (too low a salt content in the cheese moisture).

3.3 The causes of protein breakdown in the cheese

3.3.1 The ripening of the cheese in the absence of the surface flora

The most important sources of proteolytic enzyme activity in the cheese could be considered to be the surface flora, the lactic acid bacteria, the calf rennet and milk protease. The question now arose as to which sources contributed primarily to the protein breakdown and to the related changes of consistency in the cheese.

To answer this question it was first studied how the ripening of the cheese would proceed in the absence of the surface flora, although the fact that the cheese ripened from the inside to the outside and the above-mentioned relation between the consistency of the cheese and the degree of protein breakdown did not indicate any essential contribution of the surface flora to the

Table 2. Meshanger cheese ripened under anaerobic (A) and normal conditions (B). Composition of samples K, R1, R2, R3 and M at an age of the cheese of 14 (A) and of 15 days (B). Values of % SN/TN on the other sampling days of the cheeses are also given.

Cheese	Sample	pH	Moisture content (%)	NaCl (%)*	Reducing carbohydrates (%)*	Lactic acid (%)*	% SN/TN after . . . days		
							7	14	21
A	K	5.26	54.86	3.45	2.53	2.57	5.10	7.48	9.48
	R1	5.20	57.36	3.38	2.59	2.51	5.62	8.70	10.20
	R2	5.06	59.12	3.49	2.50	2.51	5.83	9.93	11.62
	R3	5.00	58.65	3.41	2.49	2.58	6.31	10.52	12.01
	M	4.98	58.28	3.41	2.32	2.58	6.82	10.31	12.04
Cheese	Sample	pH	Moisture content (%)	NaCl (%)*	Reducing carbohydrates (%)*	Lactic acid (%)*	% SN/TN after . . . days		
							8	15	22
B	K	5.13	52.92	3.17	1.55	2.49	6.80	9.94	13.91
	R1	5.02	58.80	3.10	2.07	2.62	6.35	10.14	12.57
	R2	4.97	60.40	3.06	2.19	2.54	6.99	11.02	12.03
	R3	4.92	60.03	3.02	2.29	2.53	7.33	10.99	12.12
	M	4.92	59.93	3.03	2.42	2.45	7.21	11.11	12.37

* In the moisture of the cheese.

ripening of the cheese. In the manner described in Section 2.1.2 some cheeses of a batch were ripened under anaerobic conditions, whereas the remaining cheees of the batch were ripened in the normal way.

On different days of the ripening period normally and anaerobically stored cheeses were sampled and analysed for their composition. For organizational reasons normal cheeses were sampled after 8, 15 and 22 days, and anaerobic cheeses after 7, 14 and 21 days. Anaerobic cheeses showed fairly constant salt contents in the moisture (3.4 - 3.6 %) on the successive sampling days. With the normal cheeses, however, these contents varied very considerably: 2.6 - 2.8 % in the cheese analysed after 8 days and 4.2 - 4.3 % in the 22-day old cheese. Because of the great influence of this content on the other ripening characteristics of the cheese the complete composition of the cheese is only given after two weeks of ripening. At this age, when the cheese is ready to be eaten, moisture and salt contents of both cheeses were in reasonable agreement (Table 2).

As appears from Table 2 and Section 3.1.5, the cheeses had somewhat too low a salt content in the moisture. As a result of this in both cheeses the pH was lower and the lactic acid content in the moisture somewhat higher than normal for Meshanger cheese in ripened condition (see Fig. 6 and 7).

Particularly during the third week of the storage period considerable differences occurred between the sum of the contents of reducing carbohydrates and of lactic acid in the moisture of the outer layers of the cheeses. In all parts of the anaerobically stored cheeses this sum showed an almost constant level with values between 4.90 and 5.10 % during the entire period of storage. This was also the case in the portions R3 and M of the normally stored cheeses, showing values between 4.80 and 5.00 %. In the other parts of these cheeses, however, this sum showed a continuing decrease during the ripening period. This decrease was stronger as the part was situated more closely to the surface of the cheese. Thus, the values after 8, 15 and 22 days, respectively, were as follows: in part K 4.70, 4.04 and 3.04 %, in part R1 4.81, 4.69 and 3.95 %, and in part R2 4.80, 4.73 and 4.59 %. This reflects the increasing activity of micro-organisms growing on the surface of the normally stored cheeses during continued storage of the cheese, as was assumed earlier (Section 3.2.1).

Particularly after storage for more than 14 days important differences were also observed between values of % SN/TN in the outer layers of the cheeses. Contents found on the different days of sampling are given in Table 2. Apart from small deviations in the parts R3 and M the increase of % SN/TN in the anaerobically stored cheeses occurred in such a way that the values always remained the lowest in part K and were higher towards the centre of the

cheese. On the individual days of sampling the ripening degrees of portions R3 and M of the anaerobically stored cheese almost corresponded to those of the normally ripened cheese. In agreement with earlier results (Section 3.2.3.1) a stronger increase of % SN/TN was observed in the portions K and R1 of the normally ripened cheeses, which became very clearly visible when the cheese was analysed after 21 days. At that time the degrees of ripening of the parts K and R1 exceeded those of the other parts of the cheese. This reflects the proteolytic activity of micro-organisms on the cheese surface during prolonged storage of the cheese.

In both cheeses during the entire ripening period only small differences were found between the values for % AN/TN. Moreover these values were always lower than 1 %, indicating that hardly any amino acids had been formed.

In both the normally and anaerobically ripened cheeses the changes of consistency occurred in almost the same way, resulting in the same degree of softness of the cheeses at an age of two weeks. The presence and activity of the surface flora is thus not necessary for the softening of Meshanger cheese as it is in the case of other soft cheeses such as Camembert and Brie.

3.3.2 The ripening of the cheese in the absence of bacteria (GDL cheese)

In the foregoing section it was concluded that the surface flora was at most of minor interest to the protein breakdown in the cheese. It next was studied how the ripening of the cheese would proceed in the absence of microbial enzymes, particularly those of the lactic acid bacteria. For that purpose GDL cheeses were prepared according to the method described in Section 2.1.3. Thimerosal was added as a preservative. From the observations performed on these cheeses the following example is given. At an age of 7 days a cheese of a given batch showed the following composition: moisture content 59.6 %, salt content in the cheese moisture 3.52 %, TN 2.41 %, % SN/TN 11.24. The pH of the cheese was 5.23. The total bacterial count, determined aerobically as well as anaerobically with Plate Count Agar provided with 1 % skim milk, was less than 200 per g of cheese. This indicated that bacteria had not developed in the cheese. At this age the cheese had already softened completely, its consistency being comparable to that of a normal Meshanger cheese at the stage where it is ripe for eating. On continued storage the other cheeses of the batch became so soft that they started to liquefy at an age of about ten days.

These results indicated that even if the lactic acid bacteria contribute to the protein breakdown in the cheese, this contribution is at least not crucial for the changes of consistency in the cheese.

After seven days the ripening degree of the GDL cheese (11.24 %) appeared to be about equal to that in the central portion of a normally ripened Meshanger cheese at an age of two weeks (Tables 1 and 2). This indicated a much larger proteolytic activity of rennet in the GDL cheese. The ultimate activity of milk protease in this cheese had to be considered as being less than in a normal Meshanger cheese for the reason that the pH had decreased more rapidly. At moulding the pH of the GDL cheese had already decreased to 5.45, whereas this value in the centre of a normal cheese is only reached some days after production (Fig. 6). According to the literature the activity of milk protease decreases at lower pH values (10, 11, 12).

It is not surprising that a faster degradation of the protein was found in the GDL cheese as a result of rennet activity. Although it was not established experimentally it can be safely assumed that the GDL cheese will have contained more rennet than a normal Meshanger cheese because of the fact that more rennet will have been retained in the curd as a result of the rapid decrease of the pH of the milk (13). In addition to this, shortly after production the pH of the GDL cheese was already more favourable for rennet activity than was that of a normal cheese.

Furthermore it has to be considered that the composition of the GDL cheese will not have been completely comparable with that of a normally ripened Meshanger cheese. In this connection one can consider the calcium content. As a result of the rapid decrease of the pH during production of the GDL cheese, calcium will have left the paracasein complex and will have been lost with the whey. The GDL cheese will thus have contained less calcium than a normal cheese. It may be that such differences have influenced the changes in consistency. However, this does not weaken the conclusion that the cheese softened rapidly in the absence of microbial enzymes.

Together with the faster protein breakdown in the GDL cheese an earlier softening of the cheese was observed. This observation also shows the existence of a relation between the protein degradation and the consistency of the cheese.

From the results obtained from our investigations it could be concluded that, as far as the consistency of the cheese is related with the degree of protein degradation, the proteolytic activity of the calf rennet used must be the main cause of the softening of Meshanger cheese.

4 Discussion

4.1 The regulation of the pH in Meshanger cheese

In most well known varieties of cheese the desired lactic acid fermentation

is completed or almost so before the cheese is salted. In cheeses like Gouda and Edam the pH is changed only to a very small extent during the ripening period. On the other hand, in other varieties of cheese the pH during the ripening is increased to a greater or less degree by the de-acidifying activity of a surface flora as is the case with Brie, Camembert and Limburger cheese or of an internal flora of moulds as is observed with the blue-veined cheeses.

By the use of a very slow acid-producing starter and by the other conditions during the production and ripening, the development of the pH in Meshanger cheese is markedly different from that outlined briefly above for other varieties of cheese. The main factors determining this development are mentioned in Section 3.2.2. A distinctive characteristic of the cheese is that the pH is decreasing continuously during the ripening period.

During the normal ripening period of 14 days the contribution of the surface flora to the pH regulation in the cheese is practically negligible, as deduced from the almost constant values of the sum of the contents of reducing carbohydrates and of lactic acid in the cheese moisture (Fig. 8). On continued storage the slow decrease of the pH of the cheese towards the end of the time can be changed into an increase as a result of the de-acidifying activities of micro-organisms on the cheese surface, particularly moulds. This is also reflected in a decrease of the sum of the above-mentioned contents in the cheese moisture, beginning in the superficial part of the cheese. However, these phenomena are of very limited importance to the ripening of the cheese, because they occur after the cheese has reached its correct stage of ripening.

The curd is not washed during the production of the cheese, and because of the high moisture content (about 60 %) much lactose is enclosed. The cheese still contains a considerable amount of carbohydrates when it has reached its correct stage of ripening. At that time only 40 - 50 % of the carbon sources are present as lactic acid (Fig. 7, Table 2).

The optimal pH for Meshanger cheese in ripened condition (14 days) is 5.1 - 5.2. A slow acid formation by the starter bacteria and a high moisture content of the cheese, which allows the diffusion of salt into the cheese to proceed so rapidly that salt concentrations which retard the growth of these bacteria are reached after a short period of time, are of decisive importance to the correct regulation of the pH and consequently to a successful product. These factors could provide a reasonable explanation of the fact that no starter was used at the making of the cheese on the farm and that the cheese could only be made during autumn (1). A slow rate of acid formation in the cheese first required the presence of only a small number of lactic acid bacteria in the milk. In connection with this it would also be logical for the cheese always to be made from milk as freshly drawn as possible, and not from a mixture

of creamed evening and fresh morning milk as was frequently the case in the production of Edam cheese. The evening milk, which was kept overnight at a not very low temperature, without doubt would have shown a considerable development of lactic acid bacteria. That the cheese was made only during the autumn might be accounted for by the circumstances that lactic acid fermentation in the cheese frequently did not proceed smoothly during this period and that it was difficult to obtain the desired dryness of the curd, which favoured a high moisture content of the cheese. The composition of the milk could also have been of importance, particularly the relatively high fat content and the higher acid-binding capacity of autumn milk.

4.2 Protein breakdown and consistency of the cheese

A distinctive character of Meshanger cheese is that the softening of the cheese body starts in the centre and from there proceeds to the surface of the cheese. We concluded that there is a strong relation between the degree of protein breakdown and the consistency of the cheese. This relation is not always observed. Weckx & Vanderpoorten (14) found that the size of the unripe core of Herve cheese was not decreased by the use of a microbial rennet instead of calf rennet, although the cheese made with microbial rennet showed considerably more degradation of the protein.

In Section 3.3.1 it was shown that during the normal ripening period of Meshanger cheese the contribution of the surface flora to the protein breakdown in the cheese was not of crucial importance to the changes of consistency occurring in the cheese.

Any substantial contribution of the starter bacteria to the proteolysis cannot be expected, as it is known that slow acid-producing lactic acid bacteria have only a weak proteolytic capacity (15, 16, 17).

In a subsequent paper it will be shown that milk protease may contribute to the protein breakdown in the cheese. However, this contribution is limited because of the short ripening time of the cheese, the low ripening temperature and other conditions during the ripening period which soon become less favourable for the activity of the enzyme, particularly the pH.

The results of the experiments with GDL cheeses (Section 3.3.2) strongly indicate that the protein degradation in Meshanger cheese and the changes of consistency related to it are caused by the proteolytic activity of the calf rennet used. In the meantime this has been established by de Jong (18). In a normal ripening Meshanger cheese the conditions such as the pH, the salt content and the high moisture content will determine the degree of rennet activity and thus the degree of protein breakdown and the changes of consis-

tency. However, at a given degree of protein degradation the changes of consistency are also influenced by the physical and chemical conditions in the cheese, in view of the observation that a ripened cheese becomes firmer again under conditions which allow too great a development of acidity in the cheese (Section 3.2.3.3).

The important contribution of calf rennet to protein breakdown in cheese has been established by several workers. With respect to this, the investigations with aseptic starter-less cheeses have contributed substantially to the evidence (19, 20, 21, 22). The dominant importance of rennet activity to the consistency of cheese is however insufficiently distinguished.

It can be safely assumed that in cheeses with or without a surface flora, with a high moisture content and with a pH which has usually been adjusted by washing the curd to a value found in a ripe Meshanger cheese, calf rennet activity will contribute very substantially to the consistency of the cheese as is the case in Meshanger cheese. Cheeses such as Butterkäse, Bel Paeso, the modern Saint Paulin, and Kernhem probably can be considered as belonging to this group. The main function of a surface flora of coryneform bacteria on such cheeses will be a contribution to the flavour of the cheese, which of course as such is of great importance. Calf rennet activity can equally well have a very important function in the consistency of cheeses with a lower moisture content such as Gouda and Edam cheese.

The situation seems to be more complicated in cheeses with an initially very low pH, ripening from the outside to the inside under the influence of a surface flora. With respect to these cheeses the prevailing opinion is that the ripening is caused by extracellular proteolytic enzymes formed by the surface flora. These enzymes would migrate through the cheese by diffusion, while rennet and starter bacteria would play no role or perhaps only a preparatory one. Seeler (23) and Knoop & Peters (24) share this opinion, founded on the results of their electron-microscopical investigations on the ripening of Camembert cheese. Lenoir (25) found the proteolytic activity of rennet and that of the internal flora of lactic acid bacteria in Camembert cheese to be almost negligible in comparison with that of the surface flora. Whereas a strong increase of activity was observed in the outer layer of the cheese (8 mm), that in the centre of the cheese remained weak and almost constant during the entire ripening period, and yet this part of the cheese showed a considerable increase of NPN. According to Lenoir his results could indicate that migration of enzymes from the surface to the centre of the cheese was absent. The increase of NPN in the internal part of the cheese could have been caused by a migration of soluble products of protein degradation from the surface to the centre of the cheese rather than by the local formation of NPN

by the sources of proteolytic activity present in that part of the cheese.

Soft cheeses ripening from the outside to the inside do not exhibit any visual ripening in the absence of a surface flora. Thus, there can be no doubt about the great importance of the flora to the ripening of these cheeses. However, one can wonder if the visual ripening of these cheeses is correlated with the proteolytic activity of enzymes formed by the surface flora to the extent that is generally believed. In experiments with cheese model systems showing conditions corresponding to those in Meshanger cheese, the breakdown of the protein by calf rennet at pH 4.8, a value at which the cheese shows a firm and short consistency, quantitatively as well as qualitatively (the degradation of the subcaseins), was almost equal to that at pH 5.1, at which Meshanger cheese shows a soft consistency (details to be published). In relation to this, one could suggest that the degree of protein degradation and the way in which the protein in the cheese has been broken down could allow the softening of the cheese, but that the existing fysical and chemical conditions in the cheese determine if this weakening occurs or not.

In the case of Camembert cheese, for example, the surface flora would then contribute to the visual ripening in two ways. First by a direct contribution to the protein degradation at the surface and in the cheese layers situated directly under it as a result of a possible but probably very limited diffusion of extracellular proteolytic enzymes. Secondly by a de-acidifying activity, owing to which the pH of the cheese is raised, starting at the surface and proceeding to the centre of the cheese. The potentially existing tendency of the cheese to become soft, caused by the degradation of the protein by rennet and the internal flora of lactic acid bacteria, can then manifest itself when the pH has been raised to a certain value. With respect to the explanation of the visual ripening of soft cheeses with a surface flora, ripening from the outside to the centre, such a conception would make us independent of the diffusion of proteolytic enzymes.

Owing to the uncomplicated ripening, Noordhollandse Meshanger cheese is very suitable as a model for research on cheese ripening. The simple method of fabrication and the very short ripening period of the cheese also contribute to this. The investigations are continuing.

Acknowledgment

The author is much indebted to Mr E. de Vries and Mr J. L. Uitentuis for their assistance in cheesemaking.

Samenvatting

A. Noomen, *Noordhollandse Meshanger kaas: een model ter bestudering van rijping van kaas. 2. De rijping van de kaas.*

Het artikel beschrijft de rijping van Noordhollandse Meshanger kaas. Tijdens de rijping is de kaas zeer heterogeen van samenstelling. De kaas vertoont een zeer kenmerkend en in principe van plaats tot plaats in de kaas verschillend pH-verloop. Dit wordt veroorzaakt door het gebruik van een zeer langzaam zurend zuursel, door de kaas te zouten op een tijdstip waarop de groei van de zuurselbacteriën nauwelijks is aangevangen en door de kaas direct na de bereiding te bewaren bij 13 °C, de rijpingstemperatuur. De groei van de zuurselbacteriën vindt derhalve plaaats bij lage temperatuur en onder de invloed van groeivertragende zoutconcentraties in het kaasvocht, die bovendien in het begin van de rijpingsperiode plaatselijk sterk verschillen. De groei van de bacteriën wordt verder beïnvloed door de tijdens de rijping voortdurend lager wordende pH van de kaas en door de uiteindelijke zoutconcentratie in het kaasvocht. Ter voorkoming van een te zure en korte kern dient de snelheid waarmee het zout zich vanaf het oppervlak naar binnen verplaatst, zodanig te zijn dat ook in het midden van de kaas na korte tijd een voor de melkzuurbacteriën groeivertragende zoutconcentratie in het vocht wordt bereikt. Een hoog vochtgehalte van de kaas is daartoe een vereiste. De optimale pH voor de rijpe kaas, d.w.z. wanneer de kaas ongeveer twee weken oud is, is 5,1 tot 5,2.

Doordat de wrongel tijdens de bereiding niet wordt gewassen en door het hoge vochtgehalte van de kaas bevat de rijpe kaas nog een aanzienlijke hoeveelheid suiker. Slechts 40-50 % van de lactose wordt tot melkzuur omgezet.

De oppervlakteflora, voornamelijk bestaande uit gisten en later ook uit schimmels, heeft tijdens de normale rijpingsperiode geen wezenlijke betekenis voor de pH-regeling in de kaas en evenmin voor de eiwitafbraak.

In tegenstelling tot zachte kaassoorten met een oppervlakteflora die van buiten naar binnen rijpen, rijpt Meshanger kaas van binnen naar buiten. Er bleek een sterk verband te bestaan tussen de rijpingsgraad van de kaas en de mate van consistentieverandering. Het bij de bereiding gebruikte kalfsstremsel bleek de primaire oorzaak te zijn van de eiwitafbraak in de kaas en de consistentieveranderingen bij de omstandigheden zoals die in de rijpende kaas aanwezig zijn. Het belang van kalfsstremsel voor de rijping en de consistentie van andere kaassoorten wordt bediscussieerd.

References

1. A. Noomen & H. Mulder, *Neth. Milk Dairy J.* 30 (1976) 230-241.
2. A. Noomen, *Neth. Milk Dairy J.* 31 (1977) 103-108.
3. T. J. Geurts, P. Walstra & H. Mulder, *Neth. Milk Dairy J.* 28 (1974) 102-129.
4. A. J. G. Barnett & G. Abdel Tawab, *J. Sci. Fd Agric.* 8 (1957) 437-441.
5. J. Davidson, *J. Dairy Res.* 16 (1949) 209-216.
6. A. Noomen, *Neth. Milk Dairy J.* 29 (1975) 153-161.
7. H. A. Sirks, *Versl. landbouwk. Onderz.* 49 (1943) 221-263.
8. J. Stadhouders & F. Hassing, *Off. Org. FNZ* 64 (1972) 695-699.
9. J. C. de Man, M. Rogosa & M. E. Sharpe, *J. appl. Bact.* 23 (1960) 130-135.
10. F. Kiermeier & G. Semper, *Z. LebensmUnters. u. -Forsch.* 3 (1960) 282-307.
11. S. Kaminogawa, H. Mizobuchi & K. Yamauchi, *Agric. biol. Chem.* 36 (1972) 2163-2167.
12. J. R. Dulley, *J. Dairy Res.* 39 (1972) 1-9.
13. J. Stadhouders & G. Hup, *Neth. Milk Dairy J.* 29 (1975) 335-353.

14. M. Weckx & R. Vanderpoorten, *Milchwissenschaft* 28 (1973) 332-338.
15. J. E. Citti, W. E. Sandine & P. R. Elliker, *J. Dairy Sci.* 48 (1965) 14-18.
16. D. C. Westhoff, R. A. Cowman & M. L. Speck, *J. Dairy Sci.* 54 (1971) 1253-1258.
17. F. A. Exterkate, *Neth. Milk Dairy J.* 30 (1976) 3-8.
18. L. de Jong, *Neth. Milk Dairy J.* 31 (1977) (in press).
19. B. Reiter, Y. Sorokin, A. Pickering & J. Hall, *J. Dairy Res.* 36 (1969) 65-76.
20. M. L. Green & P. M. D. Foster, *J. Dairy Res.* 41 (1974) 269-282.
21. J. C. Gripon, M. J. Desmazeaud, D. le Bars & J. L. Bergère, *Lait* 55 (1975) 502-516.
22. R. B. O'Keeffe, P. F. Fox & C. Daly, *J. Dairy Res.* 43 (1976) 97-107.
23. G. Seeler, *Milchwissenschaft* 23 (1968) 661-665.
24. A. M. Knoop & K. H. Peters, *Milchwissenschaft* 26 (1971) 193-198, 27 (1972) 153-159.
25. J. Lenoir, *Revue lait. fr.* 275 (1970) 231-243.

Neth. Milk Dairy J. 31 (1977): 103-108

Noordhollandse Meshanger cheese: a model for research on cheese ripening. 3. Manufacture of the cheese on a small scale

A. Noomen

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

Received: 5 January 1977

Summary

This article, the last one of the series, deals with the production of Noordhollandse Meshanger cheese on a small scale and describes the characteristics of the cheese when it is ripe and ready for eating.

1 Introduction

Originally the cheese could only be made in autumn from raw cow's milk (1). Our work on reconstructing the method of production and the ripening of the cheese (1, 2) allowed certain parts of the original process to be adapted to modern methods of cheesemaking, which also makes it possible to produce the cheese throughout the whole year from pasteurized milk.

This article deals with two methods of production on a small scale. In one method the cheese is made traditionally in Edam moulds and salted by rubbing the cheese surface with a paste of sodium chloride and water. In the other method the cheese is made in Gouda moulds and salted in brine.

Finally a description of the cheese is given at the stage where it is ripe and ready for consumption, i.e. at an age of about two weeks.

2 Manufacture of the cheese

2.1 Milk

The cheese milk is standardized by centrifugation to a fat content of 3 ± 0.1 %. With respect to the removal of pathogenic bacteria and of bacteria causing cheese defects, particularly coliform bacteria and in this special case

fast acid-producing lactic acid bacteria, the milk is pasteurized for 15 s at 72 °C. The presence of fast acid-producing lactic acid bacteria in the milk must be avoided because acid formation in the cheese has to proceed slowly. Of course the milk must be of good quality. The raw milk used by us normally had a bacterial count of less than 50 000/ml.

2.2 Additions to the milk

2.2.1 Nitrate. The slow acid formation and the large amount of lactose cause the cheese to be very sensitive to the early-blowing defect. Apart from adequate pasteurization of the milk, extremely good hygienic conditions are required during manufacture to prevent post-contamination, particularly with coliform bacteria. Potassium nitrate is added to the milk (20 g/100 l) as a further precaution.

2.2.2 The starter. The cheese milk is inoculated (0.01 %) with a very slow acid-producing *S. lactis* strain, cultivated for 18 hours at 30 °C in sterile skim milk. The activity of this starter in low pasteurized skim milk was 23 °N according to the method of Stadhouders & Hassing (3).

2.2.3 Rennet. For every 100 litres of milk 40 ml of commercial calf rennet are added (strength 1 : 10 800).

2.3 Renneting of the milk

The milk is renneted at 30 °C. The renneting time should amount to 20 - 25 min.

2.4 Treatment of the curd

To obtain cheese with a high moisture content the curd is worked only very slightly. The treatment is stopped at the time when the still large curd particles are beginning to appear firm. Too long a treatment of the curd is more dangerous to the cheese than a too short one.

In our 'pilot plant' productions quantities up to 200 litres of milk are normally made into cheese. The curd is cut very gently for about 5 min, followed by gently stirring the curd-whey mixture manually for 6 - 7 min. After sedimentation of the curd for 10 min the supernatant whey is taken off.

2.5 Production of the cheese in Edam moulds

2.5.1 Moulding of the cheese. The curd is brought into a 2-kg Edam mould and turned three times with intervals of about 10 min. To obtain a product as uniform as possible the weight of the curd in the vat is 'standardized' to 2250 g before it is bandaged and pressed. In spite of this 'standardization' it remains difficult to obtain a uniform weight of cheese after pressing. At that stage the weight of cheeses from one batch as well as of cheeses from different batches can vary considerably (1625 ± 125 g).

2.5.2 Pressing of the cheese. The cheese is pressed at room temperature for one hour and a half with a pressure of 30 - 35 kg/cheese. During pressing the cheese looses about one fourth of the original weight of the curd.

2.5.3 Salting of the cheese. After the removal of the rim the globular cheeses are immediately brought to a ripening room with a temperature of 13 °C and a relative humidity of about 95 %. They are at once rubbed with a paste of salt and water. This is repeated after 3, 7 and 20 hours. As a result of the very high moisture content after pressing (65 - 70 %), the cheeses sag very rapidly and finally have the shape of a small Gouda cheese. This metamorphosis is completed within 2 days (Fig. 1). To control the sagging of the cheeses they are placed against each other during the first couple of hours and frequently turned. After some days the cheeses are surrounded by a PVC ring (Ø 19 cm, height 4 cm) to prevent them from further sagging.

2.5.4 Treatment and behaviour of the cheese during the ripening period. After salting, the cheeses are washed with lukewarm water, wiped and placed on clean shelves. A flora, consisting particularly of yeast and, in the last stage of the ripening period, also of moulds, develops spontaneously on the cheese surface. The appearance of the surface of the cheese as a result of the growth of this flora has been described in an earlier report (2). Every five days the cheese surface is washed clean with lukewarm water.

During the ripening period the cheese shows a continuous loss of moisture, causing a loss of weight of about one fourth of its weight after pressing.

The softening of the cheese starts in its centre after about 7 days, and after about 14 days the cheese is ripe and ready for consumption.

2.6 Manufacture of the cheese in Gouda moulds

The production of the cheese in Edam moulds, particularly the salting and

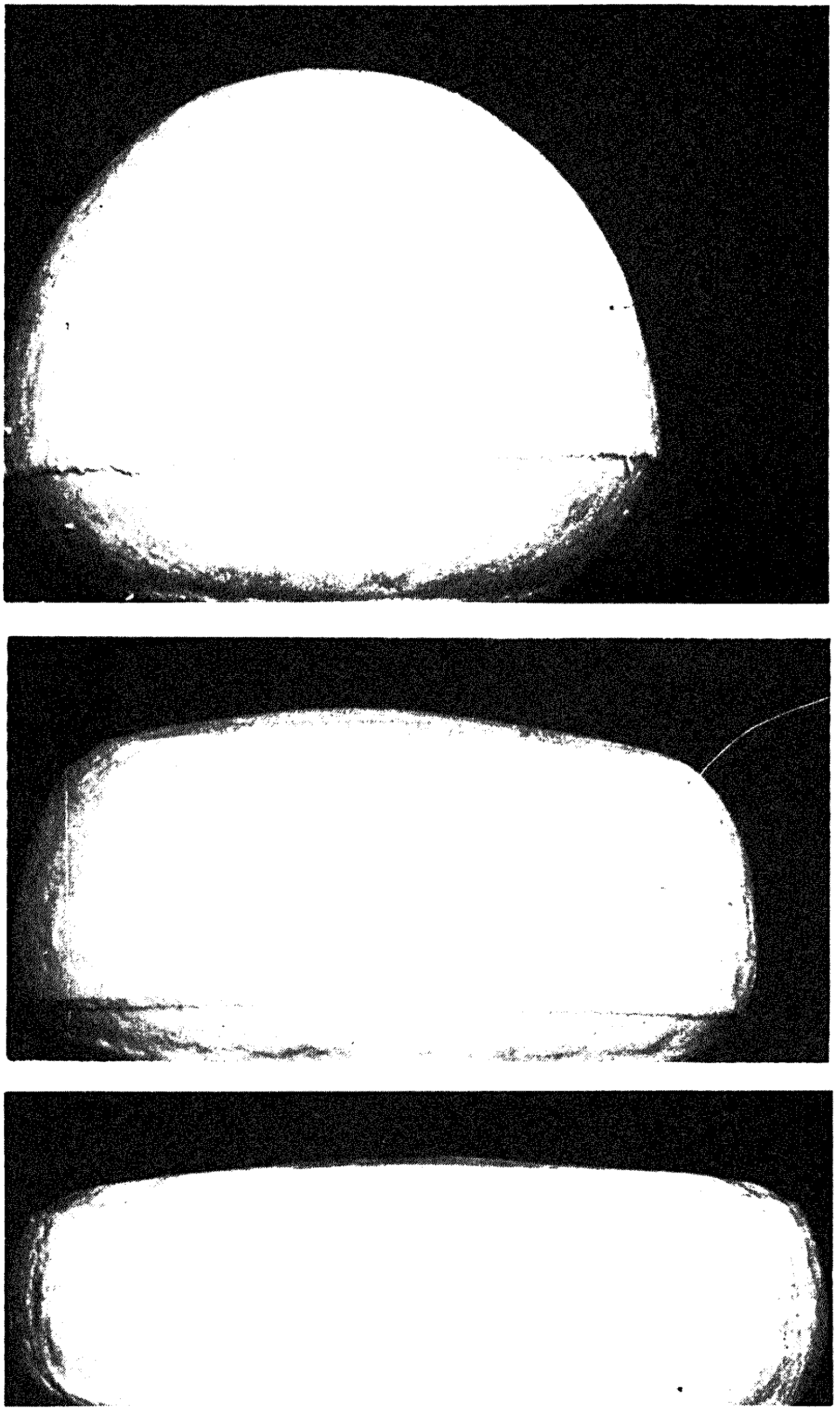

Fig. 1. Sagging of Meshanger cheese from the Edam to the Gouda shape, after pressing. Top: immediately after pressing; middle: 3 hours later; bottom: 48 hours after pressing.

the sagging of the cheese, hardly lends itself to modern production. For that reason the possibilities were considered of making the cheese directly in a Gouda shape and to salt it by brining. Cheeses were made, bandaged and pressed as described before, but in Gouda (2 kg) instead of Edam moulds. They were brined at 13 °C in a solution of 20 % NaCl and 1.2 % $CaCl_2$ (w/w). The brine was buffered with acetate (0.05 *M*) at pH 5.11. This composition of the brine was chosen in accordance with the work of Geurts et al. (4). Their work (5, 6) might also have enabled us to perform orientating calculations on the required brining time and the concentrations of salt in the different layers of the cheese, if the syneresis of a large amount of whey did not take place complicating such calculations enormously. Therefore, and as the course of the changes in salt concentration in the cheese moisture is crucial for the growth of the lactic acid bacteria, we preferred empirical experiments. After brining, the cheeses were handled as those made in Edam moulds. In these experiments, with a brining time of 20 hours, cheeses were obtained of a quality corresponding with that of traditionally made cheeses.

3 Main characteristics of the cheese in matured condition

Owing to the PVC ring the shape of the cheese is that of a flat cylinder with a diameter of 19 cm and a height of about 4 cm. The weight of the ripe cheese is 1250 ± 100 g. The composition of the cheese is as follows: moisture content 57 - 60 %, fat content in the dry matter 45 - 47 %, protein content 14 - 18 % and salt content in the cheese moisture 3.75 - 4.50 %. Optimal pH value is 5.1 - 5.2. In addition to lactic acid the cheese contains a considerable amount of non-fermented carbohydrates and frequently the contents of lactic acid and of sugar in the cheese moisture are about equal. The consistency is soft to very soft. The taste of the cheese is mildly acid and slightly yeasty. As a result of the repeated washings a distinct flora is not visible on the surface of the cheese.

Samenvatting

A. Noomen, *Noordhollandse Meshanger kaas: een model ter bestudering van rijping van kaas. 3. De bereiding van de kaas op kleine schaal*

Het artikel, het laatste van een serie van drie, beschrijft de bereiding van Noordhollandse Meshanger kaas op kleine schaal. Onderdelen van de oorspronkelijke bereidingswijze werden aangepast aan de moderne wijze van kaasbereiding, zodat het mogelijk is de kaas gedurende het gehele jaar te bereiden, uitgaande van laaggepasteuriseerde melk. Ook bleek het mogelijk te zijn de oorspronkelijke wijze van vormen van de kaas (be-

reiding in Edammer vaten, gevolgd door uitzakken tot een kleine Goudse kaas) en van zouten (insmeren van de kaas met een papje van zout en water) te vervangen door een directe bereiding in Gouds model en een pekelproces.

Ten slotte worden de kenmerken van de kaas beschreven in consumptierijpe toestand, d.w.z. op een ouderdom van ongeveer twee weken.

References

1. A. Noomen & H. Mulder, *Neth. Milk Dairy J.* 30 (1976) 230-241.
2. A. Noomen, *Neth. Milk Dairy J.* 31 (1977) 75-102.
3. J. Stadhouders & F. Hassing, *Off. Org. FNZ* 64 (1972) 695-699.
4. T. J. Geurts, P. Walstra & H. Mulder, *Neth. Milk Dairy J.* 26 (1972) 168-179.
5. T. J. Geurts, *Versl. landbouwk. Onderz., Wageningen* 777 (1972).
6. T. J. Geurts, P. Walstra & H. Mulder, *Neth. Milk Dairy J.* 28 (1974) 102-129.

Neth. Milk Dairy J. 31 (1977): 163-176

A rapid method for the estimation of the dissolved and the undissolved nitrogen compounds in cheese

A. Noomen

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

Received: 15 April 1977

Summary

The consistency of cheese is related to the undissolved N components of the cheese. This article deals with the estimation of the undissolved N compounds in cheese, based on an extraction method for estimating the dissolved N compounds, products of protein degradation and milk serum proteins. Difficulties were encountered with the clarification by centrifugation and filtration of extracts, prepared by extracting the cheese with a liquid with a composition comparable with that of cheese moisture. For that reason the possibility of an alternative clarification method was studied.

The aim was satisfactorily achieved by homogenizing the cheese under standardized conditions at 30 °C in a 0.037 *M* $CaCl_2$ solution with the aid of an Ultra Turrax. The pH of the extraction mixture was adjusted to 7.5. After centrifugation for 10 min at 40 000 *g* at 30 °C and filtration through paper, the quantity of nitrogen in the filtrate was estimated.

The method is simple and rapid, so that any further degradation of protein during the extraction is prevented. The results obtained with the method give a fair representation of the amount of N compounds soluble in cheese moisture. Moreover, they compare well with those obtained on the overall breakdown of α_{s1} and β casein in cheese as measured by quantitative polyacrylamide gel electrophoresis.

1 Introduction

Modern analytical methods, such as electrophoretic and gel filtration techniques, have contributed much to a better understanding of the proteolytic processes involved in the ripening of cheese. The methods, particularly when they are used in combination, give a detailed picture of the products formed by protein degradation. Thus, if one wants to perform detailed studies on proteolysis in cheese the use of those methods is the appropriate course. In other cases, in which the main interest is for general information on the degree of protein degradation in cheese, there is still a need for a simple, reliable and practical method.

The consistency of cheese is mainly determined by the water, the fat and the undissolved cheese protein materials. Therefore the question whether the protein components of cheese are in the undissolved or dissolved state is of dominant importance, especially for soft cheeses with a high moisture content. If information is needed about the dissolved, and consequently of the undissolved, N components with the aid of an extraction method, the conditions in the extract to be analysed have to agree as well as possible with the actual conditions in the cheese. In relation to this, experiments were made in our laboratory to perform the extraction at the pH and the temperature of the cheese and to adapt the calcium and sodium concentrations in the extraction liquid to the corresponding values found in cheese moisture. Particularly the sodium concentration proved to be of decisive importance because this factor had a large effect on peptizing by sodium chloride of the paracasein complex in strongly diluted suspensions, such as cheese extracts (1, 2).

This article is a report on our efforts concerning the development of an extraction method based on these considerations.

2 Materials and methods

2.1 Noordhollandse Meshanger cheese

The cheese was made as described in an earlier report (3, 4, 5), making use of Edam moulds.

2.2 Preparation of a cheese extract

To a weighed amount of cheese, representing 10 g of cheese dry matter, extraction liquid was added in such a quantity that the total volume of moisture present amounted to 200 ml. The mixture was homogenized under standardized conditions for 5 min with an Ultra Turrax. After adjustment of the pH the extraction mixture was centrifuged. The supernatant was filtered through paper.

Details of the conditions during the preparation of cheese extracts are mentioned further on.

2.3 Estimation of nitrogen

Nitrogen determinations were made by the micro-Kjeldahl method, with one tenth of the amounts of reagents used in the macro-Kjeldahl method according to Netherlands Standard NEN 3198.

Total nitrogen (TN) was determined in 1 g of cheese according to the method described in an earlier report (6).

Nitrogen in cheese extracts (N) was expressed as a percentage of the total nitrogen of the cheese (% N/TN).
The contents of maximal coagulable nitrogen (CN) and of non-coagulable nitrogen (NCN) of extracts were determined according to the principle of the method of Sirks (7), in which N fractions are distinguished by their sensitivity to coagulation depending on pH and heat treatment. Determinations were carried out on extracts obtained by homogenizing the cheese with a solution of 0.137 *M* $CaCl_2$ and 0.684 *M* NaCl and adjusting the pH of the mixture to 5.1 (see Section 3.1).

In a volumetric flask, to 40 ml of extract 10 ml of 0.25 *N* HCl were added (final pH about 1.6). The flask was heated at 55 °C for 30 min and kept overnight at room temperature. Nitrogen was estimated in the filtrate. NCN was expressed as a percentage of the total nitrogen of the cheese (% NCN/TN). The difference between % N/TN and % NCN/TN represented % CN/TN.

Preliminary experiments with extracts of Meshanger cheese, obtained by using the extraction liquid mentioned above, showed that the amount of 'coagulable nitrogen' increases when the pH is lowered until a value of about 2 is reached and remains almost constant at still lower pH values. These results were also obtained in this laboratory by Kleter (8) with similar extracts of Gouda cheese. This method of coagulation of nitrogen compounds is somewhat at variance with that in a water extract according to the method of Sirks. In such extracts a pH optimum is observed with respect to the degree of coagulation.

2.4 Polyacrylamide gel electrophoresis

Electrophoretic experiments were performed according to the method described by de Jong (9).

3 Experiments and results

3.1 Extraction of Meshanger cheese under conditions resembling the actual conditions in the cheese

During the ripening period a Noordhollandse Meshanger cheese is not homogeneous in composition. Different parts of the cheese show considerable and continuously changing differences in pH and salt content (4) and probably the same will be the case with the calcium concentration in the cheese moisture. For these reasons it was decided to adjust the conditions during the extraction of the cheese to the average conditions in a mature cheese. Normal values for the calcium and NaCl concentration in the cheese moisture being

0.55 and 4 %, respectively, a solution of 0.137 *M* $CaCl_2$ and 0.684 *M* NaCl was chosen as the extraction liquid. The extraction was performed at 13 °C, the ripening temperature of the cheese. The pH of the extraction mixture was adjusted to 5.1, after which the mixture was centrifuged at 40 000 *g* for 45 min at 13 °C.

The extracts obtained under these conditions appeared to be turbid to very turbid, particularly those prepared from cheese in an advanced state of ripening. The degree of turbidity of the extracts was scarcely influenced by the time of centrifugation, which was also expressed by the almost constant values for % N/TN at centrifugation times varying from 10-45 min.

The N compounds which were responsible for the turbidity of the extracts could not be considered as being dissolved, which made it necessary to achieve a better clarification of the extracts. Nevertheless, an interesting question was whether this nitrogen represented products of protein degradation or not. To gain some information about this point, the nature of the N compounds present in turbid and in clarified extracts of the cheese was studied. Clarification of turbid extracts was performed by subjecting the extracts at 13 °C to a Seitz filtration (filter EKS), which proceeded extremely slowly in most cases. Seitz filtrates obtained from very young cheeses were perfectly clear, while those from cheeses in an advanced state of ripening were more or less opalescent or, occasionally, showed a slightly turbid appearance.

On different days of the ripening period a homogeneous sample of cheese was prepared by grinding it in a mortar. An extract of the cheese was made according to the directions given in Section 2.2 in the conditions mentioned above. Part of the extract was subjected to Seitz filtration. In the turbid extract as well as in the Seitz filtrate of it the contents of N, NCN and CN were estimated. Fig. 1 shows the results of this experiment.

On every sampling day with both the extract and the Seitz filtrate the same value was found for % NCN/TN. This value increased regularly and gradually during the ripening period. In the extract % CN/TN appeared to increase gradually during the first days of ripening but later on this value increased very strongly, and so consequently did % N/TN. In contradiction to this, % CN/TN in the Seitz filtrate increased only to a small extent during the ripening period, and as a matter of course the same was the case for % N/TN. From these results it was concluded that the turbidity of the cheese extracts was caused exclusively by the presence of coagulable nitrogen.

As a consequence the nature of this CN was studied. Turbid extracts and their corresponding clear Seitz filtrates were analysed electrophoretically. In addition to products of protein degradation, the turbid extracts appeared

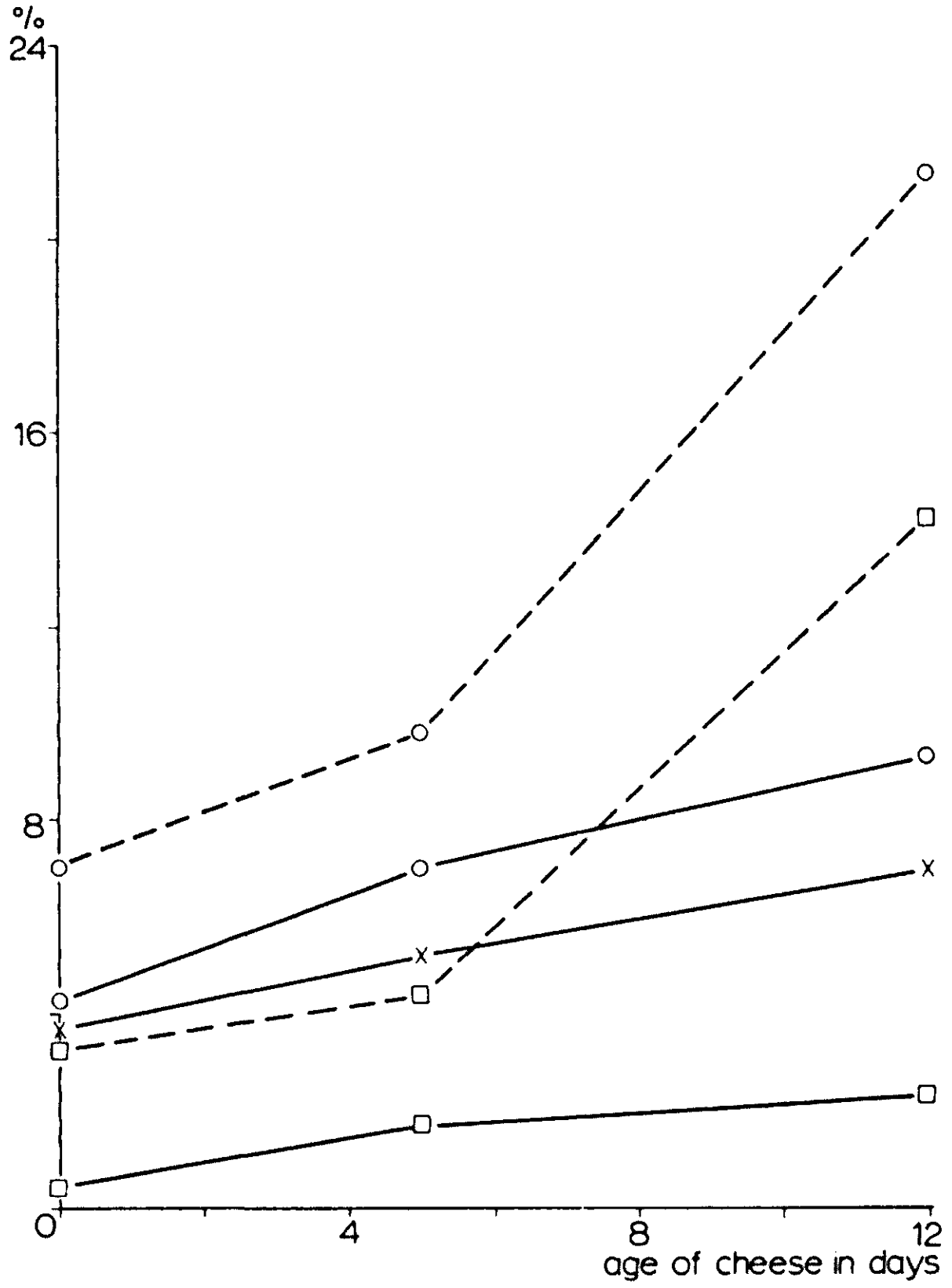

Fig 1. Extraction of Meshanger cheese with a solution of 0.137 *M* $CaCl_2$ and 0.684 *M* NaCl at 13 °C and at pH 5.1 on different days of the ripening period. Contents of N, NCN and CN of the extracts before and after Seitz filtration, expressed as a percentage of the total nitrogen of the cheese. Before: N (O - - - - - O) and CN (□ - - - - - □). After: N (O ——— O) and CN (□ ——— □). Equal values were found for NCN (x ——— x).

to contain unhydrolysed α_{s1} and β casein (see Fig. 2 for an example). From this it could be concluded that the presence of dispersed genuine protein at least was contributing considerably to the turbidity of the extracts.

In view of the aim of our investigation these results indicated once again the necessity for clarification of the extracts. In principle clarification might be performed by filtration, such as the Seitz filtration used by us. However, at 13 °C these filtrations took a long period of time and the filtrates did not always show the desired clearness. This last problem might eventually have been overcome by filtration through still finer filters. However, by the use of very fine filters dissolved N compounds can also be removed. For these reasons the possibility of a less complicated and more rapid method of clarification of the extracts was studied.

3.2 Influence of the temperature on the precipitation of dispersed protein in cheese extracts

When turbid extracts of Meshanger cheese, obtained at 13 °C by the method

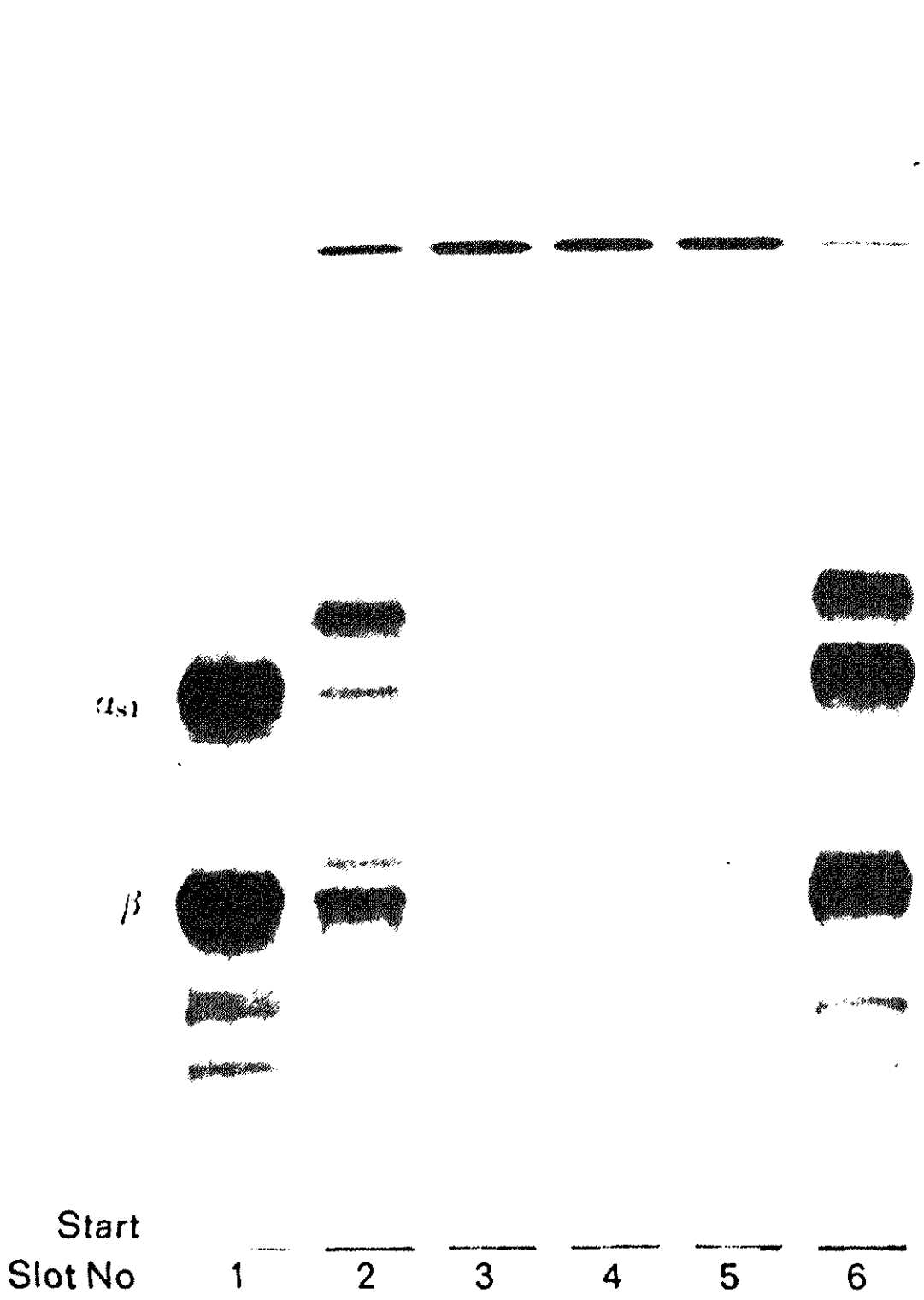

Fig. 2. Extraction of Meshanger cheese with a solution of 0.137 *M* $CaCl_2$ and 0.684 *M* NaCl at the pH of the cheese (5.34). Influence of the extraction temperature on the precipitation of nitrogen compounds, analysed by gel electrophoresis and by estimation of the content of nitrogen of the extract, expressed as a percentage of the total nitrogen of the cheese. Slot 1: sodium paracaseinate. Slots 2, 3, 4 and 5: dialysed and freeze-dried preparations of extracts obtained at 13 °C (% N/TN 28.53), 30 °C (12.59), 40 °C (11.12) and 50 °C (10.86), respectively. Slot 6: sample of the cheese.

described in Section 3.1, were kept at room temperature, the turbidity appeared to decrease as a result of the precipitation of dispersed protein. Curiously, this happened quickly in extracts from cheese in a young ripened condition and not at all or hardly in extracts prepared from cheeses in a more mature state.

This observation indicated that the presence of dispersed protein in the cheese extracts could probably be largely influenced by the extraction temperature. For that reason we performed the extraction of the cheese at various temperatures.

A homogeneous sample was prepared from Meshanger cheese. Equal portions were extracted at 13, 30, 40 and 50 °C, with a solution of 0.137 *M* $CaCl_2$ and 0.684 *M* NaCl as extraction liquid. The extraction was performed at the pH of the cheese sample. The mixtures were centrifuged at 40 000 *g* for 10 min at 13 °C (13 °C mixture) or at 30 °C (30, 40 and 50 °C mixtures). The nitrogen content of the extracts was estimated. In addition, from every extract a quantity was dialysed at 0-2 °C against distilled water for 24 hours

and freeze-dried to enable accurate electrophoretic analysis for α_{s1} and β casein in the extracts.

The results of such an analysis, performed with an 11-day old cheese, are shown in Fig. 2. The pH of the cheese was 5.34. The extracts obtained at 13 °C contained α_{s1} and β casein, while this was no longer the case in the extracts at higher temperatures. A continuous decrease of % N/TN was observed at increasing extraction temperatures, indicating that not only genuine protein but also products of protein degradation were precipitated. This is also visible in the decrease of the size of the bands representing degradation products of α_{s1} and β casein with the lowest electrophoretic mobility, particularly when the patterns at 13 and 30 °C are compared.

In some other experiments the extracts at 30 °C still showed the presence of unhydrolysed sub-caseins. At 40 and 50 °C they were no longer detected in our experiments. However, the extracts still frequently failed to show the desired clarity at these temperatures, which could not always be attributed to the presence of finely dispersed fat as was established by microscope. For this reason it was decided to study a better clarification procedure. In view of the favourable effect of the extraction temperature on the precipitation of dispersed protein, 30 °C was chosen as the extraction temperature in our further experiments.

3.3 The influence of the pH on the precipitation of dispersed protein in cheese extracts

It is well known that, at the normal pH value of milk, rennet-treated casein is easily precipitated at very small calcium concentrations and that the calcium-paracaseinate-phosphate complex of milk at this pH value is found in the undissolved state. In view of the high calcium concentration in the extraction liquid it could therefore be assumed that the dispersed protein could be precipitated by adjusting the pH of the extraction mixture to the value of milk (6.7).

Extracts of Meshanger cheese made by this procedure appeared to be clear and, as expected, did not show the presence of genuine protein.

In some of our experiments it was established in what way the precipitation of N compounds was influenced by the pH of the extract. Equal portions of a homogeneous sample of Meshanger cheese were extracted at 30 °C with a solution of 0.137 *M* $CaCl_2$ and 0.684 *M* NaCl at the pH value of the cheese. Subsequently the extraction mixtures were adjusted to increasing pH values and centrifuged for 10 min at 40 000 *g* at 30 °C. Nitrogen was estimated in the extracts obtained. Dialysed and freeze-dried preparations of the extracts were analysed electrophoretically.

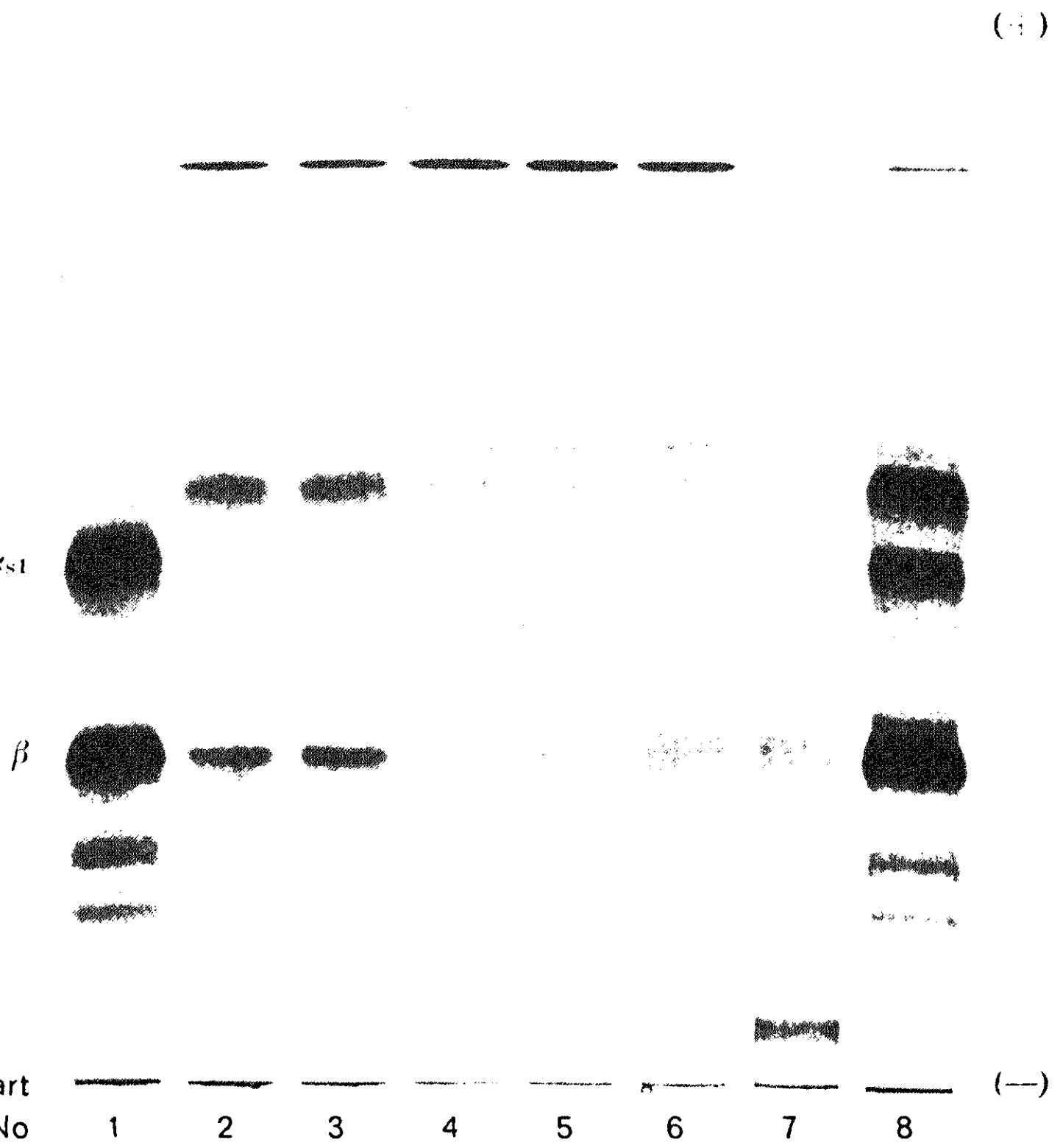

Fig. 3. Extraction of Meshanger cheese with a solution of 0.137 *M* $CaCl_2$ and 0.684 *M* NaCl at 30 °C. Influence of the pH of the extraction mixture on the precipitation of nitrogen compounds, analysed by gel electrophoresis and by estimation of the content of nitrogen of the extract, expressed as a percentage of the total nitrogen of the cheese. Slot 1: sodium paracaseinate. Slot 2 to 7 inclusive: patterns of dialysed and freeze-dried preparations of extracts obtained at pH values of 5.2 (% N/TN 24.19), 5.3 (22.52), 5.5 (14.77), 5.7 (14.69), 5.9 (13.22) and 7.5 (8.98), respectively. Slot 8: sample of the cheese.

The results of such an analysis, performed on a 16-day old cheese, are shown in Fig. 3. The pH of the cheese was 5.20. In this experiment the pH of the extraction mixtures was adjusted to the following values: 5.2, 5.3, 5.5, 5.7, 5.9 and 7.5. At pH 5.2 the extract already scarcely showed any α_{s1} casein in this experiment. Between pH 5.3 and 5.5 β casein appeared to be precipitated. However, % N/TN showed a further decrease up to pH 7.5, indicating the precipitation of protein degradation products. In this case, the amount of breakdown products precipitated between pH 5.5 and 7.5 amounted to 40 %. The removal of such products is also visible by comparison of electrophoretic patterns of the extracts at pH 5.9 and pH 7.5, showing the decrease

and finally the disappearance of bands representing degradation products of α_{s1} and β casein.

In several experiments it was established that starting from pH 7.5 the value of % N/TN remained constant. It was also observed that with cheeses in an advanced state of ripening, showing increasing values of % N/TN at pH 7.5 according as the cheese had ripened for a longer period of time, the influence of the pH on the precipitation of degradation products became less marked, constancy of % N/TN being obtained at much lower pH values than 7.5, frequently already at pH 6.5. Apparently with prolonged ripening more and more degradation products are formed which are not sensitive to the pH of the extract.

At pH values where the unhydrolysed caseins had just precipitated, the extracts showed a turbidity which was apparently caused by the presence of products of protein degradation with higher molecular weight. This turbidity decreased at increasing pH values up to about 6.5. At still higher pH values the extracts were perfectly clear. This and the constancy of % N/TN at those pH values made it attractive to regard this N fraction as an index of the protein breakdown in cheese as far as it concerns the formation of soluble degradation products, and so the amount of undissolved N compounds can be obtained. With respect to this the reliability of the fraction was established in experiments with Meshanger cheese and with model substrates of calcium-paracaseinate-phosphate complex in which the degradation of protein was also followed by quantitative polyacrylamide gel electrophoresis. In these experiments results obtained with the extraction method compared well with those obtained on the overall breakdown of α_{s1} and β casein as measured by densitometry of the gels. Examples of this will be given in subsequent papers on cheese ripening.

3.4 Extraction of Meshanger cheese with a 0.037 M $CaCl_2$ solution

In view of the possible use of extracts for analytical purposes, in which the high $CaCl_2$ and NaCl concentrations of the extracts might cause problems (for example, in gel filtration techniques), it was studied whether it was possible to perform the extraction of the cheese with a liquid with lower calcium and sodium concentrations. These experiments showed that the omission of NaCl from the extraction liquid hardly influenced the values of % N/TN at pH $\geqslant$ 7. The same results were obtained when in addition to the omission of NaCl the calcium concentration of the extraction liquid was lowered from 0.55 to 0.05 %. At still lower calcium concentrations (tested values 0.01 and 0.001 %) there was no longer any constancy observed for the values of % N/TN. The results of an experiment in which the influence of the calcium

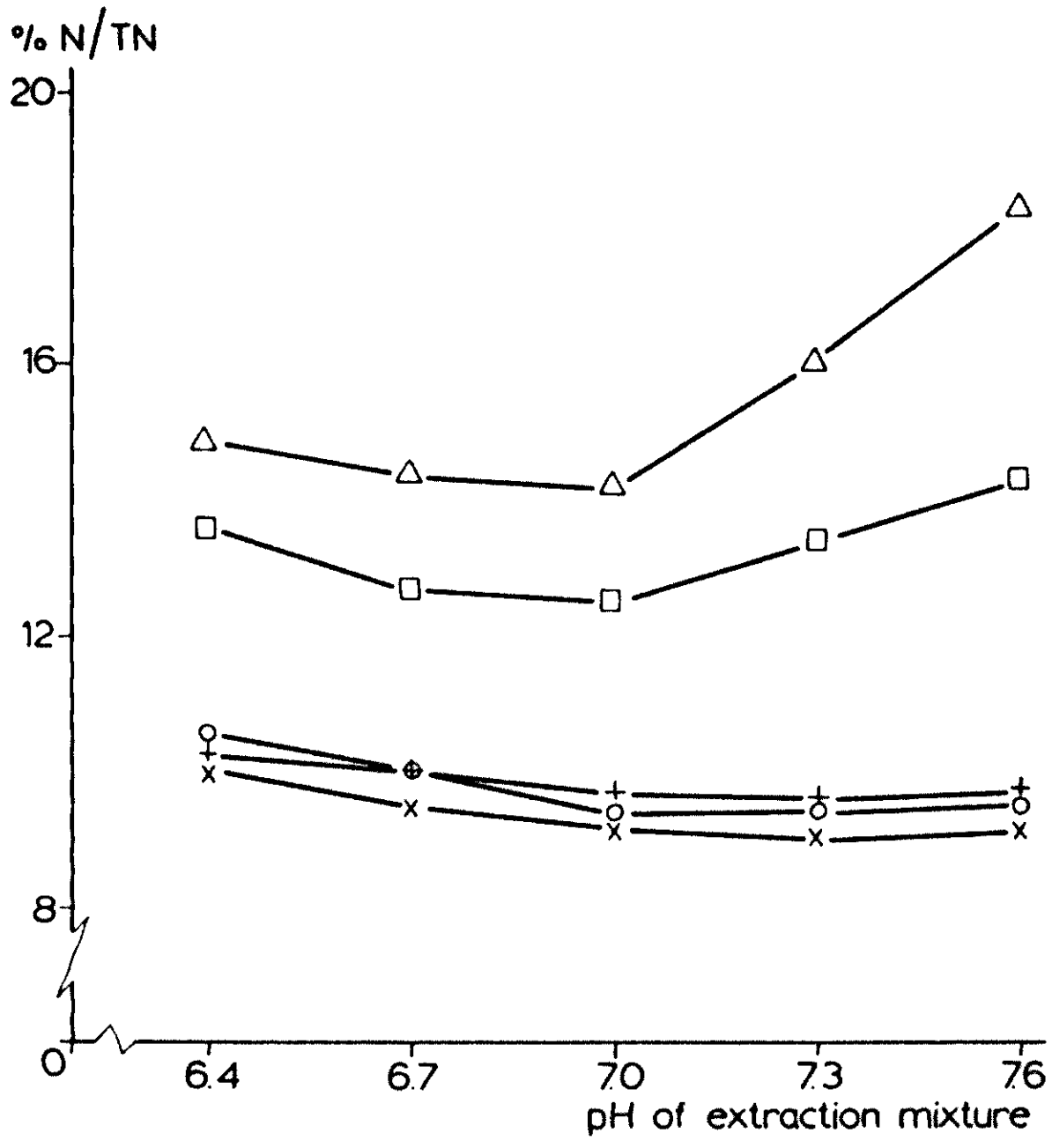

Fig. 4. Extraction of Meshanger cheese with a $CaCl_2$ solution at 30 °C. Influence of the calcium concentration of the extraction liquid on the nitrogen content of extracts, expressed as a percentage of the total nitrogen of the cheese, at pH values of the extraction mixture ranging from 6.0 to 7.6. Calcium concentrations: 0.001 % (△——△), 0.01 % (□——□), 0.05 % (○——○), 0.15 % (x——x) and 0.55 % (+——+).

concentration of the extraction liquid on % N/TN at high pH values was studied are shown in Fig. 4.

Although convenient results seemed to be obtained at a calcium concentration of 0.05 %, as a matter of safety it was decided to perform the extraction of the cheese with a solution of 0.037 *M* $CaCl_2$ (0.15 % Ca).

From all the results obtained it could be concluded that the degradation of protein in Meshanger cheese could be followed in a rapid, simple and reliable way by the use of the values of % N/TN at pH 7.5 as an index of the overall protein breakdown in the cheese. Nitrogen is estimated in the cheese extract obtained according to the description given in Section 2.2 under the following conditions: the homogenization is performed at 30 °C with a 0.037 *M* $CaCl_2$ solution and the pH of the extraction mixture is adjusted to pH 7.5. Subsequently the mixture is centrifuged at 40 000 *g* for 10 min at 30 °C.

3.5 Application of the extraction method to different varieties of cheese

With respect to the general applicability to cheese of the extraction method developed for measuring the degree of protein degradation, an experiment was performed in which the influence of the pH of the extraction mixture on % N/TN was studied for different varieties of cheese. Extraction mixtures

derived from equal portions of each individual cheese were adjusted to an increasing pH, and nitrogen was estimated in the extracts obtained. The pH values used were chosen within the range pH 6.0-7.6. Gouda, Meshanger, Kernhem, Camembert, Port Salut, Cheddar, Emmental and Roquefort cheeses were involved in this experiment. The results are illustrated in Fig. 5. With all cheeses constancy of % N/TN was observed at pH values from about 6.5.

About the general appearance of cheese extracts the following can be

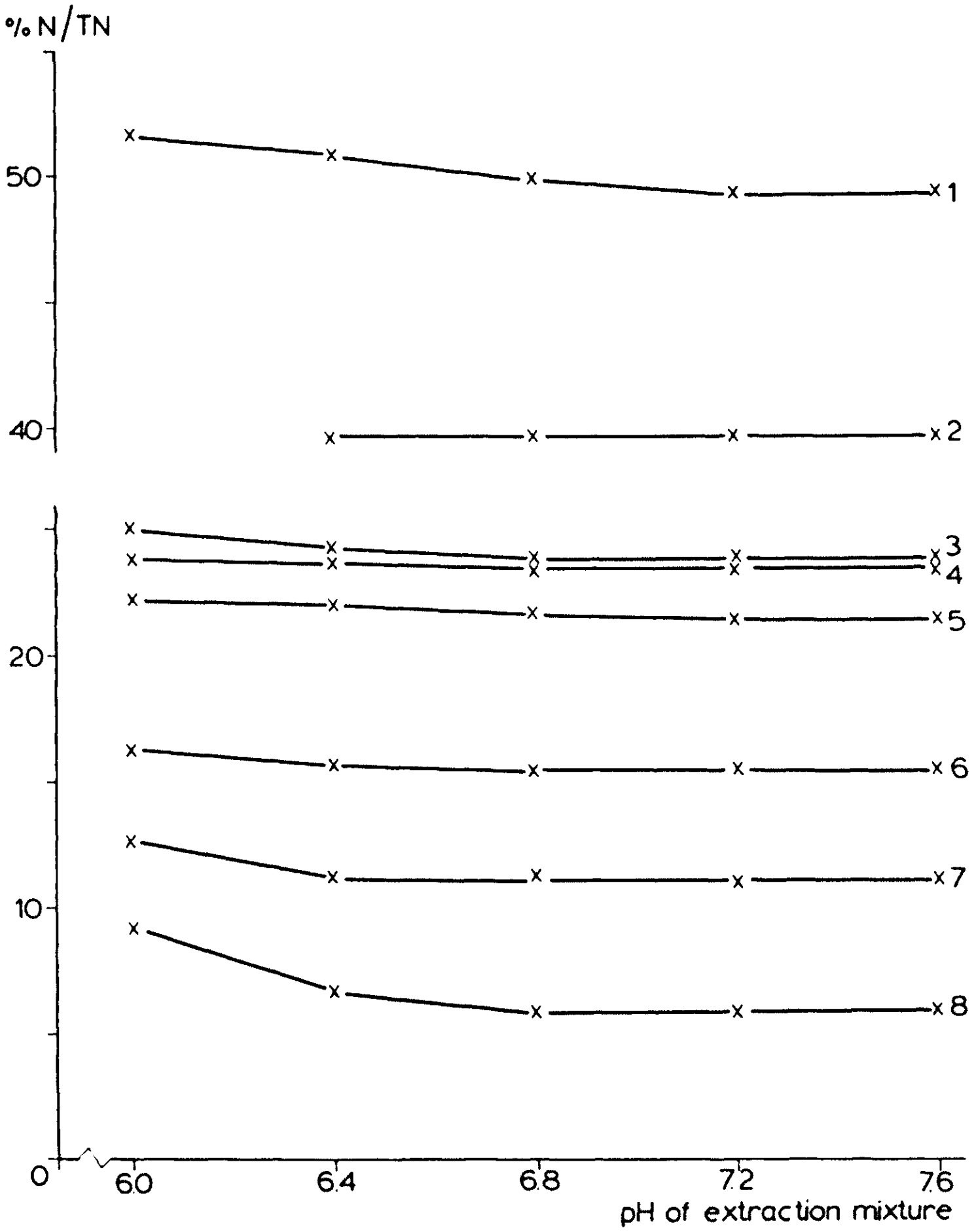

Fig. 5. Extraction of different varieties of cheese with a 0.037 *M* $CaCl_2$ solution at 30 °C. Contents of nitrogen of extracts, expressed as a percentage of the total nitrogen of the cheese, at pH values of the extraction mixtures ranging from 6.0 to 7.6.
1 = Roquefort; 2 = Cheddar; 3 = Emmental; 4 = Camembert; 5 = Kernhem; 6 = Gouda; 7 = Port Salut; 8 = Noordhollandse Meshanger cheese.

Table 1. Values of soluble nitrogen in different varieties of cheese, expressed as a percentage of the total nitrogen of the cheese, estimated with the extraction method and with clear Seitz filtrates of the cheese, obtained under conditions resembling those in the moisture of a ripe Meshanger cheese.

Variety of cheese	% N/TN	
	extraction method	Seitz filtrates
Meshanger	4.85	6.15
Gouda	16.87	17.73
Emmental	28.11	27.24
Kernhem	23.80	24.07
Cheddar	30.08	28.79

mentioned. Extracts of cheeses in a very young state of ripening are mostly perfectly clear. Those obtained from more ripened cheeses can show a more or less distinct opalescence depending on the degree of protein degradation in the cheese. The extracts may also show a slightly turbid appearance as a result of the presence of very finely dispersed fat, which could be confirmed by microscope.

3.6 The representability of the N fraction at pH 7.5 for the amount of N substances soluble in cheese moisture

In our opinion the amount of nitrogen in a clear filtrate of an extract of cheese, prepared under conditions largely corresponding to those actually present in the cheese, had to be considered as the most reliable index of the soluble N compounds in the cheese.

The following experiment was performed. From samples of different varieties of cheese, two extracts were prepared. A first extract was made at 13 °C under conditions resembling those present in a ripe Meshanger cheese, with a solution of 0.137 *M* $CaCl_2$ and 0.684 *M* NaCl as extraction liquid. After adjustment of the pH of the extraction mixture to 5.1, the mixture was centrifuged for 10 min at 40 000 *g*. The supernatant liquid was filtered through a Seitz EKS-filter. A second extract was made according to the extraction method described in Section 3.4. In both the extracts % N/TN was estimated.

The results of this experiment are given in Table 1. With some of the cheeses investigated it appeared to be impossible to obtain clear filtrates with the Seitz filter used, and for that reason they have been omitted. It is obvious that relative small differences were observed between the values of % N/TN obtained by the two methods, indicating that the N fraction at pH 7.5 is a

fair reflection of the amount of N compounds soluble in the moisture of cheese.

It might be objected that all cheeses were extracted under conditions present in a ripe Meshanger cheese, while the conditions in the other cheeses undoubtedly somewhat differed from that. However, preliminary experiments indicated that changes in the conditions (pH, calcium and sodium concentration) within a range normally found with different varieties of cheese, had no great effect on the amount of nitrogen in clear Seitz filtrates of the cheese.

As a general conclusion it can be stated that the extraction method developed satisfied the aim of our investigation. The method is simple and rapid, so that a further degradation of protein during the extraction is prevented. The results of the method give a good estimate of the amount of N compounds soluble in the moisture of the cheese. Moreover, they compare well with those obtained on the overall breakdown of α_{s1} and β casein in cheese as measured by quantitative polyacrylamide gel electrophoresis, as far as it concerns cheeses in a relative young state of ripening. With well matured cheeses, which scarcely show the presence of genuine casein any more, as a matter of course the correlation between the results obtained by the extraction method and by gel electrophoresis becomes less distinct. Like all other extraction methods, the method has an empirical character.

Acknowledgment

The author is indebted to Mrs M. A. C. de Dreu-Baarends and Mrs G. C. van Roekel-Jansen for their help in performing the analyses.

Samenvatting

A. Noomen, *Een snelle methode ter bepaling van de hoeveelheid opgeloste en onopgeloste stikstofverbindingen in kaas*

De consistentie van kaas staat in relatie tot de onopgeloste stikstofverbindingen in de kaas. Het artikel handelt over de ontwikkeling van een extractiemethode ter bepaling van de in het kaasvocht opgeloste stikstofbestanddelen, vooral eiwitafbraakprodukten. De hoeveelheid onopgeloste stikstofverbindingen in kaas is dan eveneens bekend. Er werden moeilijkheden ondervonden bij het klaren van de extracten door middel van centrifugeren en filtreren, wanneer deze extracten werden gemaakt met een vloeistof waarvan de samenstelling vergelijkbaar was met die van kaasvocht. Om deze reden werd naar een andere klaringsmethode gezocht.

Het doel werd bevredigend bereikt door de kaas onder gestandaardiseerde omstandigheden bij 30 °C te homogeniseren met een oplossing van 0.037 *M* $CaCl_2$ met behulp van een Ultra Turrax. Het extractiemengsel wordt ingesteld op pH 7.5 en gedurende 10 minuten gecentrifugeerd bij 40 000 *g* en een temperatuur van 30 °C. In het gefiltreerde centrifugaat wordt de hoeveelheid stikstof bepaald.

De methode is eenvoudig en snel, zodat tijdens de extractie geen verdere eiwitafbraak kan optreden. De met de methode verkregen resultaten geven een goede afspiegeling van de hoeveelheid stikstofverbindingen die opgelost is in het kaasvocht bij de in kaas heersende omstandigheden. Bovendien zijn zij goed vergelijkbaar met die welke worden verkregen wanneer de eiwitafbraak in kaas wordt gevolgd met behulp van quantitatieve polyacrylamide-gel-electroforese.

References

1. H. Mulder & A. F. Monib, *Proc. 16th int. Dairy Congr.* B (1962) 539-544.
2. A. M. M. F. Monib, *Meded. LandbHogesch., Wageningen* 62(10) (1962) 1-76.
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. A. Noomen, *Neth. Milk Dairy J.* 29 (1975) 153-161.
7. H. A. Sirks, *Versl. landbouwk. Onderz.* 49 (1943) 221-263.
8. G. Kleter, *Neth. Milk Dairy J.* 30 (1976) 254-270.
9. L. de Jong, *Neth. Milk Dairy J.* 29 (1975) 162-168.

Neth. Milk Dairy J. 32 (1978): 26-48

Activity of proteolytic enzymes in simulated soft cheeses (Meshanger type). 1. Activity of milk protease

A. Noomen

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

Received: 27 October 1977

Key words: cheese ripening, milk protease, soft cheese, protein breakdown

Summary

The activity of milk protease in soft cheese was studied with simulated cheeses, wherein milk protease was acting under conditions (ratio of protein to water, pH, salt content, etc.) which approached as nearly as possible those existing in Noordhollandse Meshanger cheese during its ripening. The cheeses were prepared with (1) rennet-free cheese curd, or with (2) a rennet-free calcium paracaseinate-calcium phosphate complex. Both preparations contained the milk protease already present in the milk from which they were made. Protein breakdown was followed by quantitative polyacrylamide gel electrophoresis and by the estimation of the amounts of soluble nitrogenous compounds liberated. In almost all tested conditions, the results of both methods for the total degradation of protein were well correlated.

Apart from differences in a quantitative sense, in all cheeses milk protease acted in a similar manner in corresponding test conditions. Quantitative differences may have been caused, for example, by differences in protease activity, protease content, the substrates, etc.

Enzyme activity appeared to be strongly influenced by the pH, the NaCl concentration in the cheese moisture and the ripening temperature of the cheese.

Protein breakdown was most extensive in cheeses with a low acidity. Whereas at a high pH (e.g. pH 6.2) β casein was much more quickly degraded than α_{s1} casein, at a low pH (e.g. pH 5.4) α_{s1} casein was attacked somewhat more than β casein. It is suggested that in addition to alkaline milk protease an acid protease, preferably acting on α_{s1} casein, under favourable conditions contributes to protein breakdown in certain soft cheeses.

Enzyme activity against both caseins was stimulated by low concentrations of NaCl in the cheese moisture, but was reduced by high concentrations. Under the conditions tested, maximum breakdown was found at about 2 % NaCl.

At pH 6.0, both α_{s1} and β casein were increasingly degraded at higher temperatures within the range of 5 - 37 °C. At pH 5.2, the degradation of α_{s1} casein showed a similar tendency, whereas β casein degradation remained at a constant and low level at temperatures above 20 °C.

In view of the conditions in a ripening Meshanger cheese and the activity of milk protease observed under comparable conditions in the simulated cheeses, the contribution

of milk protease to protein breakdown in this cheese is considered to be of little importance for the normal ripening (see also Part 2 of this series). This contribution may be more important in soft cheeses with a surface flora, showing ripening conditions which favour in particular the activity of alkaline milk protease.

1 Introduction

The presence of a proteolytic enzyme in milk was first reported by Babcock & Russell (1). Since then, several workers have contributed to the evidence that cow's milk contains a proteolytic enzyme of non-bacterial origin (2, 3, 4, 5, 6, 7, 8). In this laboratory, we irrefutably demonstrated a considerable activity of bovine milk protease in aseptically drawn milk (9).

Until now relatively little attention has been paid to the contribution of milk protease to protein degradation in cheese. Stadhouders (10) considered the enzyme to be of no importance in the production of amino acids in Edam cheese. Reiter et al. (11) ascribed milk protease to be responsible for the low concentration of free amino acids in aseptic starter-free cheese of the Edam type. Green & Foster (12), although observing a perceptible activity of the enzyme in skim milk, assumed that this activity at the low pH of ripening cheese was negligible compared with that of the coagulating enzymes used in the production of their experimental cheeses. Creamer (13) identified, during the ripening of Gouda and Cheddar cheese, increasing amounts of peptides of high molecular weight and low electrophoretic mobility as γ, TS and R caseins. These 'minor caseins' are thought to originate from the action of milk protease on β casein (14, 15).

In our laboratory, studies on cheese ripening led to the development of a gel electrophoretic and of an extraction method to follow protein degradation in cheese quantitatively (16, 17), the latter method being more sensitive for the determination of milk protease activity than other precipitation methods used before (9). Using these methods, Visser (18, 19) in our laboratory studied the degradation of protein in cheeses of the Gouda type, which exclusively contained milk protease as active proteolytic enzyme. During ripening, the cheeses showed relatively very low, but gradually increasing levels of soluble and amino acid N and the cleavage of β casein, liberating typical 'minor caseins'.

Recently we reported in detail about soft Noordhollandse Meshanger cheese (20, 21, 22). Among other things, this cheese is characterized by the differing and continuously changing physical and chemical conditions during the ripening period (21), which locally in the cheese might be more favourable to milk protease activity than those found, for instance, in Gouda and Cheddar cheese. In preliminary experiments it was observed that in preserved

Meshanger cheese of pH 6.6 the protein was considerably degraded. The way in which the various caseins had been decomposed strongly suggested the action of milk protease. This led us to study the contribution of milk protease to protein degradation under various conditions occurring in Meshanger cheese.

The relative rates of degradation of the different caseins and the nature of the products formed may be significantly influenced by the conditions of the substrate used. These factors have been studied merely in connection with the action of rennet on casein components, and therefore relevant literature information will be given in a subsequent report dealing with the proteolytic activity of rennet in Meshanger cheese. Consequently, the experiments needed to be carried out under conditions resembling as closely as possible those existing in the cheese.

Enzyme activity was studied with preserved simulated cheeses, in which various conditions such as the pH, the NaCl content and the ratio of protein to water could be easily and reproducibly obtained by artificial acidification and by the addition of proper amounts of NaCl and water. The cheeses were made up with rennet-free cheese curd and with a freeze-dried rennet-free calcium paracaseinate-calcium phosphate complex, respectively. Preliminary experiments indicated that this paracasein complex showed considerable milk protease activity, which was not unexpected for the reason that at the normal pH of milk the protease is predominantly associated with the casein micelles (7). Both rennet-free preparations were made by the technique of Visser (23), which is based on the heat inactivation of rennet by pasteurization (20 s, 72 °C) after it has completed its primary action on $\varkappa$ casein in milk of which the calcium has been lowered by means of ion exchange, thus preventing the milk from coagulation. After pasteurization the milk is cooled down to 4 - 5 °C. A rennet-free coagulum is subsequently obtained by the addition of a solution of calcium chloride and lactic acid and by warming the milk to 30 °C.

Since the cheeses prepared with 'curd' contained cheese whey and fat whereas those made with paracasein complex did not, the simulated cheeses differed in composition. Other factors may have contributed to this as well. For instance, the cheeses may have shown differences in enzyme concentration and in the chemical conformation of the calcium. Furthermore, the properties of normal milk differ from those of milk treated according to Visser's technique. Among other things, this was demonstrated by the necessity to increase the 'calcium' content of the ion exchanged milk to 0.155-0.160 %, and to decrease its pH to 6.30-6.34 to obtain a stable milk and a firm gel after warming (23). Thus the conditions for enzyme action in the simulated cheeses

were not exactly the same as in cheese. The cheeses were however thought to approach the situation in cheese sufficiently closely and therefore to make a suitable model.

Simulated cheeses, made from a paracasein complex made from milk which had been freed from milk protease activity by a heat treatment, do not show any degradation of protein (24). It would thus be logical also to study milk protease activity by the addition of enzyme preparations to cheeses made with milk protease-free paracasein complex. However, in preliminary experiments that kind of preparation, although considerably active against sodium caseinate, failed to show any activity in such cheeses. Investigations on this subject are continuing.

2 Materials and methods

2.1 Aseptically drawn milk

Bulk cow's milk was obtained from the herd of the University in the manner normally used in our laboratory and described by Kleter & de Vries (25). Aerobic and anaerobic bacterial counts of the milk amounted to about 3 and 1 per ml, respectively.

2.2 Preparation of rennet-free cheese curd

The curd was prepared from aseptically drawn milk according to the technique developed in this laboratory by Visser (23). The same Dutch commercial rennet was used as applied by that author.

After the rennet-treated and subsequently pasteurized milk had been cooled down to 4 °C, thimerosal – ((carboxyphenyl)thio) ethylmercury sodium salt – was added as a preservative in a concentration of 100 mg per litre. The curd was cut and about 40 % of whey were taken off. The curd/whey mixture was stirred for 2½ hours at 37 °C to obtain a curd sufficiently dry to allow it to be used for the preparation of simulated cheeses with the desired composition (Section 2.4). The curd was moulded, bandaged, pressed, and stored at –20 °C until used. Aerobic and anaerobic bacterial counts of the fresh curd were zero per g.

The relevant composition of the defrosted and homogenized curd was as follows: moisture content 50.62 %; nitrogen content 3.24 %, corresponding to a protein content of 20.67 %; soluble nitrogen, expressed as a percentage of the total nitrogen, 1.47 %; calcium content 0.80 %; pH 6.31.

2.3 Preparation of a rennet-free calcium paracaseinate-calcium phosphate complex

The complex was prepared from skimmed milk, derived from the same batch of milk as used in the preparation of rennet-free curd. Rennet-free curd particles were obtained by the procedure followed in Section 2.2. To obtain finely divided particles, stirring was applied while raising the temperature of the milk from 4 to 30 °C. After sedimentation of the particles the whey was taken off and the sediment was washed with pasteurized (25 s, 85 °C) tap water to about a 1000-fold dilution of milk serum constituents. Subsequently the complex was freeze-dried and milled with an impact mill (mesh sieve 0.8 mm), with which the milling could be performed at a temperature sufficiently low to prevent the sticking of the complex. The very fine powder obtained was well mixed, tinned and stored at room temperature. No thimerosal was added at any stage during the preparation of the complex.

The powder had a moisture content of 3.42 %; a nitrogen content of 13.31 %, corresponding to a protein content of 84.92 %; and a calcium content of 2.52 %. Aerobic and anaerobic bacterial counts of the complex amounted to 800 and 500 per g, respectively.

2.4 Preparation of simulated cheeses

2.4.1 Cheeses made with 'curd'. Cheeses were prepared with a ratio of protein to water of 1 : 3.5, which is normally present in Noordhollandse Meshanger cheese.

The 'curd', which had been stored at –20 °C, was defrosted, milled twice and ground in a mortar. From its composition (Section 2.2) it was calculated that 21.7 g of water had to be added to 100 g to achieve the desired ratio.

Cheeses were made as follows. In a mortar to 20 g of 'curd' were added:
– 4.35 ml of a thimerosal solution in water (100 mg/litre). Taking into account the quantity of thimerosal already added to the milk during the preparation of the 'curd' (Section 2.2), by this addition the cheeses contained 100 mg of this preservative per litre of moisture.
– a weighed amount of NaCl, the quantity depending on the NaCl concentration in the moisture of the cheese to be adjusted.
– a weighed amount of gluconic acid δ-lactone. In preliminary experiments it was established what approximate amounts of lactone had to be added to achieve the desired final pH values of the cheeses.

After mixing the ingredients thoroughly with the 'curd', the mixture was transferred quantitatively to a 50-ml glass beaker and firmly pressed together.

Subsequently the cheeses were stored anaerobically for a given period of time at 13 °C, the normal ripening temperature of Meshanger cheese. Anaerobic conditions were created first by flushing the contents of an anaerobic jar with N_2 gas, complete anaerobic conditions being obtained after that with the BBL Gas Pak System.

2.4.2 Cheeses made with paracasein complex. Cheeses were made with a ratio of protein to water of 1 : 3.5. Each cheese was composed with 5.18 g of complex, which corresponded to 5 g of dry matter. According to the composition of the complex (Section 2.3), the desired ratio was achieved by the addition of 15.4 g of liquid.

The cheeses were made as follows. A beaker glass of 50 ml was provided with:

– a weighed amount of NaCl.
– an amount of 10 or 20 % lactic acid solution. The amounts of solution needed to adjust the pH values desired were established in preliminary experiments.
– 2 ml of a thimerosal solution in water (0.77 g/litre). The final concentration of this preservative in the moisture of the cheeses was 100 mg per litre, corresponding to that in the cheeses prepared with 'curd'.
– water in such an amount that, together with the moisture derived from the complex, the total amount of moisture present in the cheeses amounted to 15.4 ml.
– 5.18 g of complex. The complex was very well mixed with the solution and compressed.

The cheeses were stored for a given period of time at a given temperature under anaerobic conditions as described in Section 2.4.1.

2.4.3 General remarks about the cheeses. Preliminary experiments established that the pH of the cheeses had almost equilibrated within a few hours after preparation. At the end of the storage period differences in pH values of duplicate or triplicate cheeses were mostly less than 0.05 and never exceeded 0.1 pH unit.

The use of thimerosal, in combination with anaerobic storage, proved to be very effective with respect to the microbial preservation of the cheeses, even when they were kept for a very long period of time. Bacterial numbers after storage were mostly zero and never more than 500 per g of cheese or per ml of cheese serum. The activity of milk protease was not significantly influenced by the preservative in the concentration applied, as was tested with aseptically drawn and low-temperature pasteurized milk, incubated for

3 days at 37 °C with and without preservative. Proteolysis was about 10 % less in the preserved milk. The unpreserved milk showed considerable bacterial growth, and this may have contributed to the small difference in the degree of degradation observed.

During the preparation of the cheeses, asepsis was maintained as much as possible.

2.5 Estimation of bacterial counts

For the estimation of bacterial numbers in dry complex and in cheeses made with 'curd', 5 g of material were dissolved in 45 ml of a 2 % Na-citrate solution, preheated at 45 °C. Serial dilutions in quarter strength Ringer solution were plated in Plate Count Agar (Difco) supplemented with 1 % skim milk, and incubated aerobically and/or anaerobically for three days at 30 °C. In the case of cheeses prepared with paracasein complex, counts were made on the cheese serum, which could be easily obtained by slightly pressing the cheese. Determinations of bacterial counts were performed on cheeses which were made separately for this purpose. Plates showing the presence of particles were checked by stereomicroscope for their number of colonies.

2.6 Estimation of the pH

Measurements of the pH of the cheeses were performed at the storage temperature of the cheese with a combined glass electrode.

2.7 Estimation of protein breakdown

The degradation of proteins was studied in separate cheeses by the estimation of the amounts of soluble nitrogenous compounds liberated and by quantitative polyacrylamide gel electrophoresis.

2.7.1 Nitrogen soluble in the cheese moisture. The determinations were performed by the extraction method described by us previously (17). To the cheeses 0.037 *M* $CaCl_2$ solution was added to a total volume of 200 ml of moisture present. After homogenization of the mixture, nitrogen was estimated in the extract obtained at pH 7.5 at 30 °C and expressed as a percentage of the total nitrogen of the cheese (% SN/TN).

Nitrogen determinations were made by a micro-Kjeldahl method, with one tenth of the amounts of reagents used in the macro-Kjeldahl method according to Netherlands Standard NEN 3198.

2.7.2 Polyacrylamide gel electrophoresis. Electrophoretic experiments and densitometry of the gels were performed by the methods described by de Jong (16). Cheeses prepared with paracasein complex were dissolved in a Tris-HCl buffer with 8 *M* urea (pH 8.5) and diluted in this buffer to a concentration of 0.5 % of protein. In the case of cheeses prepared with 'curd', this protein concentration was obtained by dissolving a calculated weighed amount of homogenized cheese in buffer to a total volume of 25 ml.

3 Experiments and results

3.1 Activity of milk protease in cheeses prepared with 'curd'

3.1.1 Influence of the pH. The effect was studied with cheeses showing pH values ranging from about 4.9 to 6.2 and containing 4 % NaCl in the moisture. This concentration is normally found in Meshanger cheese when the salt has become uniformly distributed (21). The cheeses were stored for 28 days at 13 °C. This relatively long period, when compared to the normal ripening time of Meshanger cheese of about two weeks, was chosen to obtain more definite information about the activity of milk protease at low pH values,

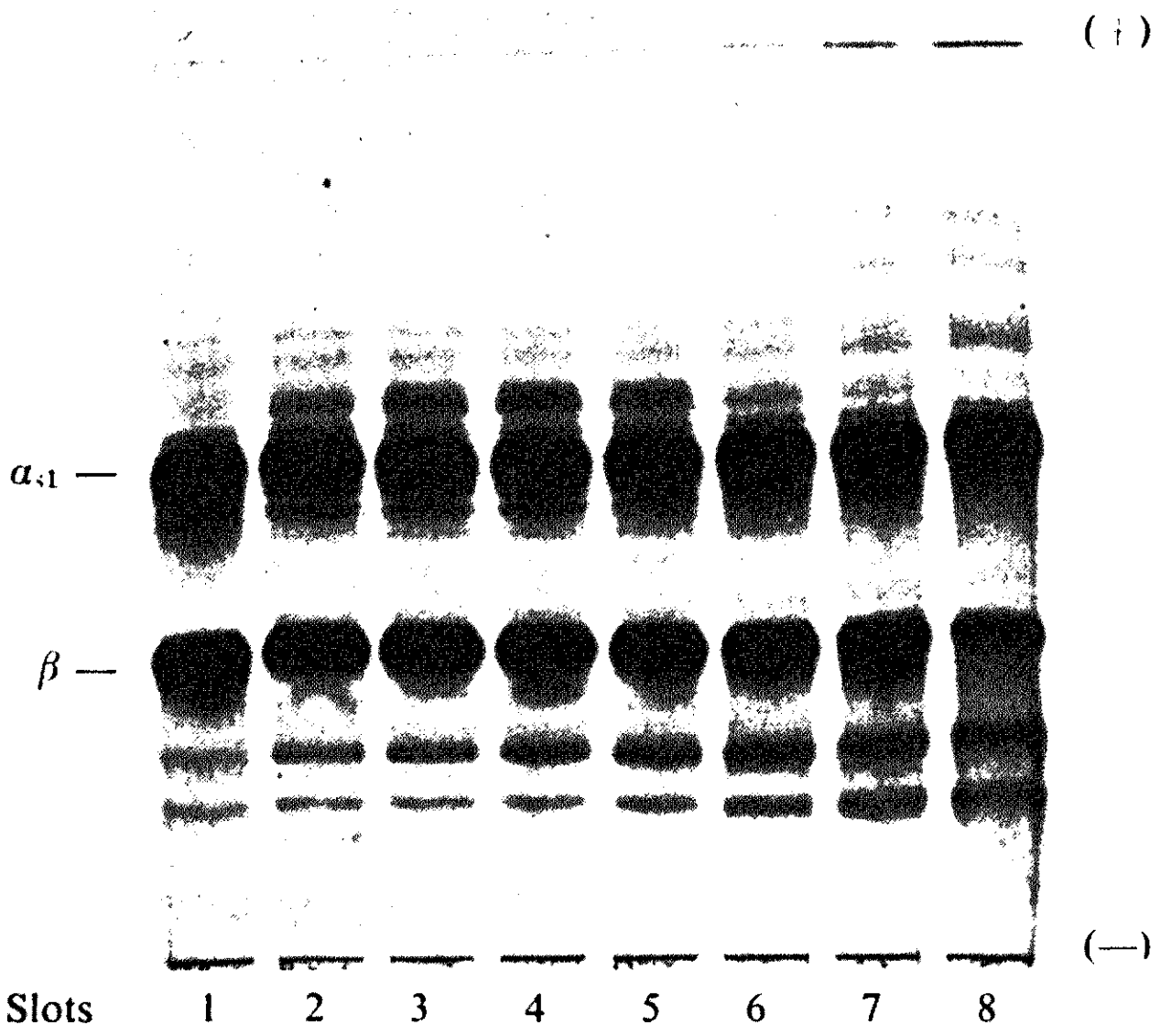

Fig. 1. Patterns of protein degradation by milk protease in simulated cheeses prepared with 'curd', depending on the pH. The cheeses contained 4 % NaCl in the moisture and were stored for 28 days at 13 °C. Slot 1: 'curd' (blank). Slots 2 to 8 inclusive: cheeses stored at pH 4.9, 5.1, 5.4, 5.6, 5.85, 6.0 and 6.2, respectively.

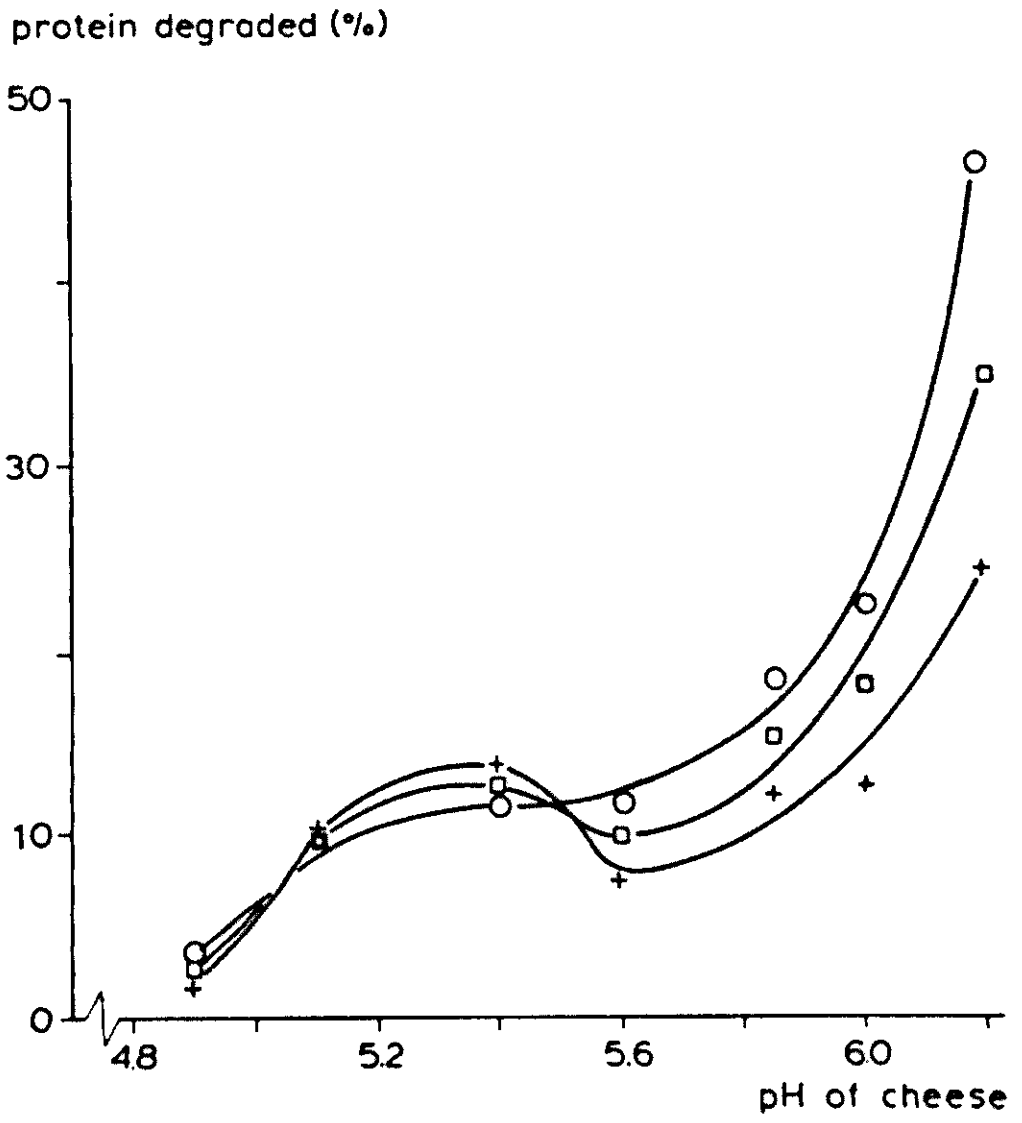

Fig. 2. Protein breakdown in the cheeses shown in Fig. 1, estimated by densitometry. +: α_{s1} casein; ○: β casein; □: α_{s1} + β casein.

where this activity was expected to be low.

Patterns of protein degradation in the cheeses are shown in Fig. 1, and densitometric results are represented in Fig. 2. The latter were generally in accordance with those to be expected by visual observation of the gel.

At pH 4.9, β casein had remained almost unattacked. A small (about 10 %) and only very slightly increased degradation of this casein was found at pH values between 5.1 and 5.6. At still higher pH values the breakdown appeared to increase very strongly, about 46 % being degraded at pH 6.2. This was also reflected by the development and the increase of the size of bands representing 'minor caseins' with decreasing acidity of the cheese.

The degradation of α_{s1} casein showed approximately the same tendency. This casein was also scarcely attacked at pH 4.9 and increasingly, but slightly, at pH values up to 5.4, which also became visible in the formation of a well developed band in front of the α_{s1} band at a position corresponding to that of α_{s1}-I casein. At these low pH values α_{s1} casein seemed to be almost equally or even slightly more attacked than β casein. Between pH 5.4 and 5.8 the increase in the α_{s1} degradation seemed to be interrupted, the degradation at approximately pH 5.6 being found somewhat smaller than at pH 5.4, whereas at higher pH values the casein again appeared to be increasingly degraded. At pH 6.2 about 24 % of this casein was found to be broken down, being about half of that of β casein at the same pH value. At high pH values the degradation of the paracasein complex by milk protease in the cheeses ap-

parently became more comparable to that of casein in milk, the breakdown of β casein in milk being reported by us as two to three times faster than that of α_{s1} casein (9).

In contrast to cheeses with low pH values, those with the highest pH did not show the presence of a well developed band in front of the α_{s1} band. This might indicate that the degradation products representing this band are not formed in those conditions.

At very low levels of protein degradation, a limited accuracy must be attributed to the densitometric results obtained. In addition, the dissolution of simulated cheese in the buffer did not always proceed smoothly and equally well. Thus it was questionable whether the differing ratio of the rates of degradation of α_{s1} and β casein at low and high pH values reflected the actual situation in the cheeses. However, in cheeses made with the same 'curd', which were stored for much longer than one month, the degradation of α_{s1} casein at low pH values had clearly surpassed that of the β casein, whereas a distinct minimum for the breakdown of α_{s1} casein was found at pH 5.8 in that experiment. Moreover, in cheeses made with paracasein complex the degradation of the caseins at different pH values was found to show a similar course at that in cheeses made with 'curd' (Section 3.2.2).

For the time being, the differing ratios of the rates of breakdown of the caseins according to the pH of the cheeses is difficult to explain. They might be caused by some changes within the protein depending on the reigning physico-chemical conditions, altering the susceptibility of α_{s1} casein particularly to being attacked by one milk protease enzyme present. One can also suggest the presence of two proteolytic enzymes, one being strongly active at high pH values and degrading β casein much more than α_{s1} casein, and the other being active at low pH values and preferably degrading α_{s1} casein. This suggestion does not necessarily imply the presence of more than one native milk protease. Since rennet was used in the preparation of the 'curd' and the paracasein complex, it could theoretically have been possible that rennet activity had not been completely destroyed by the pasteurization of the milk for 20 s at 72 °C, causing a very limited but in the long run clearly demonstrable protein degradation in the cheeses, particularly that of α_{s1} casein at low pH values. The results of the experiments of Visser (23) concerning the inactivation of rennin in ion-exchanged milk (pH 6.9) submitted to that heat treatment, make this very unlikely however. In addition to rennin, commercial rennet may contain other proteolytic enzymes. Recently it has been reported that, when expressed in terms of the clotting power at pH 6.5, present-day Dutch commercial rennet may contain 18-20 % bovine pepsin (26). This enzyme is however reported to be less heat-resistant than rennin (27). We

did not observe any clotting of milks containing 10 % rennet-free curd or paracasein complex and thimerosal (100 mg/litre) during an incubation of up to 35 days at 35 °C. The absence of proteolytic activity in the cheese originating from the rennet used was also strongly supported by the fact that Visser (23) did not find any formation of soluble N when milk, freed from milk protease activity by a heat treatment and subsequently provided with 2.5 % rennet, was treated according to his method and incubated for 2 weeks at 30 °C at pH 5.4 (details additional to the literature derived from personal communication). Finally, the formation of the band in front of α_{s1} casein as observed with simulated cheeses of a low pH may result from the action of a milk protease. Electrophoresis of pasteurized (20 s at 72 °C) and preserved aseptically drawn milk, which had been stored for 28 days at 13 °C at different pH values, among other things showed the presence of a corresponding band, particularly at pH values lower than 6. In our opinion, the above-mentioned considerations allowed the conclusion to be drawn that only indigenous milk protease was active in the simulated cheeses.

The formation of soluble N is illustrated in Fig. 3. Protein degradation appeared to have increased with decreasing acidity of the cheese. The existence of a slight minimum in the total degradation of protein at a pH of about 5.6 as indicated by densitometry was not reflected in the values of % SN/TN. With cheeses stored additionally for 56 days under conditions corresponding to those in the previous experiment, it was however observed that the curve showed a distinct shoulder at pH values 5.4-5.9 (Fig. 3), which might be

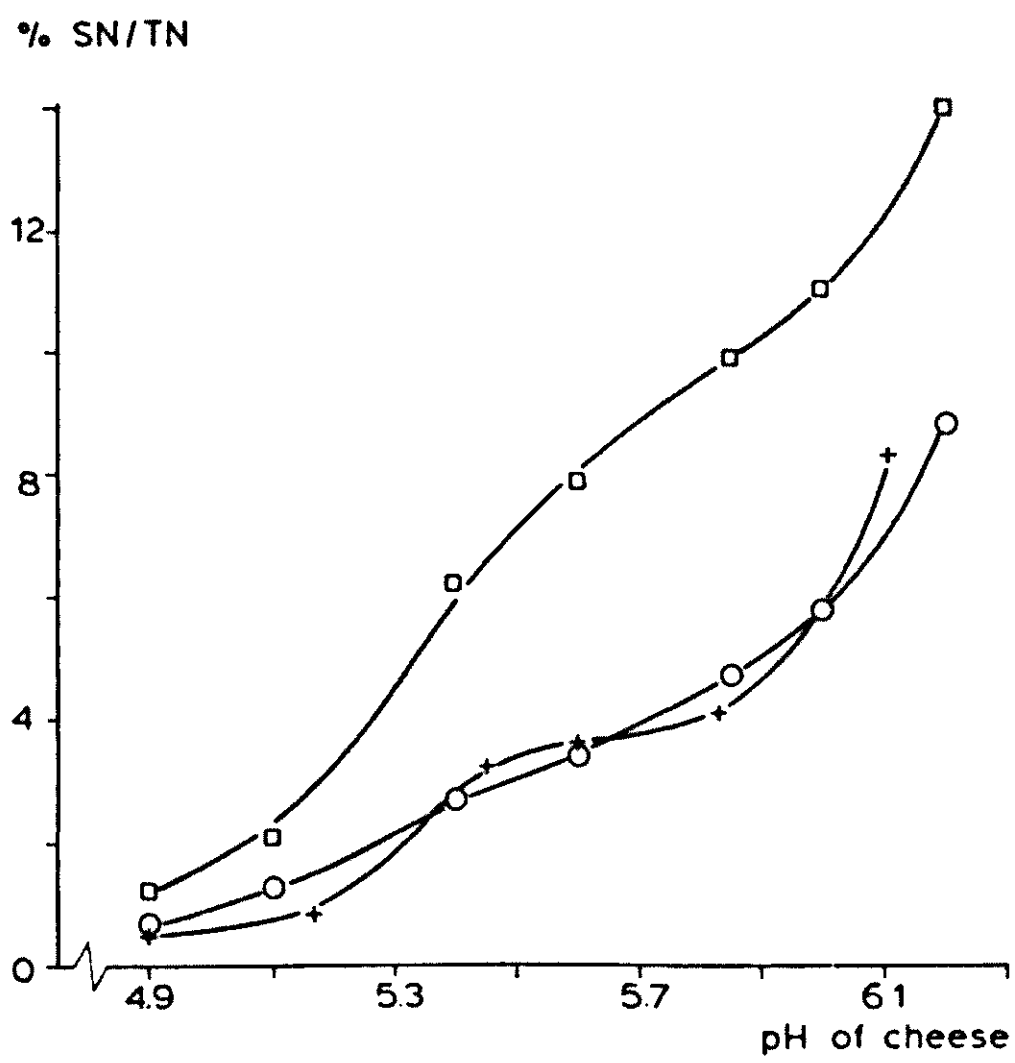

Fig. 3. Influence of the pH on the degradation of protein by milk protease in simulated cheeses at 13 °C, represented by the amount of soluble nitrogenous compounds, expressed as a percentage of the total nitrogen. The cheeses contained 4 % NaCl in the moisture and showed pH values corresponding to those referred to in Fig. 1. ○: cheeses prepared with 'curd', stored for 28 days; □: idem, cheeses stored for 56 days; +: cheeses made with paracasein complex, stored for 28 days.

related to the reversing ratio of the levels of degradation of α_{s1} and β casein in that pH traject.

With some exceptions, particularly with cheeses stored at the lowest pH values and with the lowest levels of degradation, the degrees of total protein breakdown estimated by the extraction method and the electrophoretic method, respectively, were well correlated, showing a ratio of 1 : 4 to 5.

3.1.2 Influence of the NaCl content. The effect of NaCl was studied with cheeses containing 0, 2, 4 and 8 % NaCl in the moisture, and at the pH values 6.3 and 5.4. The cheeses were stored for 28 days at 13 °C. The results are shown in Figs 4 and 5. The cheeses without NaCl at pH 5.4 got lost by accident, and for that reason protein degradation was not followed electrophoretically in those conditions.

Densitometric evaluation of the gels showed that the degradation of protein at pH 6.3 had been stimulated by low concentrations of NaCl, maximum breakdown of the caseins being observed at 2 % NaCl. At higher salt contents both caseins became less degraded, the degradation of β casein being more influenced by NaCl than that of α_{s1} casein. As could be expected, at

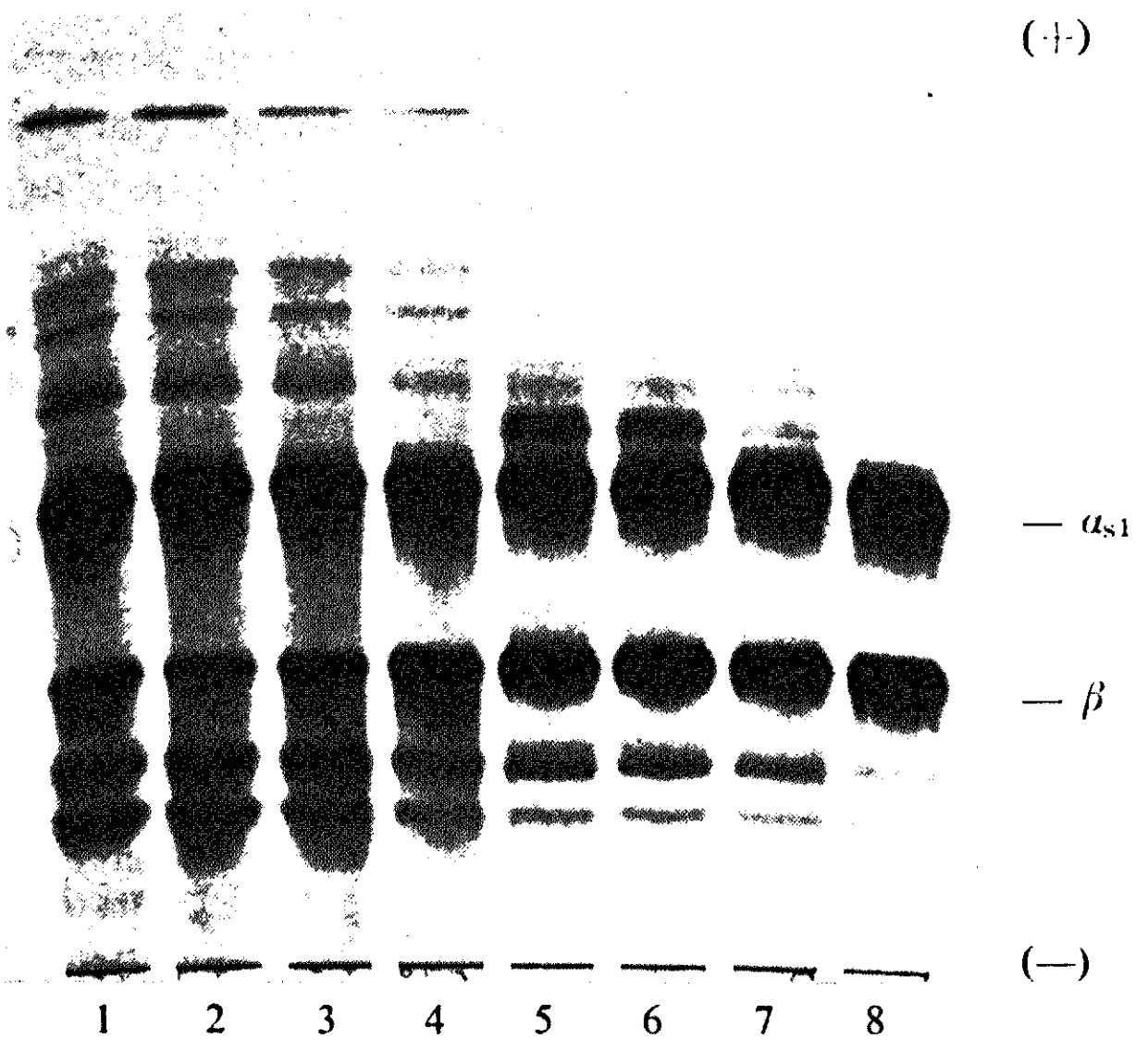

Fig. 4. Patterns of protein degradation by milk protease in simulated cheeses prepared with 'curd', depending on the NaCl concentration in the moisture of the cheese at pH 6.3 and 5.4. The cheeses were stored for 28 days at 13 °C. Slots 1 to 4 inclusive: cheeses with 0, 2, 4 and 8 % NaCl, respectively, at pH 6.3. Slots 5 to 7 inclusive: cheeses with 2, 4 and 8 % NaCl, respectively, at pH 5.4. Slot 8: 'curd' (blank).

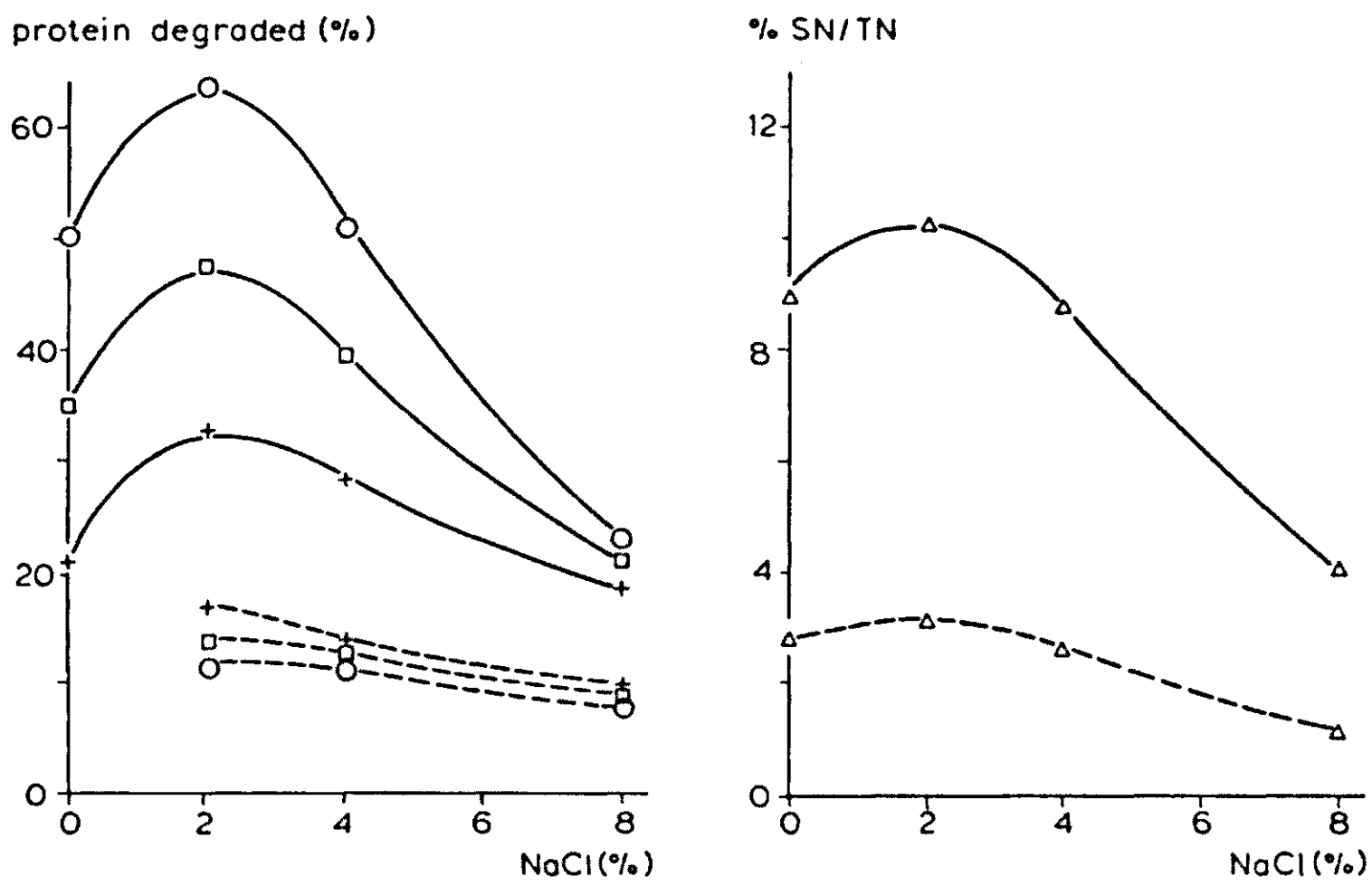

Fig. 5. Protein breakdown in the cheeses shown in Fig. 4, estimated by densitometry and by the amount of soluble nitrogenous compounds, expressed as a percentage of the total nitrogen. +: α_{s1} casein; ○: β casein; □: α_{s1} + β casein; △: values of soluble N. Solid lines represent the results at pH 6.3, dotted lines those at pH 5.4.

pH 5.4 the caseins were much less degraded and consequently the differences in degradation were less distinct. However, the influence of the NaCl concentration at pH 5.4 seemed to show a similar tendency to that observed for pH 6.3. Corresponding to the observations in Section 3.1.1, at pH 5.4 α_{s1} casein was found to be slightly more attacked than β casein, whereas at pH 6.3 β casein was much more extensively degraded.

As illustrated by the course of the relevant curves in Fig. 5, the formation of soluble N was well related to the total degradation of protein calculated by densitometry. With the exception of the cheese containing 8 % NaCl, the ratio of these criteria amounted to 1 : 4 to 5.

3.2 Activity of milk protease in cheeses prepared with paracasein complex

3.2.1 Influence of the pH. Cheeses containing 4 % NaCl in the moisture and with pH values ranging from 4.9 - 6.1 were stored at 13 °C for 28 days. Protein breakdown was followed by the estimation of soluble N. Electrophoretic analyses were not performed in this experiment. Patterns of protein degradation as influenced by the pH of such cheeses are however given in Section 3.2.2 (Fig. 6).

Values of % SN/TN are represented in Fig. 3. It can be seen that, at the pH values tested, the protein had been broken down to an extent corre-

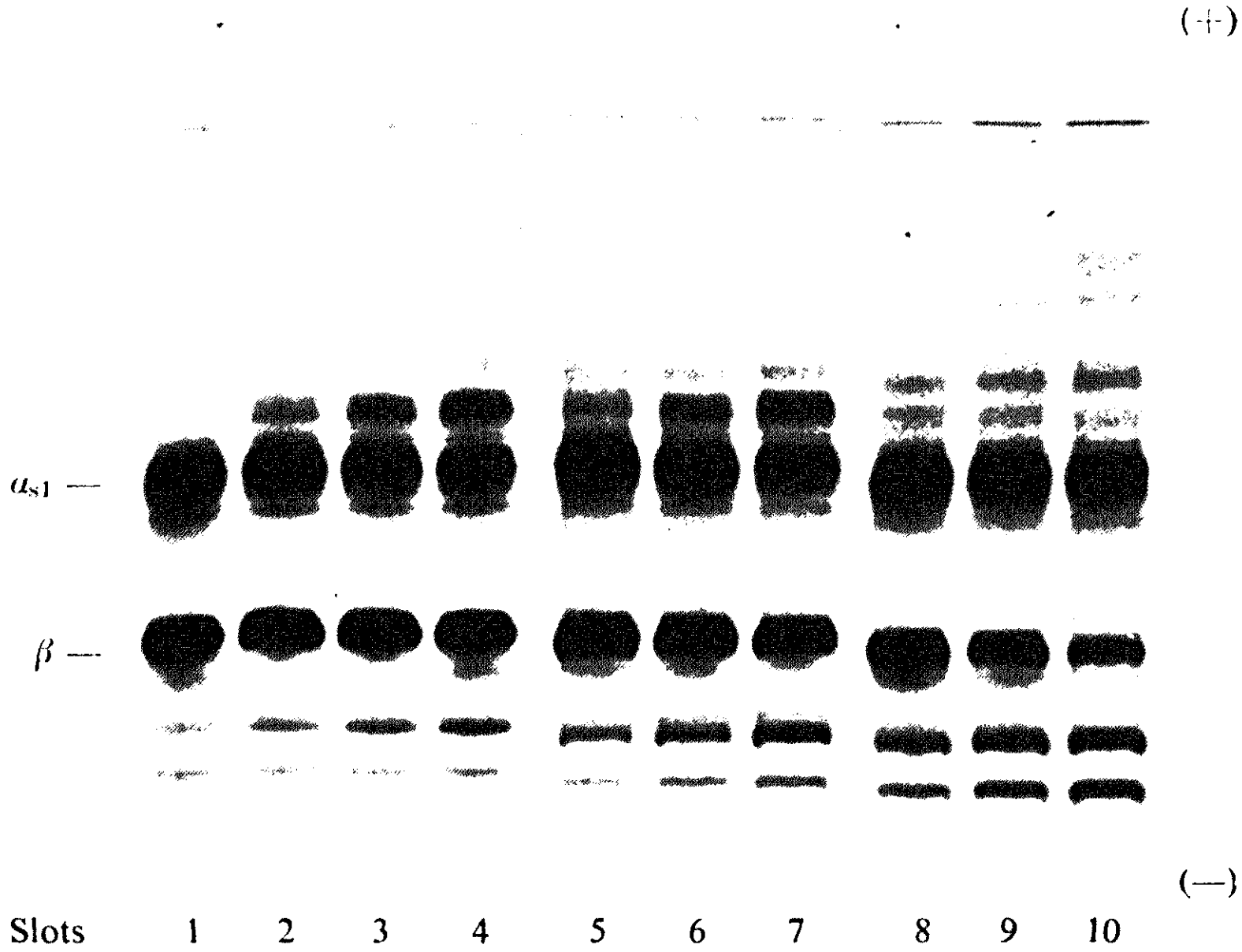

Fig. 6. Patterns of protein degradation by milk protease in simulated cheeses made with paracasein complex, depending on the pH and the storage time at 13 °C. Cheeses contained 4 % NaCl in the moisture. Slot 1: complex (blank). Slots 2, 3 and 4: cheeses stored for 2, 4 and 6 weeks, respectively, at pH 5.1. Slots 5, 6 and 7: idem, at pH 5.5. Slots 8, 9 and 10: idem, at pH 6.0.

sponding well to that found in cheeses made with 'curd'. As was also observed in similar experiments with cheeses made with paracasein complex, the curve showed a distinct shoulder in the traject pH 5.4 - 5.9, which might be related to the changing situation regarding the relative rates of degradation of the caseins at those pH values, as discussed in Section 3.1.1.

3.2.2 Influence of the storage time of cheeses. Cheeses of pH 6.0, 5.5 and 5.1, containing 4 % NaCl in the moisture, were stored for 2, 4 and 6 weeks at 13 °C. The results of the experiments are represented in Figs 6, 7 and 8.

As shown by visual observation (Fig. 6) and by the densitometric evaluation of the gels (Fig. 7), the caseins were more degraded as the cheeses had been stored for an increasing period of time. Whereas β casein had been more broken down with decreasing acidity of the cheeses, the degradation of α_{s1} casein was found to be less at pH 6.0 than at pH 5.5, which approached the degradation of this casein in cheeses made with 'curd' at different acidities (Section 3.1).

After 4 weeks of storage, the patterns of protein degradation at the respec-

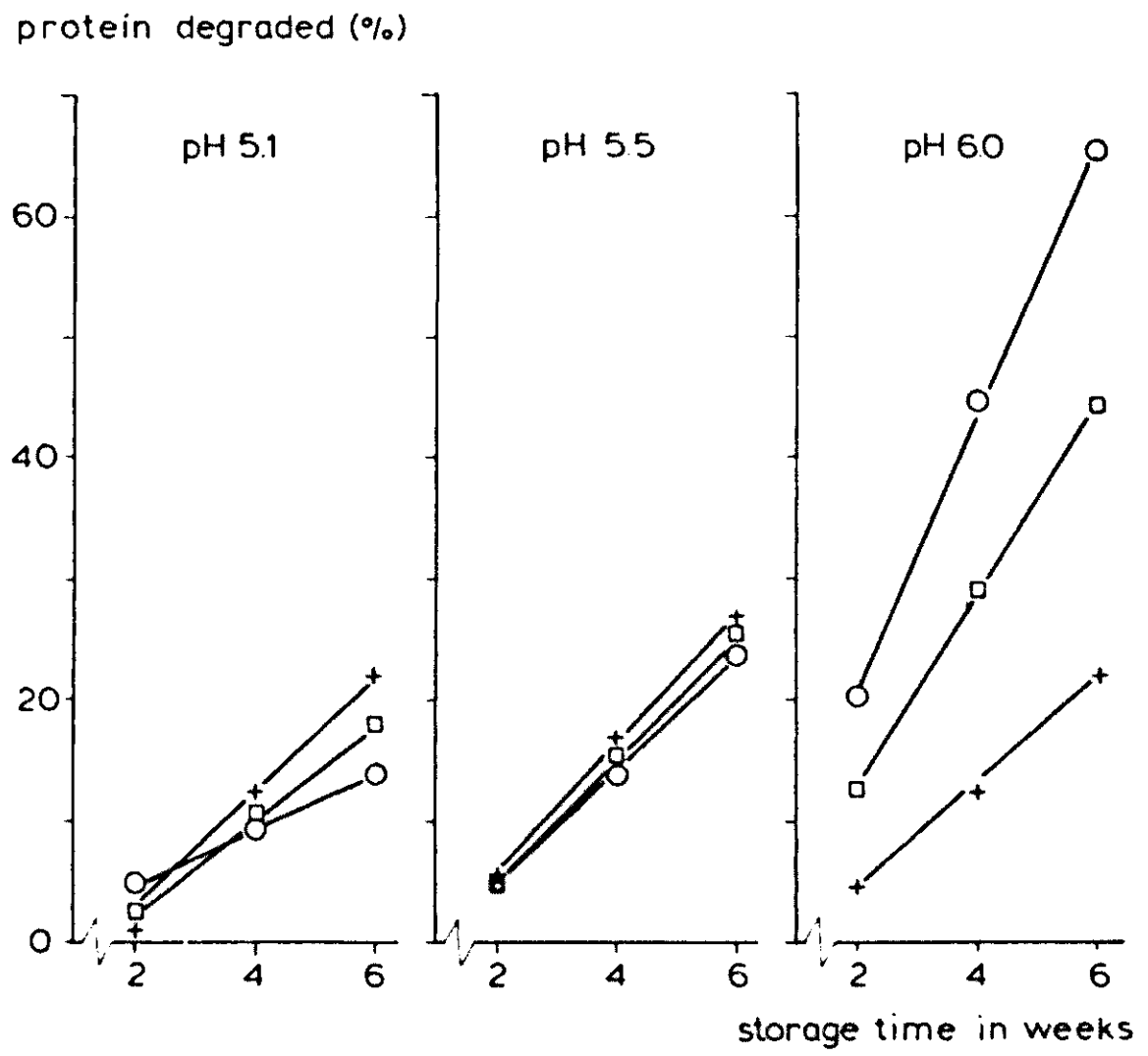

Fig. 7. Protein breakdown in the cheeses shown in Fig. 6, estimated by densitometry. +: α_{s1} casein; ○: β casein; □: α_{s1} + β casein.

tive pH values showed an appearance similar to those of cheeses made with 'curd', stored in corresponding conditions (see Fig. 1). With respect to the degree of degradation of the caseins after this period, corresponding results were obtained with both simulated cheeses at pH 5.1 and 5.5. The same was true for the degradation of α_{s1} casein at pH 6.0. At this pH value, however,

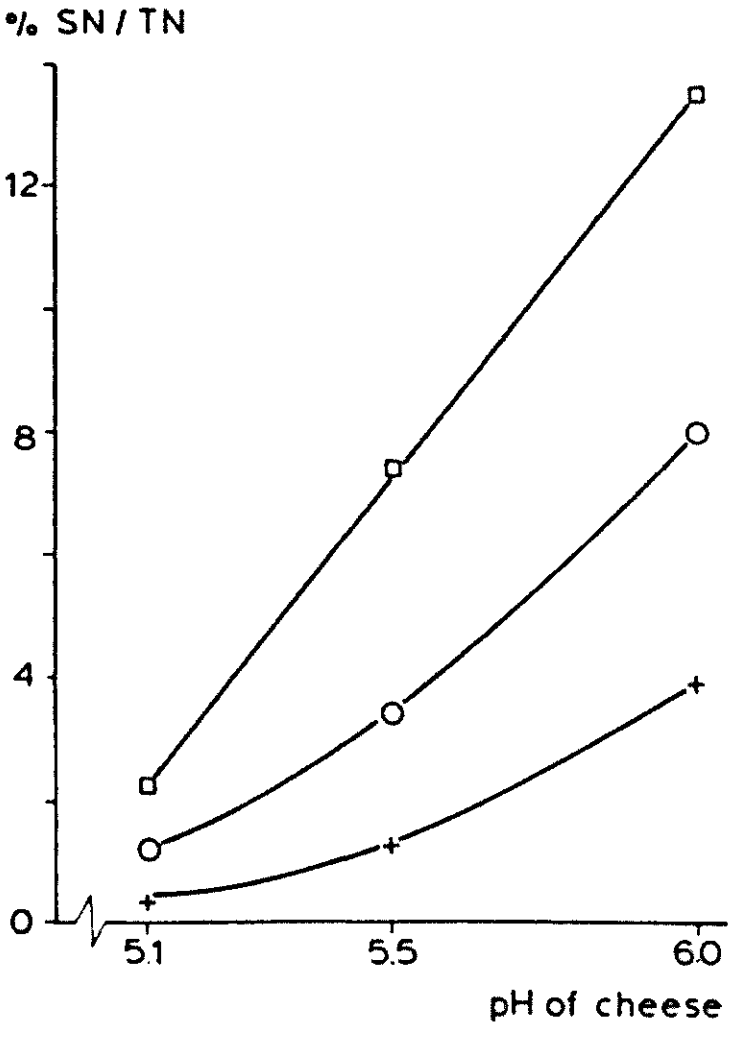

Fig. 8. Protein breakdown in the cheeses of Fig. 6, estimated by the amount of soluble nitrogenous compounds, expressed as a percentage of the total nitrogen. +: cheeses stored for 2 weeks; ○: cheeses stored for 4 weeks; □: cheeses stored for 6 weeks.

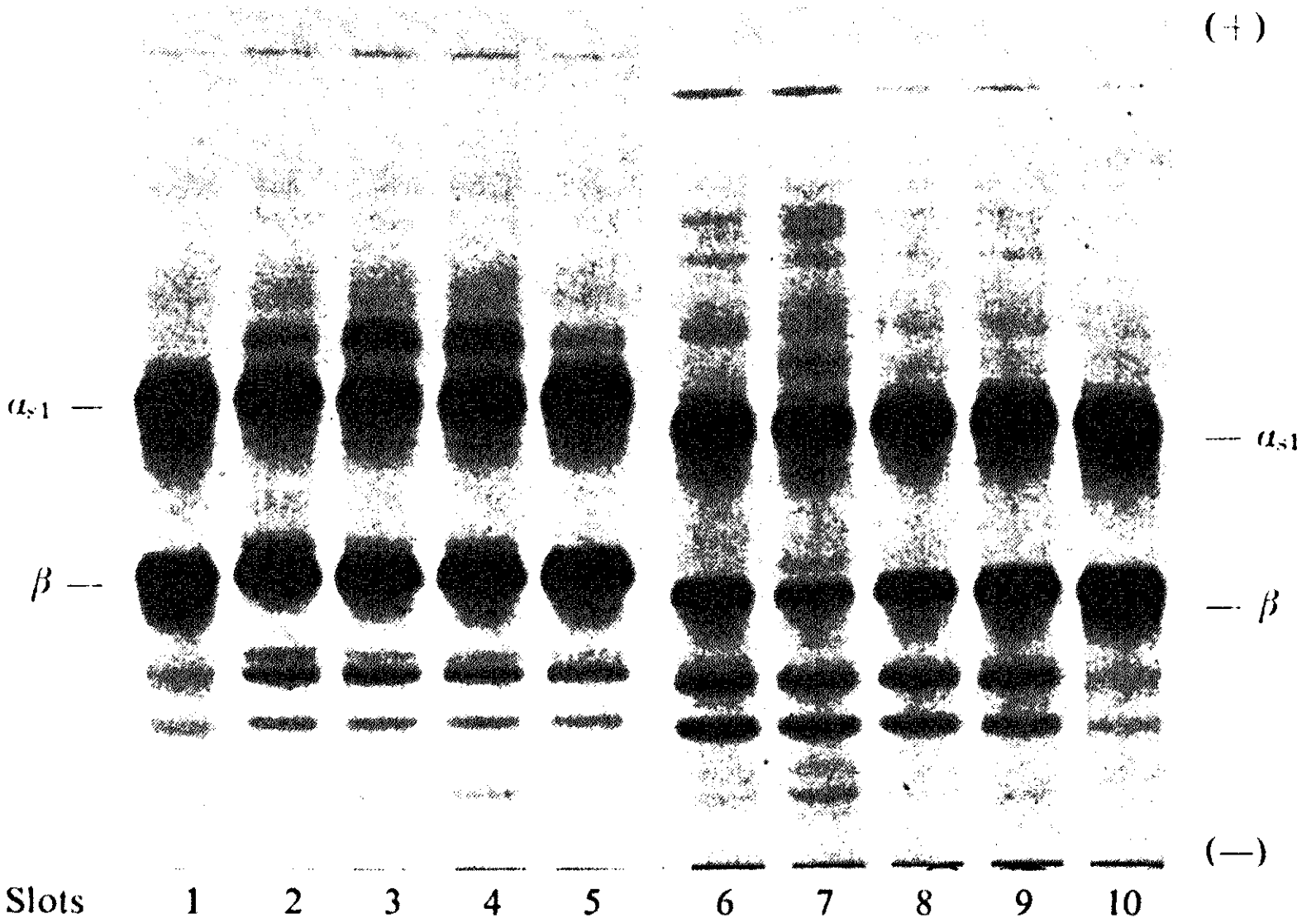

Fig. 9. Patterns of protein degradation by milk protease in simulated cheeses made with paracasein complex, depending on the NaCl concentration in the moisture at pH 6.1 and 5.4. Cheeses were stored for 28 days at 13 °C. Slots 1 and 10: complex (blank). Slots 2 to 5 inclusive: cheeses with 0, 2, 4 and 8 % NaCl, respectively, at pH 5.4. Slots 6 to 9 inclusive: idem, at pH 6.1.

β casein was found more vigorously attacked in the cheese made with paracasein complex than in that made with 'curd' (see Fig. 7 and Fig. 2).

In the test conditions, the magnitude of protein degradation as measured by densitometry was very well reflected by the amounts of soluble N liberated (Fig. 8). With the exception of cheeses with the lowest levels of protein degradation (cheeses at pH 5.1 after 2 and 4 weeks of storage), in all cases the ratio between % SN/TN and the percentage of total degradation amounted to 1 : 4 to 5, in all cases in accordance with our findings with cheeses made with 'curd' (Section 3.1).

3.2.3 Influence of the NaCl content. Cheeses containing 0, 2, 4 and 8 % NaCl in the moisture were stored for 28 days at 13 °C. The experiments were performed at pH values of 6.1 and 5.4. The results are represented in Figs 9 and 10. They were generally in accordance with those obtained with cheeses made with 'curd'.

As can be seen by the comparison of Figs 9 and 4, at a corresponding pH the patterns of protein degradation in both simulated cheeses showed a similar appearance. Furthermore, milk protease activity was again found to be

stimulated by low concentrations of NaCl, maximum activity being observed near 2 % NaCl at both pH values tested (Fig. 10). The curves representing the levels of protein degradation as estimated by the two methods used, to a large extent showed a corresponding tendency with both cheeses (Figs 10 and 5). Apart from some deviation, the results tended to indicate that protein degradation in the same period of time had been more advanced in cheeses made with paracasein complex, particularly when it is taken into account that the highest pH value of the cheeses made with 'curd' (pH 6.3) was more favourable for milk protease activity than that of the cheeses made with paracasein complex (pH 6.1).

In accordance with earlier findings, the levels of total protein degradation in the cheeses as estimated by the extraction and the electrophoretic method showed a fairly constant ratio in almost all conditions of test. This ratio was 1 : 4 to 5 for the cheeses of pH 6.1 and about 1 : 6 for those of pH 5.4.

3.2.4 Influence of the temperature. Cheeses of pH 6.0 and 5.2, which contained 4 % NaCl in the moisture, were stored for 14 days at 5, 13, 20, 30 and 37 °C.

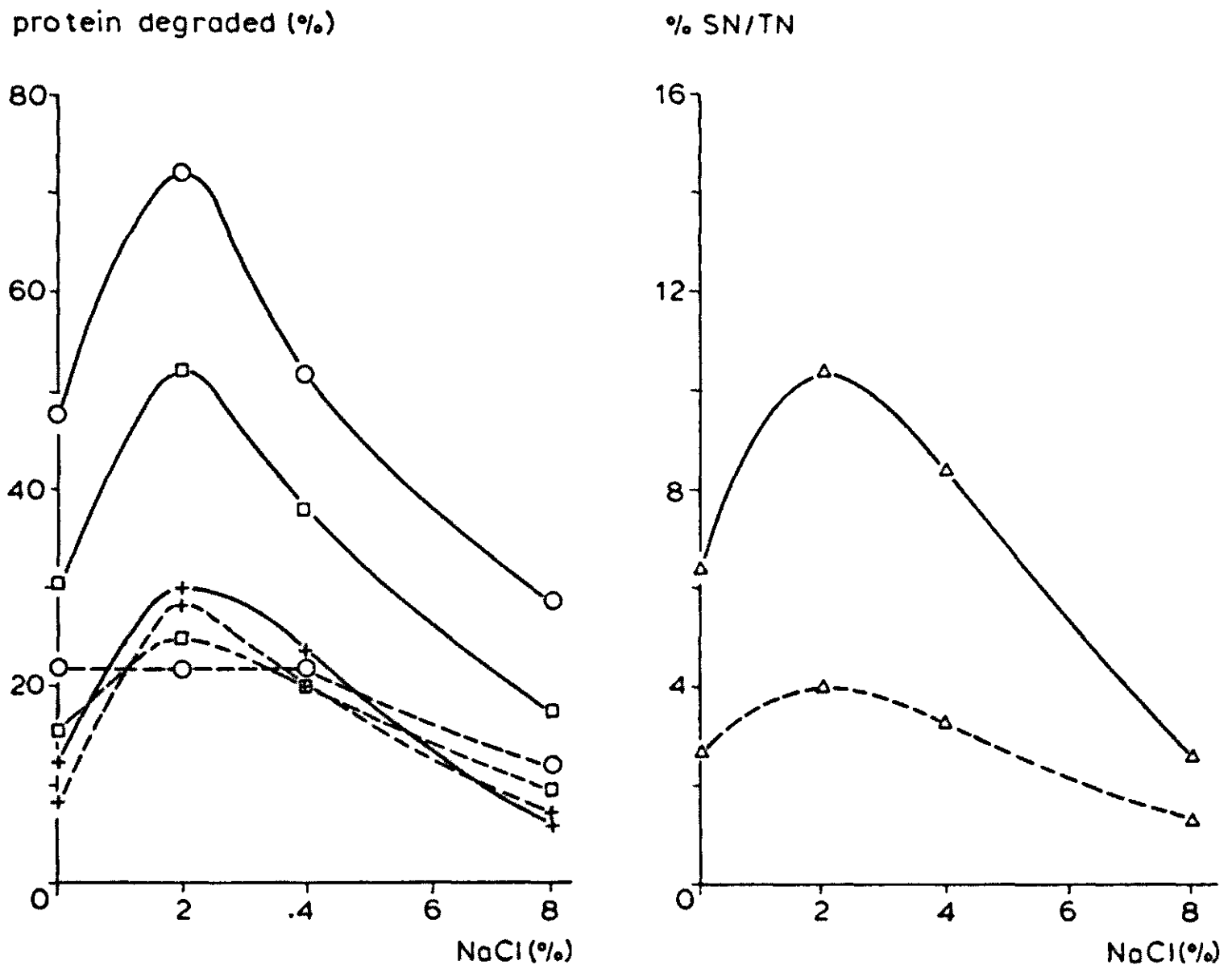

Fig. 10. Protein breakdown in the cheeses shown in Fig. 9, estimated by densitometry and by the amount of soluble nitrogenous compounds, expressed as a percentage of the total nitrogen. +: α_{s1} casein; ○: β casein; □: α_{s1} + β casein; △: values of soluble N. Solid lines represent the results at pH 6.1, dotted lines those at pH 5.4.

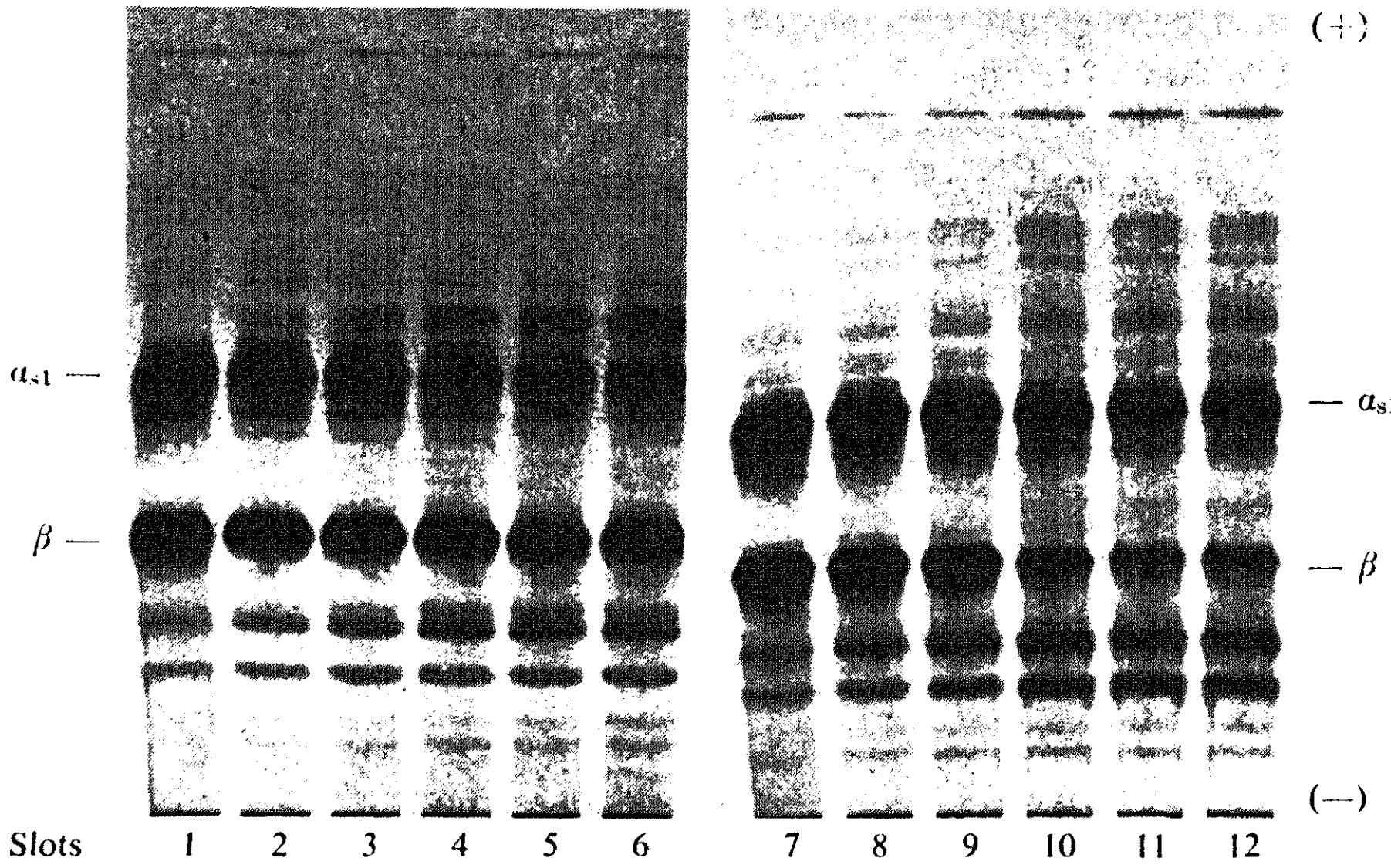

Fig. 11. Patterns of protein degradation by milk protease in simulated cheeses made with paracasein complex, depending on the storage temperature at pH 6.0 and 5.2. The cheeses contained 4 % NaCl in the moisture and were stored for 14 days. Slots 1 and 7: complex (blank). Slots 2 to 6 inclusive: cheeses stored at 5, 13, 20, 30 and 37 °C, respectively, at pH 5.2. Slots 7 to 12 inclusive: idem, cheeses at pH 6.0.

Electrophoretic patterns of cheeses of pH 6.0 (Fig. 11) showed a decrease of α_{s1} and β casein at increasing temperature, also characterized by a slight increase of the size of bands in front of α_{s1} casein and a strong increase of those below the β band. At pH 5.2, α_{s1} casein evidently became more degraded at a higher temperature as reflected by the formation of a well developed band in front of this casein. At this acidity, the degradation of β casein remained weak and did not seem to increase at temperatures above 20 °C.

These observations were completely confirmed by densitometry (Fig. 12). At pH 6.0, both caseins had been increasingly broken down at higher temperatures, about 70 % of the β casein and 40 % of the α_{s1} casein being degraded at 37 °C. The results generally confirmed the earlier results that at high pH values and moderate NaCl contents β casein was degraded two to three times more rapidly than α_{s1} casein (see the Figs 2, 5, 7 and 10). Cheeses of pH 5.2 showed an increasing degradation of α_{s1} casein with increasing temperature, about 45 % of it being broken down at 37 °C, whereas β casein breakdown gradually increased to a level of about 15 % at approximately 20 °C, and remained constant at higher temperatures.

According to the densitometric results, at 5 °C the caseins seemed to be

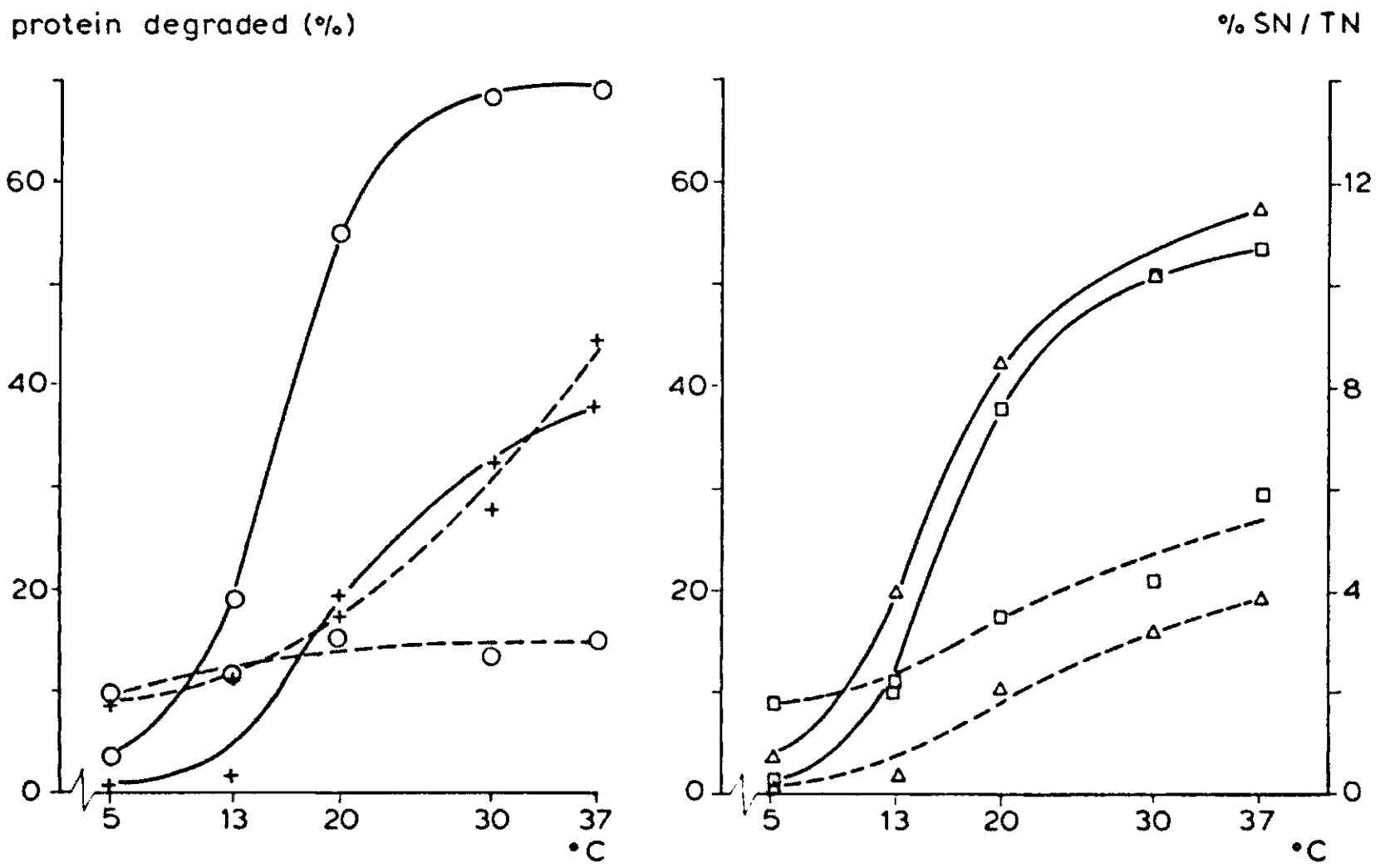

Fig. 12. Protein breakdown in the cheeses shown in Fig. 11, estimated by densitometry and by the amount of soluble nitrogenous compounds, expressed as a percentage of the total nitrogen. +: α_{s1} casein; ○: β casein; □: α_{s1} + β casein; △: values of soluble N. Solid lines represent the results at pH 6.0, dotted lines those at pH 5.2.

more degraded at pH 5.2 than at pH 6.0. However, the limited accuracy of that method at very low levels of protein degradation should be kept in mind before drawing conclusions. Values of % SN/TN indicated the opposite.

As can be seen in Fig. 12, the curves representing the values of % SN/TN and the percentages of total protein degraded followed an almost similar course. With the exception for the cheeses stored at 5 °C, the results of both methods again showed a fairly constant ratio of 1 : 4 to 5 for the cheeses of pH 6.0 and 1 : 7 to 9 for those of pH 5.2.

4 Discussion

Our study established the activity of milk protease in simulated cheeses made from rennet-free cheese curd or from a rennet-free calcium paracaseinate-calcium phosphate complex.

As indicated by both methods used for the estimation of proteolytic activity, milk protease showed a very similar action in both cheeses in comparable conditions (pH, NaCl content). The calcium concentration, which may influence proteolysis (28), was also comparable, being 0.66 % in cheeses made with 'curd' and 0.63 % in those prepared with paracasein complex. In a quantitative sense, however, indications were obtained that at correspond-

ing conditions the protein became more degraded in the cheeses made with paracasein complex than in those prepared with 'curd' (Sections 3.2.2 and 3.2.3). This may perhaps be due to the presence of protease inhibitors in the latter cheeses, which have been reported to occur predominantly in the milk serum (29). It might be worth while to study the effect of whey on milk protease activity in more detail.

Our experiments indicated that milk protease may contribute to both α_{s1} and β casein degradation in soft cheese, to an extent which is largely determined by the pH, the NaCl content, the ripening time and the ripening temperature of the cheese.

In the cheeses stored at 13 °C, the relative rates of breakdown of the different caseins appeared to be strongly influenced by the pH (Sections 3.1 and 3.2). Whereas at high pH values β casein was more degraded than α_{s1} casein, at low pH values the opposite appeared to occur, which became particularly apparent after a prolonged period of storage. An increased vulnerability to attack of in particular α_{s1} casein at higher acidities in soft cheese might contribute to an explanation of this phenomenon. It might also be explained by the presence of different bovine milk proteases, whose existence has been reported in the literature (30, 31, 32). In view of our findings, the occurrence of an acid protease as reported by Kaminogawa & Yamauchi (32) is of particular interest. The properties of this enzyme were quite different from those of alkaline milk protease. When acid protease acted on casein in solution at pH 5.5 at 37 °C, the electrophoretic patterns of α_{s1} degradation were similar to those obtained by the action of rennet, showing the formation of a strong α_{s1}-I casein-like band. Moreover, α_{s1} casein was more susceptible to the acid protease than was β casein. This manner of protein degradation thus resembled that in our cheeses at low pH values. It is therefore suggested that, in addition to alkaline milk protease, an acid protease contributed to protein degradation in the cheeses. However, further studies are required to confirm this view.

Under most conditions at 13 °C, the values of % SN/TN and the percentages of total degradation of protein estimated by densitometry showed a fairly constant ratio of 1 : 4 to 5. This supported our earlier statements concerning the usefulness of the soluble N fraction, obtained by the extraction method developed, as an index of the protein breakdown in cheese (17). The very few deviating ratios were almost exclusively observed with cheeses showing very low levels of protein breakdown, and may be ascribed predominantly to the limited accuracy of particularly the densitometric method at those circumstances.

The influence of NaCl on the activity of milk protease has been scarcely

studied. Thatcher & Dahlberg (33) performed experiments with preserved skim milk. They reported the activity to decrease with increasing salt concentrations and found it completely inhibited by 15 % NaCl. In our simulated cheeses we found the activity of milk protease to be considerably stimulated in the presence of low concentrations of NaCl, maximum activity being observed near 2 % NaCl under the conditions of test (Sections 3.1.2 and 3.2.3).

The experiments with cheeses made with paracasein complex revealed that, within the temperature range of 5 to 37 °C, the total degradation of protein increased very considerably when the cheeses were kept at a higher temperature (Fig. 12). Striking differences were observed in the relative rates of breakdown of the caseins at low and high pH values, respectively. At pH 6.0, both caseins became increasingly degraded. At pH 5.2, the degradation of α_{s1} casein showed a similar behaviour, whereas β casein breakdown remained at a constant low level above 20 °C. The observation that α_{s1} casein was almost equally degraded at both pH values, whereas the activity of alkaline milk protease could be reasonably expected to decrease with increasing acidity of the cheese, supports our idea concerning the presence of a second proteolytic enzyme in the cheeses.

With cheeses stored at pH 6.0, values of % SN/TN and the percentages of total degradation estimated by densitometry showed a ratio of 1 : 4 to 5 at all temperatures tested. At pH 5.2, however, this ratio amounted to 1 : 7 to 9. Taking into consideration the rates of degradation of both caseins at the respective pH values, this might indicate that degradation products of β casein were more abundant in the cheese extracts than those of α_{s1} casein. This would explain the results of Section 3.1.1, showing the existence of a minimum in the total degradation of protein as measured by densitometry, whereas this minimum was not reflected in the values of % SN/TN.

In an earlier report, we described the conditions during the ripening of Meshanger cheese in detail (21). In view of those conditions and our observations on the activity of milk protease under comparable conditions in the simulated cheeses, the contribution of milk protease to protein breakdown in Meshanger cheese can be assumed to be very limited. The cheese has a very short ripening time and ripens at 13 °C. In the interior part of the cheese (about 30 mm), the pH decreases to values below 5.6 within one week after fabrication. During this period of time the pH of the outer 7 mm of the cheese remains above 5.8, at which a greater action of milk protease would be possible. However, during the first days of ripening the high salt concentration in this part (more than 6 % in the moisture) renders the conditions less favourable to enzyme activity. Our opinion was also supported by the patterns of protein degradation during the ripening of the cheese, indicating a very slight

activity of milk protease when related to the formation of typical 'minor caseins' from β casein. However, in other varieties of cheese with a high moisture content, particularly in those ripening under the influence of a surface flora, milk protease may possibly contribute to protein degradation to a greater extent. In Camembert cheese, for instance, during some weeks of the ripening period the outer parts show conditions (pH 6.0, NaCl content in the moisture about 5 %) which may allow a considerable action of alkaline milk protease to take place (34).

Our experiments were of a tentative character, and further studies are required to raise the veil on the action of milk protease in cheese.

Acknowledgment

The author wishes to express his gratitude to Miss A. P. Stasse and Mrs A. E. A. de Groot-Mostert for technical assistance.

Samenvatting

A. Noomen, *De activiteit van proteolytische enzymen in kunstkaasjes met een hoog vochtgehalte (Meshanger type). 1. De activiteit van melkprotease*

De activiteit van melkprotease in zachte kaas werd bestudeerd met kunstkaasjes, waarin melkprotease werkzaam was onder omstandigheden die zo goed mogelijk de omstandigheden benaderden die tijdens de rijping van Noordhollandse Meshanger kaas voorkomen. De kaasjes werden samengesteld met (1) stremselvrije kaaswrongel, of (2) een stremselvrij calciumparacaseïnaat-calciumfosfaat-complex. In beide preparaten was melkprotease reeds van nature aanwezig. De eiwitafbraak werd nagegaan met behulp van kwantitatieve polyacrylamide-gelelectroforese en door bepaling van de gevormde hoeveelheid opgeloste stikstofverbindingen. De met beide methoden verkregen resultaten aangaande de totale eiwitafbraak bleken onder nagenoeg alle onderzochte omstandigheden goed gecorreleerd te zijn.

Afgezien van verschillen in kwantitatieve zin bleek melkprotease onder vergelijkbare omstandigheden in alle kaasjes op gelijke wijze werkzaam te zijn. Kwantitatieve verschillen kunnen onder meer zijn veroorzaakt door verschillen in enzymactiviteit, in enzymconcentratie en in samenstelling van de kaasjes.

De enzymactiviteit bleek in sterke mate te worden beïnvloed door de pH, de NaCl-concentratie in het vocht en de rijpingstemperatuur van de kaas.

Het eiwit werd het meest afgebroken in kaasjes met een hoge pH. Terwijl bij hoge pH-waarden (b.v. pH 6,2) β-caseïne veel sneller werd afgebroken dan α_{s1}-caseïne, werd bij een lage pH (b.v. pH 5,4) α_{s1}-caseïne wat meer aangetast. Er wordt verondersteld dat naast alkalisch melkprotease een zuur melkprotease, dat bij voorkeur α_{s1}-caseïne afbreekt, onder gunstige omstandigheden bijdraagt tot de eiwitafbraak in zachte kaas.

Lage zoutconcentraties bevorderden de afbraak van beide caseïnes, terwijl hoge zoutconcentraties remmend werkten. Onder de onderzochte omstandigheden bleek de eiwitafbraak maximaal te zijn bij aanwezigheid van 2 % NaCl.

Bij pH 6,0 werden bij stijgende temperaturen in het trajekt 5 - 37 °C zowel α_{s1}- als

β-caseïne in toenemende mate afgebroken. Bij pH 5,2 was dit met α_{s1}-caseïne eveneens het geval, doch de afbraak van β-caseïne bleef op een constant en laag niveau bij temperaturen boven 20 °C.

Gelet op de tijdens de rijping in Meshanger kaas heersende omstandigheden en de in kunstkaasjes onder daarmee vergelijkbare omstandigheden gevonden activiteit van melkprotease, wordt de bijdrage van melkprotease tot de eiwitafbraak in deze kaas van weinig belang geacht voor de normale rijping (zie ook het volgende deel van deze artikelenserie). Deze bijdrage kan mogelijk belangrijker zijn in andere zachte kaassoorten, waarin tijdens de rijping omstandigheden voorkomen die gunstig zijn voor de activiteit van met name alkalisch melkprotease, zoals in zachte kaassoorten met een oppervlakteflora.

References

1. S. M. Babcock & H. L. Russell, *A. Rep. Wisc. agric. Exp. Stn* 14 (1897) 161-193.
2. R. C. Warner & E. Polis, *J. Am. chem. Soc.* 67 (1945) 529-532.
3. A. B. Storrs & M. E. Hull, *J. Dairy Sci.* 39 (1956) 1097-1103.
4. W. J. Harper, J. A. Robertson Jr & I. A. Gould, *J. Dairy Sci.* 43 (1960) 1850-1851.
5. F. Kiermeier & G. Semper, *Z. LebensmUnters. u. -Forsch.* 3 (1960) 282-307.
6. J. R. Dulley, *J. Dairy Res.* 39 (1972) 1-9.
7. E. H. Reimerdes & H. Klostermeyer, *Milchwissenschaft* 29 (1974) 517-522.
8. K. Yamauchi & S. Kaminogawa, *Agric. biol. Chem.* 36 (1972) 249-254.
9. A. Noomen, *Neth. Milk Dairy J.* 29 (1975) 153-161.
10. J. Stadhouders, *Neth. Milk Dairy J.* 14 (1960) 83-110.
11. B. Reiter, Y. Sorokin, A. Pickering & A. J. Hall, *J. Dairy Res.* 36 (1969) 65-76.
12. M. L. Green & P. M. D. Foster, *J. Dairy Res.* 41 (1974) 269-282.
13. L. K. Creamer, *J. Dairy Sci.* 58 (1975) 287-292.
14. S. Kaminogawa & K. Yamauchi, *Agric. biol. Chem.* 36 (1972) 255-260.
15. S. Kaminogawa & K. Yamauchi, *Agric. biol. Chem.* 38 (1974) 2343-2348.
16. L. de Jong, *Neth. Milk Dairy J.* 29 (1975) 162-168.
17. A. Noomen, *Neth. Milk Dairy J.* 31 (1977) 163-176.
18. F. M. W. Visser, *Neth. Milk Dairy J.* 31 (1977) 210-239.
19. F. M. W. Visser, *Neth. Milk Dairy J.* 31 (1977) 247-264.
20. A. Noomen & H. Mulder, *Neth. Milk Dairy J.* 30 (1976) 230-241.
21. A. Noomen, *Neth. Milk Dairy J.* 31 (1977) 75-102.
22. A. Noomen, *Neth. Milk Dairy J.* 31 (1977) 103-108.
23. F. M. W. Visser, *Neth. Milk Dairy J.* 30 (1976) 41-54.
24. A. Noomen, *Neth. Milk Dairy J.* 32 (1978) 49-68.
25. G. Kleter & Tj. de Vries, *Neth. Milk Dairy J.* 28 (1974) 212-219.
26. Jaarverslag 1976, Nederlands Instituut voor Zuivelonderzoek (NIZO), Ede, the Netherlands, p. 52.
27. J. W. Duersch & C. A. Ernstrom, *J. Dairy Sci.* 57 (1974) 590.
28. L. de Jong & A. E. A. de Groot-Mostert, *Neth. Milk Dairy J.* 31 (1977) 296-313.
29. E. H. Reimerdes, H. Klostermeyer & E. Sayk, *Milchwissenschaft* 31 (1976) 329-334.
30. V. Moreno & F. V. Kosikowski, *Milchwissenschaft* 27 (1972) 433-436.
31. E. H. Reimerdes, H. Klostermeyer & J. Thomasow, *Kieler milchw. ForschBer.* 26 (1974) 373-384.
32. S. Kaminogawa & K. Yamauchi, *Agric. biol. Chem.* 36 (1972) 2351-2356.
33. R. W. Thatcher & A. C. Dahlberg, *J. agric. Res.* 11 (1917) 437-450.
34. J. Lenoir, *Lait* 43 (1963) 154-165.

Neth. Milk Dairy J. 32 (1978): 49-68

Activity of proteolytic enzymes in simulated soft cheeses (Meshanger type). 2. Activity of calf rennet

A. Noomen

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

Received: 27 October 1977

Key words: cheese ripening, calf rennet, milk protease, soft cheese, protein breakdown

Summary

The proteolytic activity of calf rennet in soft cheese was studied with simulated cheeses under various conditions. The cheeses were prepared with (1) a calcium paracaseinate-calcium phosphate complex, made from milk freed from milk protease activity by a heat treatment, or with (2) a calcium paracaseinate-calcium phosphate complex prepared from low-temperature pasteurized milk, or with (3) rennet-free cheese curd. The latter two cheeses contained milk protease. The cheeses contained about 2.2 ml of rennet per kg of protein, being approximately 50 % more than the concentration in Meshanger cheese in a ripe condition. Protein breakdown was studied by quantitative polyacrylamide gel electrophoresis and by the estimation of the amounts of soluble nitrogenous compounds formed. The results of both methods for the total degradation of protein were well correlated.

Apart from differences in a quantitative sense, rennet acted in a corresponding manner in the different cheeses in comparable test conditions and degraded the protein very extensively under favourable circumstances. For example, in cheeses of pH 5.0 and containing 4 % NaCl in the moisture, 35 to 50 % of the total protein (65 to 90 % of the α_{s1} casein) became degraded in two weeks at 13 °C. Under all conditions, rennet degraded α_{s1} casein much more than it did β casein.

Maximum activity of rennet against α_{s1} casein was found at a pH near to 5.0. Optimum pH for β casein breakdown did not become clear. Total breakdown of protein was at a maximum at pH 4.9-5.0.

The degradation of α_{s1} casein was stimulated by NaCl concentrations in the moisture up to about 4 %, and retarded by higher salt contents. The breakdown of β casein was maximal in the absence of NaCl and was already considerably reduced at low salt contents. This may explain our observations that the total breakdown of protein decreased with increasing salt content.

Under the conditions tested, rennet did not liberate amino acids in any detectable amount.

Under the conditions of a ripening Meshanger cheese, rennet degrades α_{s1} casein so

extensively that it causes the main ripening of the cheese. Since it has been also established that the changes of consistency of the cheese are strongly related to the degree of degradation of α_{s1} casein, the characteristic and complicated way of softening of the cheese, which proceeds from the inside to the outside, can be explained. During the ripening of the cheese the most favourable conditions for the action of rennet against α_{s1} casein (pH, NaCl content) are those which are most rapidly present in the centre of the cheese. β casein is only slightly degraded in Meshanger cheese.

The results of the investigation strongly supported our conception that rennet is just as crucial in the ripening of other cheeses with a high moisture content. This concerns cheeses without a surface flora such as Butterkäse and St. Paulin cheese as well as those with a surface flora, such as Kernhem, Brie, Camembert and Limburger cheese.

1 Introduction

Our investigations on the ripening of soft Noordhollandse Meshanger cheese indicated that the protein breakdown in the cheese was to be attributed to the calf rennet used and that the softening of the cheese body, which starts in the centre and from there proceeds to the surface, was very closely related to the degree of protein degradation in the different layers of the cheese (1). With respect to the further verification of these findings, rennet activity was studied under various conditions occurring in the cheese during its ripening period.

Factors as the pH value, the concentration of the various constituents and the temperature, may influence both the relative rates of breakdown of the different caseins and the nature of the degradation products formed. Relevant literature to this subject has been reviewed recently by Green (2). With respect to the actual progress of proteolytic processes involved in the ripening of cheese, these factors render the interpretation of results of studies, performed with substrates showing conditions far remote from those in cheese, very difficult. In fact, conclusions from such studies can only be drawn reliably when the model used is correct in the essential points. Recently, this necessity was once more clearly demonstrated by de Jong & de Groot-Mostert (3) in their investigation on the breakdown by rennet of sodium paracaseinate in solution. Among other things, they found that the rates of degradation of α_{s1} and β casein were drastically changed in the presence of calcium.

We developed simulated cheese as a model that fitted as well as possible the actual situation in cheese (4). The proteolytic activity of calf rennet was studied in those models, which were made from a milk protease-free calcium paracaseinate-calcium phosphate complex and a milk protease-containing calcium paracaseinate-calcium phosphate complex, and with rennet-free cheese curd, respectively.

2 Materials and methods

2.1 Milk

The milk was obtained from the dairy herd of the University. Aseptically drawn milk was obtained in the manner normally used in our laboratory and described by Kleter & de Vries (5).

2.2 Rennet

Dutch commercial calf rennet (strength 1 : 10 000) was used.

2.3 Preparation of a calcium paracaseinate-calcium phosphate complex from low-temperature pasteurized milk

Freshly drawn milk was skimmed by centrifugation and pasteurized for 20 s at 72 °C. Rennet was added at 30 °C (40 ml/100 litres milk). From the start of visible coagulation, vigorous stirring was applied for 10 min to obtain finely divided curd particles and to facilitate the separation of whey. The curd/whey mixture was diluted twice with pasteurized tap water (25 s, 85 °C) at 30 °C, and the stirring repeated for another 10 min. After sedimentation of the curd particles the whey was siphoned off. The washings were repeated until about a 1000-fold dilution of milk serum constituents was obtained. The particles were homogenized in water with the aid of an Ultra Turrax, and spray-dried. The particles were milled with an impact mill (mesh sieve 0.8 mm) at a temperature below 40 °C. The powder was well mixed, tinned and stored at room temperature.

The paracasein complex showed the following composition: moisture 8.0 %; nitrogen 12.7 %, corresponding to a protein content of 81.0 %; soluble nitrogen expressed as a percentage of the total nitrogen (% SN/TN) 0.20; calcium 2.38 %; aerobic and anaerobic bacterial numbers were less than 40 000 per gram of complex. The preparation showed considerable milk protease activity (Section 3.2).

2.4. Preparation of a milk protease-free calcium paracaseinate-calcium phosphate complex

Ten litres of freshly drawn milk were skimmed, heated for 30 min at 80 °C to destroy milk protease activity, and cooled to 30 °C. $CaCl_2 \cdot 2H_2O$ (5.6 g) and 4 ml of rennet were added. From the start of visible coagulation the

mixture was stirred for 30 min. The very fine curd particles were collected by centrifugation. The sediment was homogenized in water with an Ultra Turrax and stirred for 15 min in 200 litres of pasteurized tap water (25 s, 85 °C), resulting directly in about a 1000-fold dilution of milk serum constituents. The complex was again collected by centrifugation and frozen at –20 °C. Several portions of milk were treated this way. Defrosted batches of complex were combined, homogenized in water and spray-dried. The complex was milled and stored as indicated in Section 2.3.

The composition of the powder was: moisture 2.81 %; nitrogen 13.95 %, corresponding to a protein content of 89.0 %; % SN/TN 0.20; calcium 2.46 %; aerobic and anaerobic bacterial numbers amounted to 3000 and 1000 per gram of complex, respectively.

2.5 Preparation of rennet-free cheese curd

The curd was prepared from aseptically drawn milk according to the technique developed in this laboratory by Visser (6) and handled as described in the foregoing report (4).

The curd had the following composition: moisture 51.27 %; nitrogen 3.59 %, corresponding to a protein content of 22.8 %; % SN/TN 1.77; pH 6.25; calcium 0.78 %; aerobic and anaerobic bacterial numbers were less than 30 per gram.

2.6 Preparation of simulated cheeses

2.6.1 Cheeses without rennet. Cheeses with various pH values and NaCl concentrations in the moisture, and with a ratio of protein to water of 1 : 3.5, were made and stored anaerobically according to the directions given in the earlier report (4). The cheeses were made in such a way that all of them contained about 16 ml of moisture. Unless otherwise indicated, the cheeses were stored for 14 days at 13 °C.

2.6.2 Cheeses with rennet. The cheeses were provided with 0.01 ml of rennet, being the quantity approximately present in 16 ml of moisture of Meshanger cheese just after production. The cheeses therefore contained about 2.2 ml of rennet per kg of protein, being approximately 50 % more than the concentration in a ripe Meshanger cheese.

With regard to the rennet-free materials, their preparation was adjusted as follows. In the case of cheeses made with paracasein complex, 2 ml of water was replaced by a dilution of rennet in water (1 : 200). To prevent the even-

tual damage to enzyme activity at very low pH values, the rennet was added at the end of the preparation, after the mixture of lactic acid solution and complex had been left for about 15 min. The rennet was very well mixed with the other ingredients. With respect to cheeses made with 'curd', 4 ml of water was replaced by rennet dilution (1 : 400). The rennet was mixed with the 'curd' prior to the addition of the lactone.

The thimerosal, added as a preservative in a concentration of 100 mg per litre of cheese moisture, hardly influenced the activity of the rennet used. As estimated by the amount of soluble nitrogenous compounds formed by rennet in milk protease-free simulated cheeses made with and without thimerosal, protein breakdown had decreased by not more than 2 % in the cheeses which contained the preservative.

The cheeses were stored in the same conditions as those without rennet.

2.7 Analytical methods

Estimations of soluble nitrogen, electrophoretic experiments and densitometry of gels, pH measurements and determinations of bacterial numbers were performed by the methods described in the previous paper (4). For electrophoretic purposes, the cheeses were completely dissolved in the buffer and diluted to 0.5 % of protein. After the storage period, bacterial numbers were mostly zero and never more than 500 per g of cheese or per ml of cheese serum. This established once again the suitability of the preservation method applied.

In one experiment, the formation of amino acids was followed by the method used by us previously (1).

3 Experiments and results

3.1 Protein breakdown by rennet in cheeses prepared with a milk protease-free calcium paracaseinate-calcium phosphate complex

Preliminary experiments showed the absence of proteolytic activity in cheeses without rennet. Cheeses with various pH values (4.5 - 6.5) and NaCl concentrations in the moisture (0 - 8 %) were stored for 14 days at 13 °C. With all cheeses, values for % SN/TN amounted to 0.20 ± 0.03, and those for % AN/TN (amino acid nitrogen expressed as a percentage of total nitrogen) to 0.12 ± 0.05; both corresponding to blank values.

The results of an experiment concerning the influence of the pH and the NaCl concentration in the moisture of the cheese on rennet activity are represented in Fig. 1. Protein degradation was estimated by the amount of

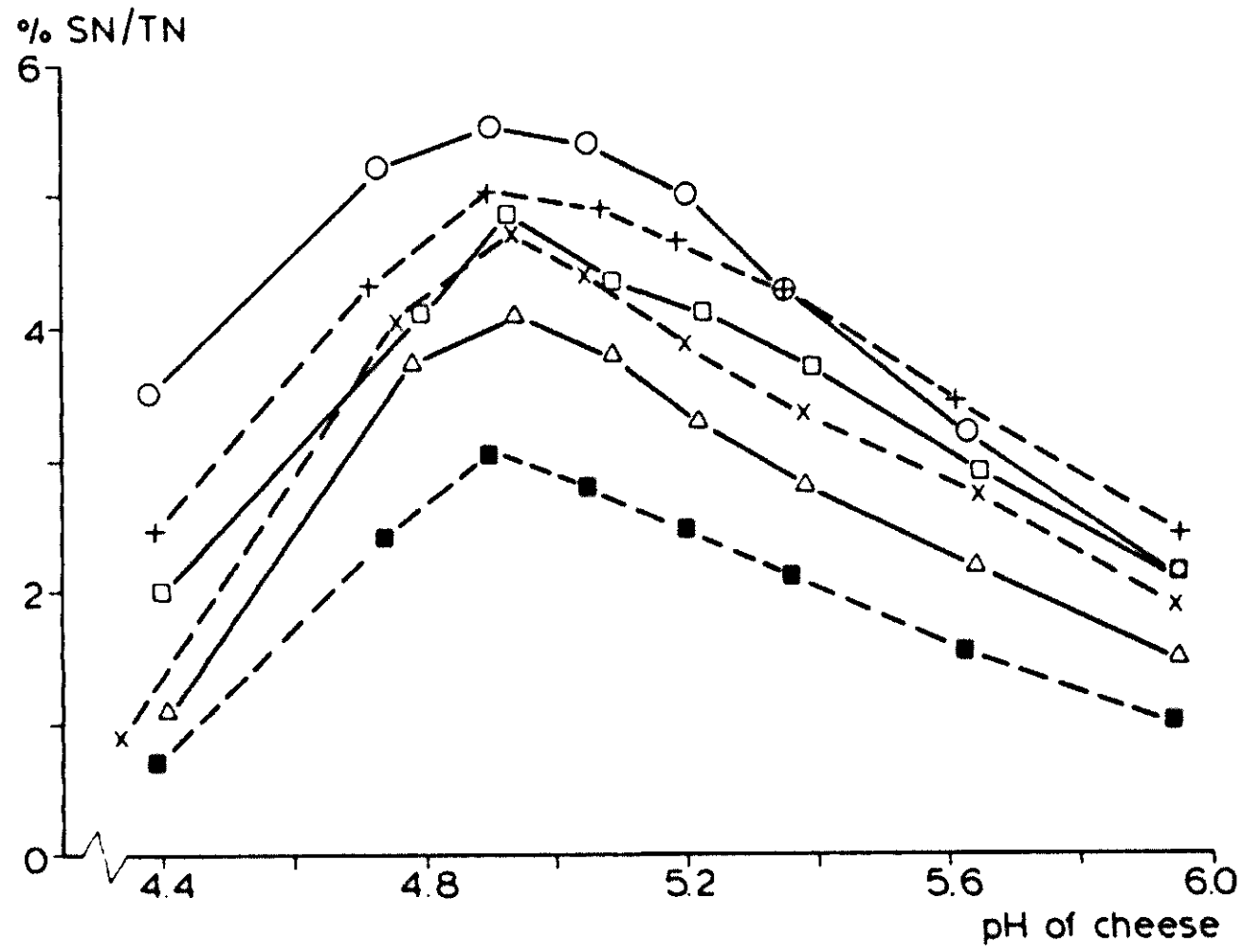

Fig. 1. Proteolytic activity of rennet in cheeses prepared with a milk protease-free paracasein complex, depending on the pH and the NaCl concentration in the moisture of the cheese. Protein breakdown was estimated by the amount of soluble nitrogenous compounds, expressed as a percentage of the total nitrogen of the cheese. The cheeses were stored for 14 days at 13 °C. NaCl concentrations: (○——○): 0 %; (+ — — +): 2 %; (□ —— □): 3 %; (× — — ×): 4 %; (△——△): 5 %; and (■ — — ■): 8 %.

soluble N and of amino acid N liberated. As shown by the values of % SN/TN, rennet had shown maximum activity at pH 4.9 - 5.0 at all NaCl concentrations tested, and this activity had decreased with increasing salt content. With all cheeses, the values of % AN/TN were found within the normal variation of values obtained with cheeses without rennet. This indicated that the formation of amino acids had been absent or negligibly small.

In some experiments the activity of rennet was also studied by gel electrophoresis and densitometry of gels. Densitometric calculations were made against blank values of a 0.5 % sodium paracaseinate solution. The influence of the pH was studied with a series of cheeses with pH values ranging from 4.85 to 5.75, and containing 4 % NaCl in the moisture (Fig. 2 and 3). At all pH values, α_{s1} casein appeared to be strongly degraded to α_{s1}-I casein. Rennet action against this casein had been maximally at pH 5.05. β casein was only very slightly degraded, the β-I band being hardly visible at the lowest pH values and becoming somewhat more distinct with decreasing acidity of the cheese. Densitometric calculations suggested a maximum in the breakdown of this casein at pH 5.5. At low levels of degradation, however, those results should be interpreted cautiously.

The influence of NaCl was studied with cheeses containing 0, 2, 3, 4, 5

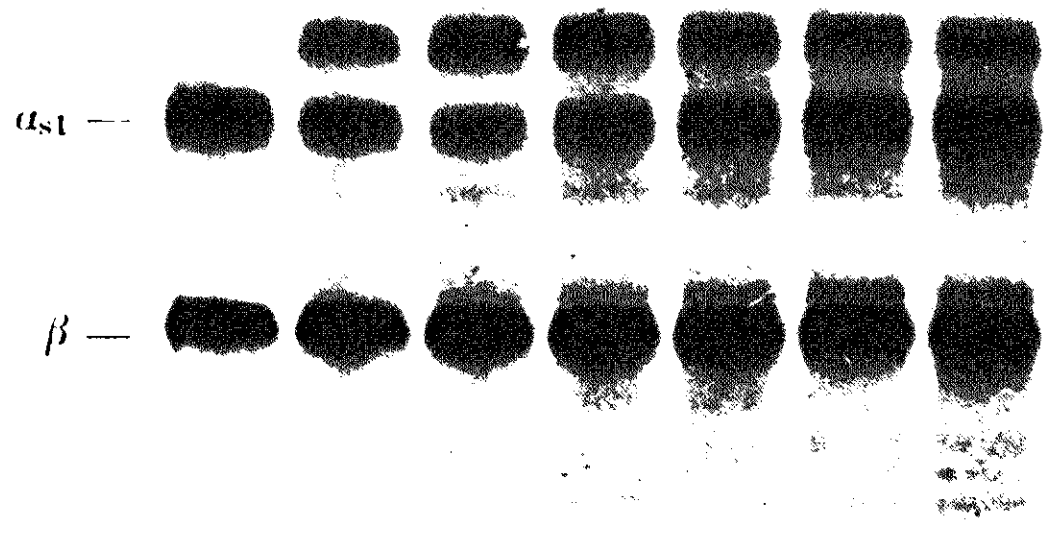

Fig. 2. Patterns of protein degradation by rennet in cheeses prepared with a milk protease-free paracasein complex, depending on the pH. The cheeses contained 4 % NaCl in the moisture and were stored for 14 days at 13 °C. Slot 1: sodium paracaseinate (blank); Slots 2 to 7 inclusive: patterns at pH 4.85, 5.05, 5.20, 5.30, 5.50 and 5.75, respectively.

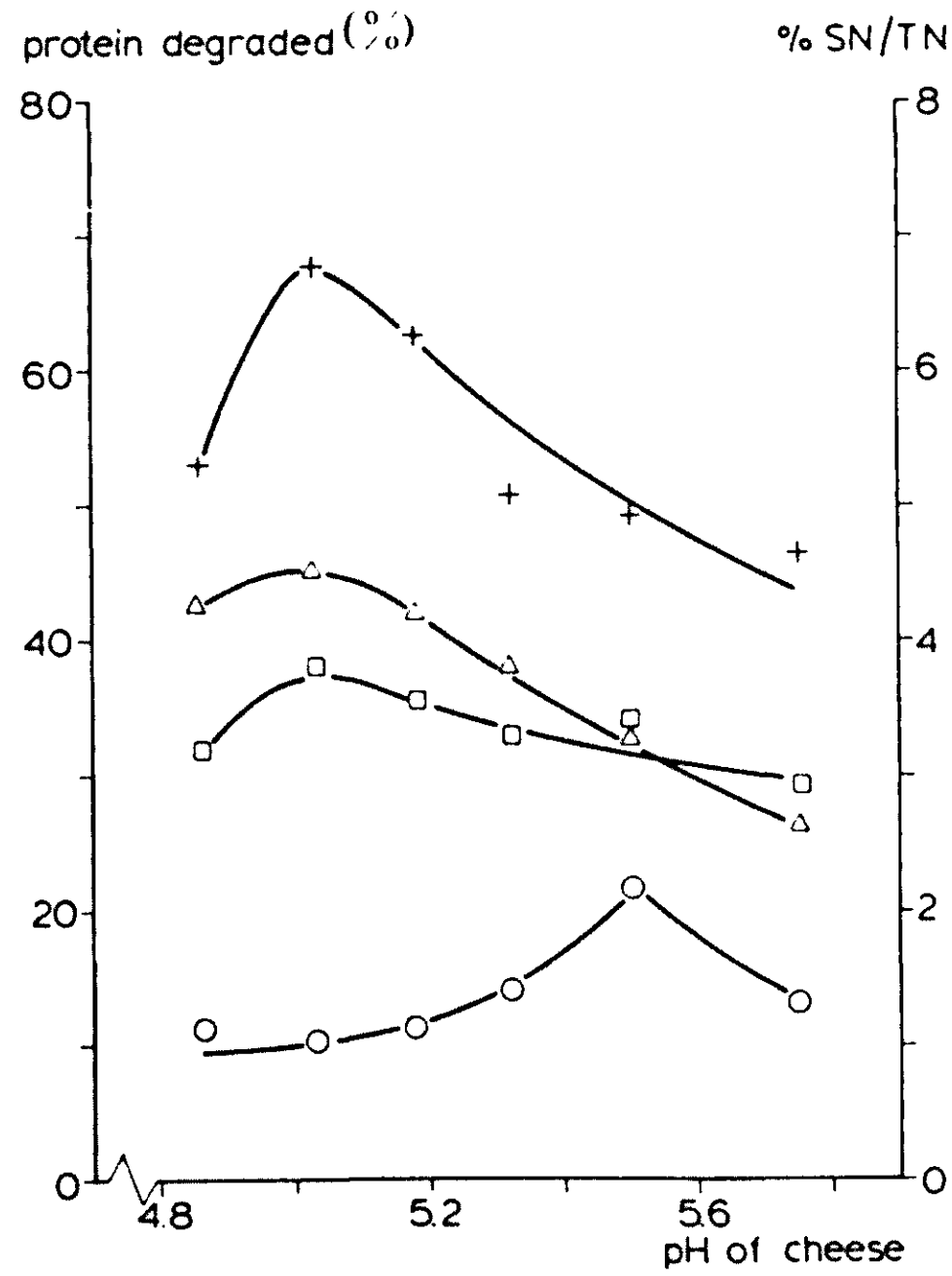

Fig. 3. Protein breakdown by rennet in the cheeses shown in Fig. 2, estimated by densitometry and by the amount of soluble nitrogenous compounds, expressed as a percentage of the total nitrogen of the cheese.
(+ —— +): α_{s1} casein; (○——○): β casein; (□ —— □): α_{s1} + β casein; (△——△): values of soluble N.

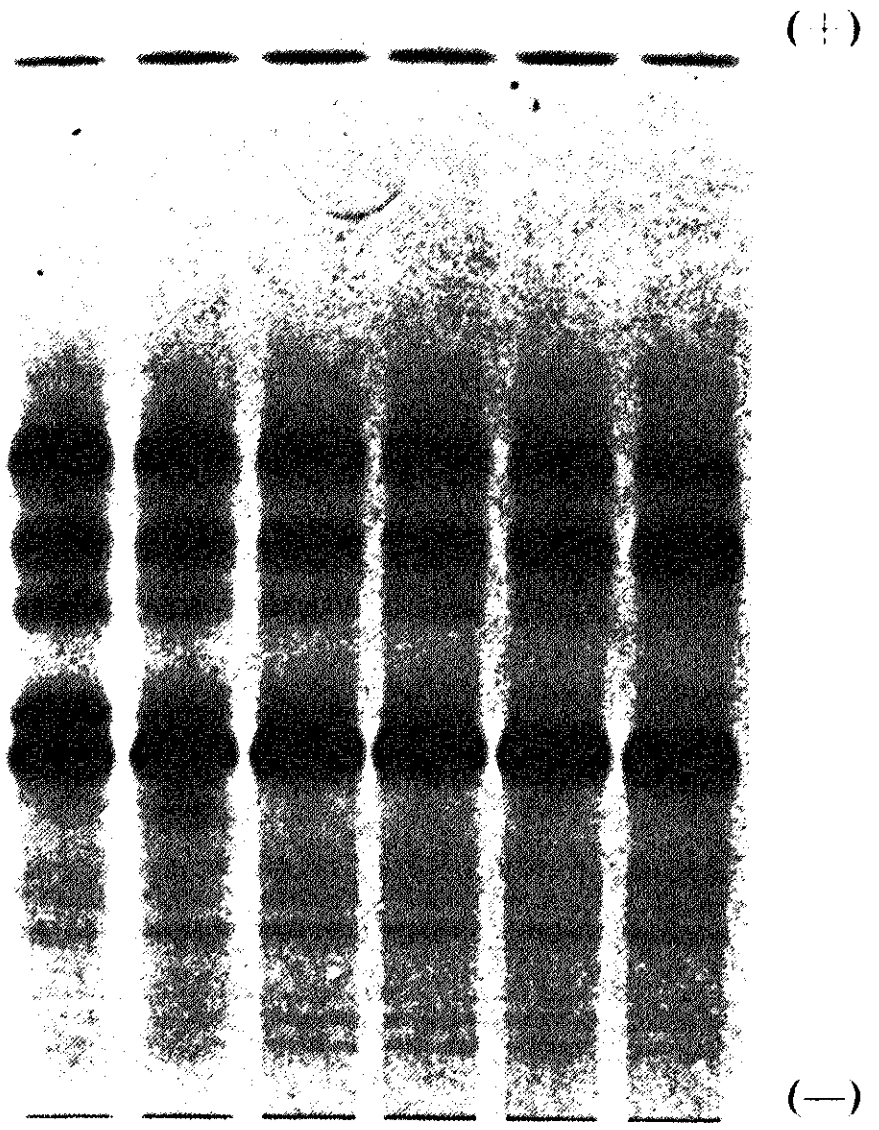

Fig. 4. Patterns of protein degradation by rennet in cheeses prepared with a milk protease-free paracasein complex at pH 5.0, depending on the NaCl concentration in the moisture of the cheese. Cheeses were stored for 14 days at 13 °C. Slots 1 to 6 inclusive: patterns at 0, 2, 3, 4, 5 and 8 % NaCl, respectively.

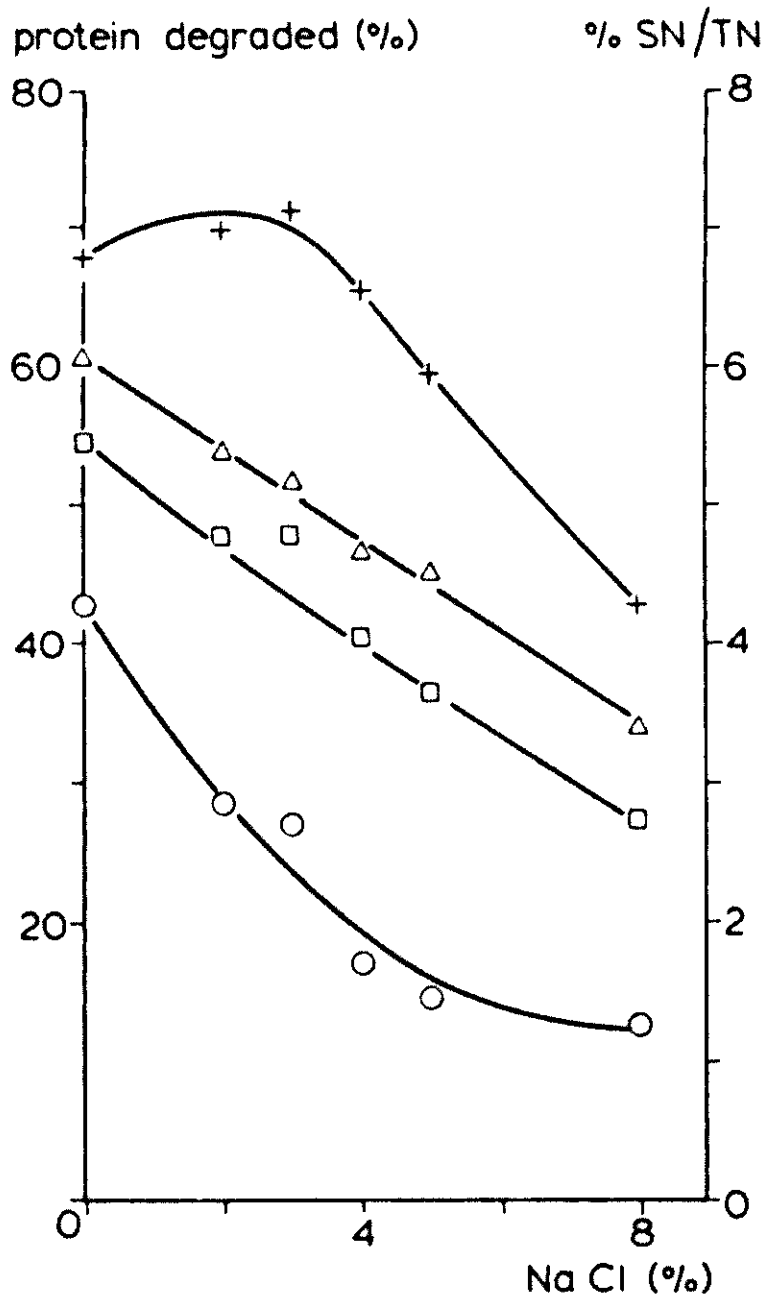

Fig. 5. Protein breakdown by rennet in the cheeses shown in Fig. 4, estimated by densitometry and by the amount of soluble nitrogenous compounds, expressed as a percentage of the total nitrogen of the cheese. (+ —— +): α_{s1} casein; (○ —— ○): β casein; (□ —— □): α_{s1} + β casein; (△——△): values of soluble N.

and 8 % NaCl in the moisture. The experiments were performed at pH 5.0, at which the differences in rennet activity were assumed to be most marked (see Fig. 1). The results are represented in the Figs 4 and 5. The degradation of α_{s1} casein showed a tendency to be stimulated by increasing NaCl concentrations up to about 4 %, and was reduced at higher concentrations. The breakdown of β casein was very considerable in the cheese without NaCl, but already decreased rapidly in the presence of low salt concentrations.

Both methods used to estimate protein degradation indicated that the total breakdown of protein had been at a maximum in the absence of NaCl and at a pH near to 5.0. The decrease of this breakdown at higher and lower pH values was predominantly caused by a decreased α_{s1} degradation, whereas the decrease at increasing NaCl concentrations up to about 4 % was due to the fact that the degradation of α_{s1} casein was less stimulated than that of β casein was inhibited by these salt contents. At higher salt concentrations a decreased breakdown of both caseins contributed to that (Figs 3 and 5).

The absence of milk protease activity in the cheeses demonstrated itself by the lack of formation of typical bands below the β casein band in all conditions tested (Figs 2 and 4).

The percentages of total degradation of protein as measured by densitometry were well correlated with the amounts of soluble N formed (ratio 8 : 1 to 9 : 1), as is also shown by the course of the relevant curves in Figs 3 and 5.

3.2 The simultaneous action of rennet and milk protease in cheeses made with a calcium paracaseinate-calcium phosphate complex prepared from low-temperature pasteurized milk

When compared to the conditions in cheese, in particular the severe heat treatment applied to the milk in the preparation of milk protease-free paracasein complex, there might have been some change in the susceptibility of the different caseins to degradation by rennet in cheeses made from it.

We found that milk protease was strongly associated with the paracasein complex made from low-temperature pasteurized milk (4). As a consequence, studies on rennet activity in simulated cheeses showing conditions which approach more nearly those existing in cheese, must be performed in the presence and eventually interfering action of milk protease. The contribution of rennet then must be calculated from the difference in the degree of protein degradation in cheeses which have been subjected to the action of milk protease and those which have experienced the simultaneous activities of milk protease and rennet. In our opinion, this was to be justified particularly under conditions at which milk protease activity had been very slight.

The first experiments were performed with cheeses made with a paracasein

complex prepared from low-temperature pasteurized milk. Cheeses of various pH values and NaCl concentrations in the moisture were prepared with and without rennet. Protein breakdown was estimated by the amount of soluble N. Results of an experiment with cheeses containing 4 % NaCl in the moisture are represented in Fig. 6.

As a result of milk protease activity the rennet-free cheeses showed a clear and increasing degradation of protein with decreasing acidity. In agreement with earlier results (4), the curve showed a distinct shoulder at pH values near to 5.4. The curve representing the combined activities of rennet and milk protease showed a maximum at a pH of about 5.2 and a minimum at a pH of about 5.5. This minimum was evidently caused by the fact that the decreasing activity of rennet at higher pH values was outrunned by the increasing activity of milk protease in those conditions. The activity of rennet, calculated by subtraction of the values of % SN/TN of cheeses with a corresponding pH, showed a maximum at pH 5.05. With similar experiments performed with cheeses containing 0, 2, 3, 5 and 8 % NaCl in the moisture,

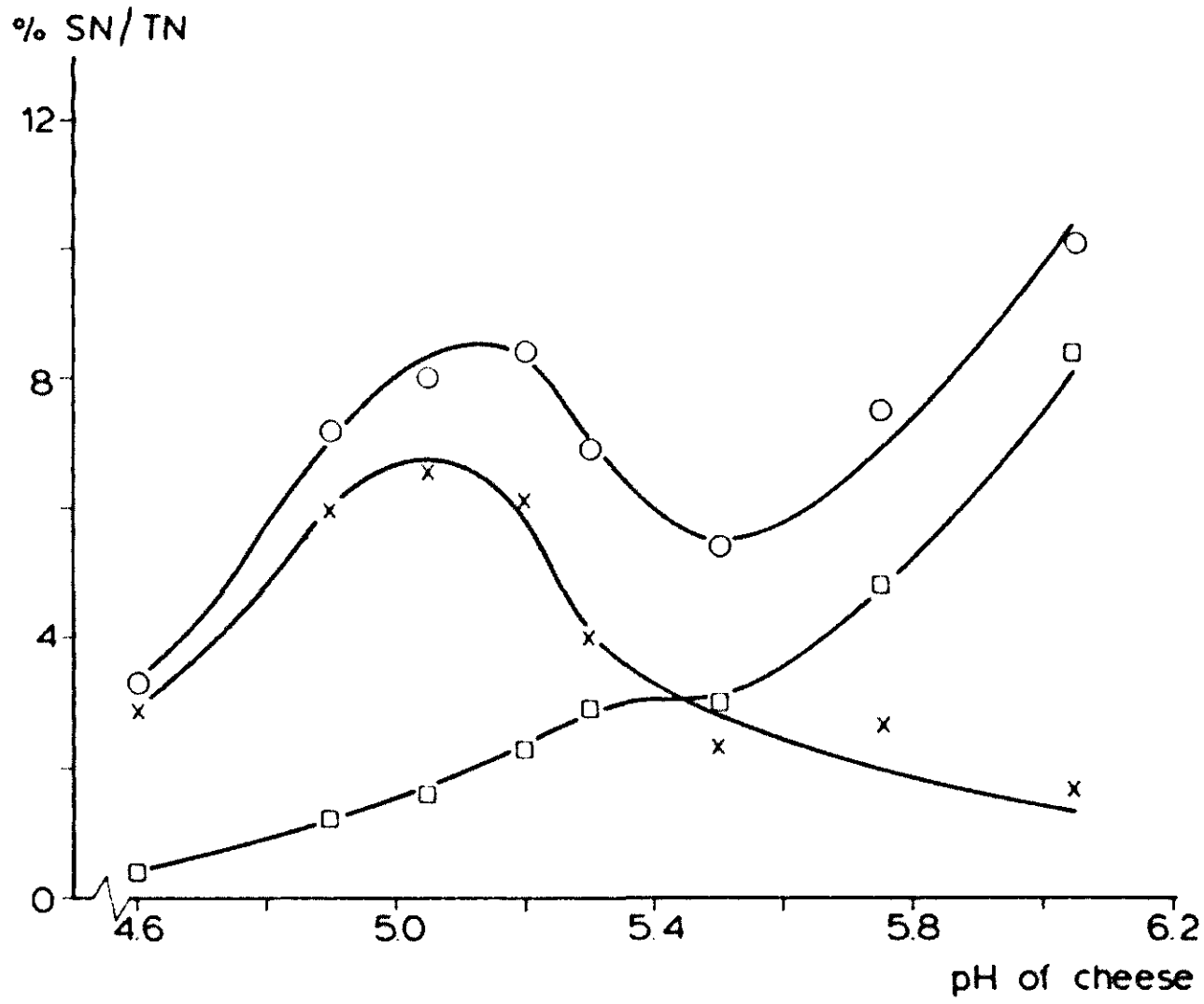

Fig. 6. Proteolytic activity of rennet and/or milk protease in cheeses prepared with a paracasein complex made from low-temperature pasteurized milk, depending on the pH. Protein breakdown was estimated by the amount of soluble nitrogenous compounds expressed as a % of the total nitrogen of the cheese. The cheeses contained 4 % NaCl in the moisture and were stored for 14 days at 13 °C. (○——○): simultaneous action of milk protease and rennet; (□——□): action of milk protease; (×——×): calculated action of rennet.

rennet activity was found to be at a maximum at pH 4.9 to 5.05, whereas protein breakdown decreased with increasing salt concentrations.

3.3 The simultaneous action of rennet and milk protease in cheeses prepared with 'curd'

Cheeses with and without rennet were made according to the directions given in Section 2.6. The influence of the pH on rennet activity was studied with cheeses containing 4 % NaCl in the moisture. The influence of the NaCl concentration in the moisture was studied at pH 5.0. At this acidity, rennet could be assumed to show a very high and milk protease a very low activity. The results are given in Figs 7, 8, 9 and 10.

As indicated by the values of % SN/TN, in cheeses without rennet milk protease had degraded the protein increasingly at lower acidities (Fig. 7A). The course of the curve representing the combined action of rennet and milk protease showed a tendency corresponding to that observed with cheeses

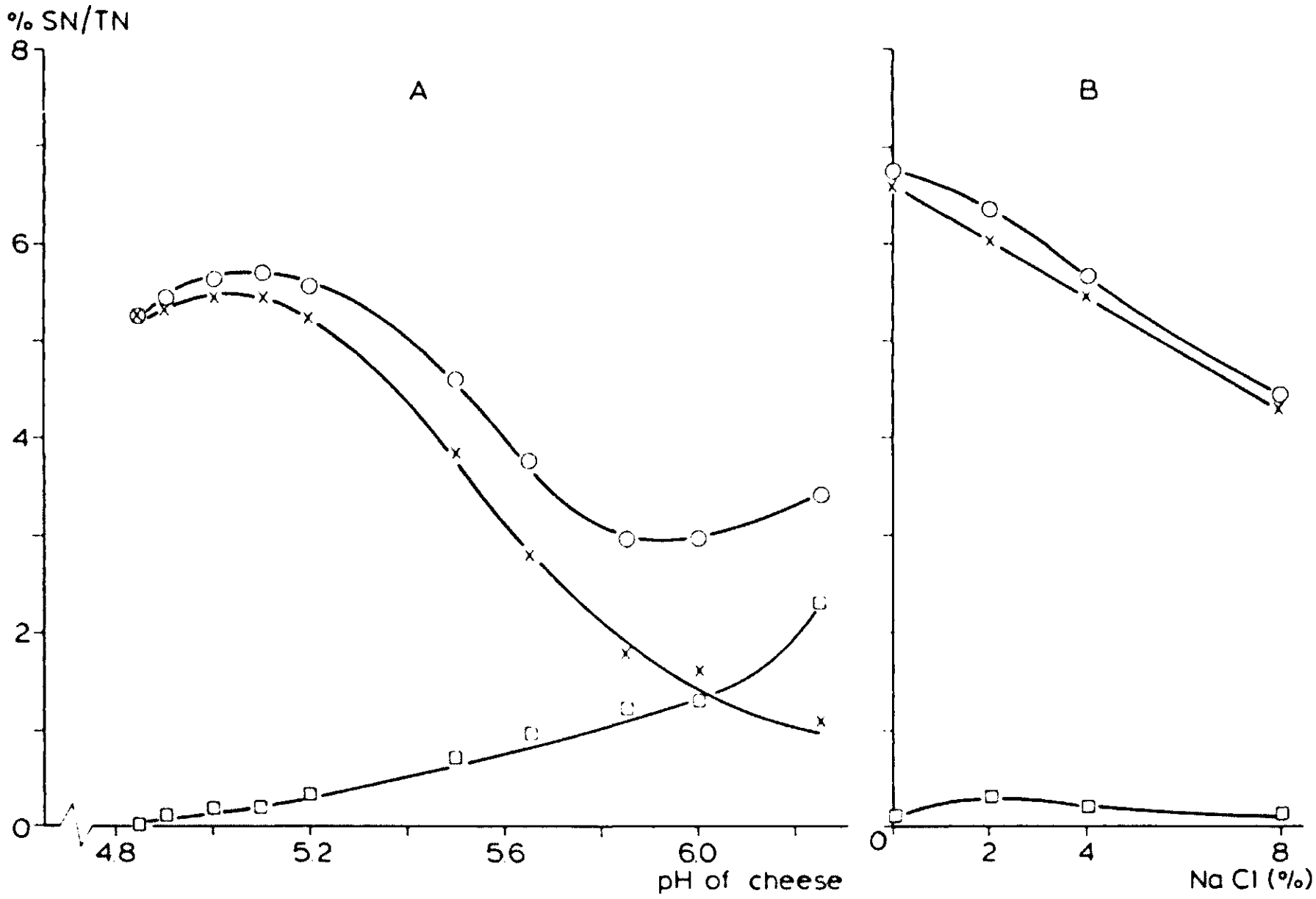

Fig. 7. Proteolytic activity of rennet and/or milk protease in cheeses prepared with 'curd', depending on the pH of cheeses containing 4 % NaCl in the moisture (A) and the NaCl concentration in the moisture of cheeses at pH 5.0 (B). Protein breakdown was estimated bij the amount of soluble nitrogenous compounds expressed as a percentage of the total nitrogen of the cheese. The cheeses were stored for 14 days at 13 °C. (○——○): simultaneous action of rennet and milk protease; (□——□): action of milk protease; (×——×): calculated action of rennet.

made with paracasein complex (Fig. 6). The curve showed a maximum at a pH of approximately 5.1 and a minimum near to pH 5.9. The situation of this minimum at a higher pH value than that estimated at pH 5.5 with cheeses made with paracasein complex can be explained by the greater activity of milk protease in the latter cheeses. The calculated activity of rennet showed its maximum at a pH of about 5 and decreased rapidly at higher pH values.

At pH 5.0, the activity of milk protease had been very small at all NaCl concentrations tested (Fig. 7B). In accordance with our earlier findings (4), this activity had been stimulated by low concentrations of NaCl, being at a maximum at 2 % NaCl. The results established that in the presence of rennet and milk protease, the protein had been less degraded according as the cheeses contained more NaCl in the moisture. The calculated activity of rennet showed the same tendency.

Patterns of protein degradation in the cheeses with rennet, as influenced by the pH, are shown in Fig. 8. Particularly at the lower pH values the densitometric results concerning the degradation of α_{s1} and β casein in the cheeses without rennet and of those of the breakdown of β casein in the cheeses with rennet were rather confusing as a result of the very weak breakdown of these caseins. This made it impossible to perform reliable calculations on the total degradation of protein by rennet. However, since β casein was scarcely broken down at low pH values, it could reasonably be assumed that the total breakdown under those conditions had been nearly determined by the magnitude of the degradation of α_{s1} casein. When related to that, rennet had shown its maximum activity at a pH of about 5 (Fig. 10A).

The cheeses without rennet did not show any detectable degradation of the caseins at any of the NaCl concentrations tested. In the cheeses with rennet (Figs 9 and 10B), the degradation of α_{s1} casein had been clearly stimulated by low salt concentrations. The degradation of β casein had been at a maximum at 0 % NaCl and became rapidly reduced at higher concentrations. As was also indicated by the amounts of soluble N (Fig. 7B), the total degradation of protein had decreased with increasing salt contents in the moisture. In the experiment described, this tendency was somewhat interrupted by the slightly deviating results obtained at 0 % NaCl.

A fairly constant ratio of 1 : 7 to 1 : 9 was found between the values of % SN/TN and the percentages of total protein degraded, as estimated by densitometry.

Other experiments generally confirmed the results mentioned above concerning the activity of rennet under various conditions (pH, NaCl content) in the cheeses. As an illustration, patterns of protein degradation by the combined action of rennet and milk protease in cheeses, made from an other

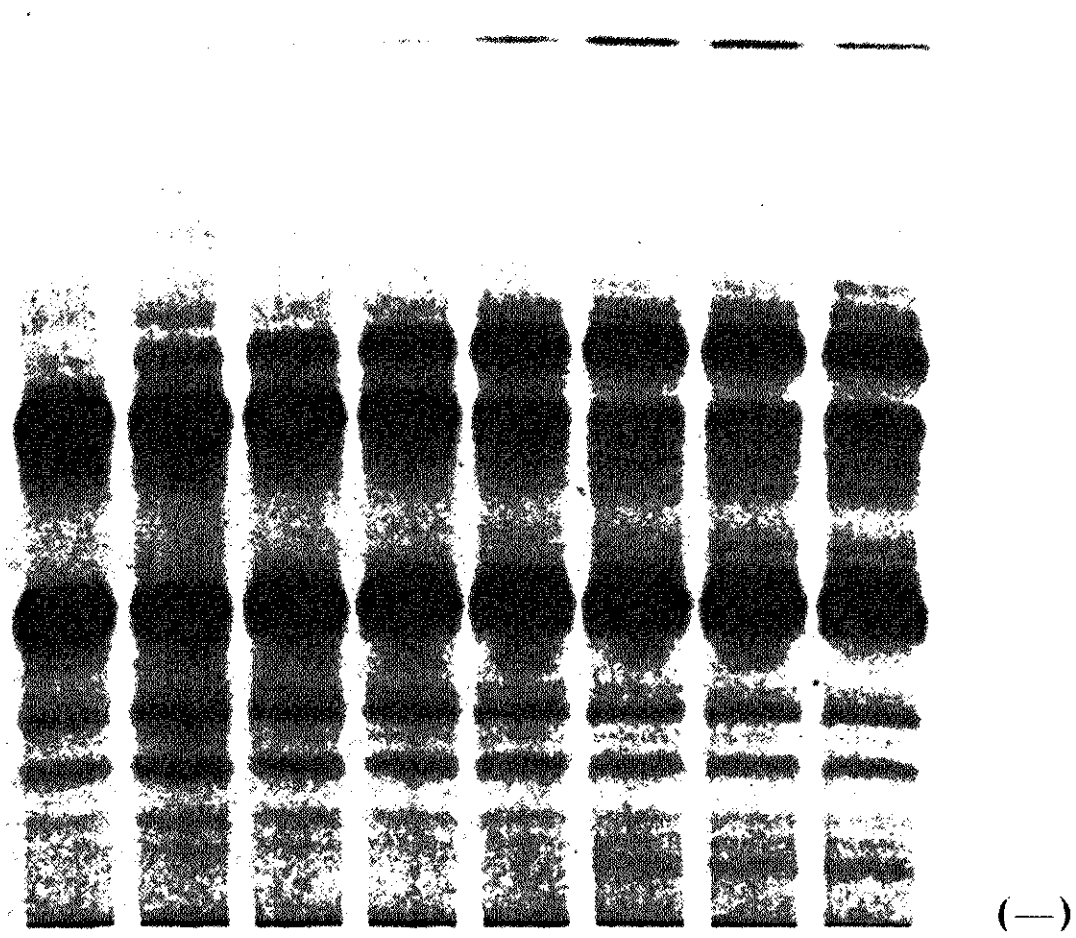

Fig. 8. Patterns of protein degradation in the cheeses with rennet of Fig. 7A. Slot 1: 'curd', as a blank. Slots 2 to 8 inclusive: patterns at pH 6.25, 5.85, 5.65, 5.20, 5.10, 5.0 and 4.85, respectively.

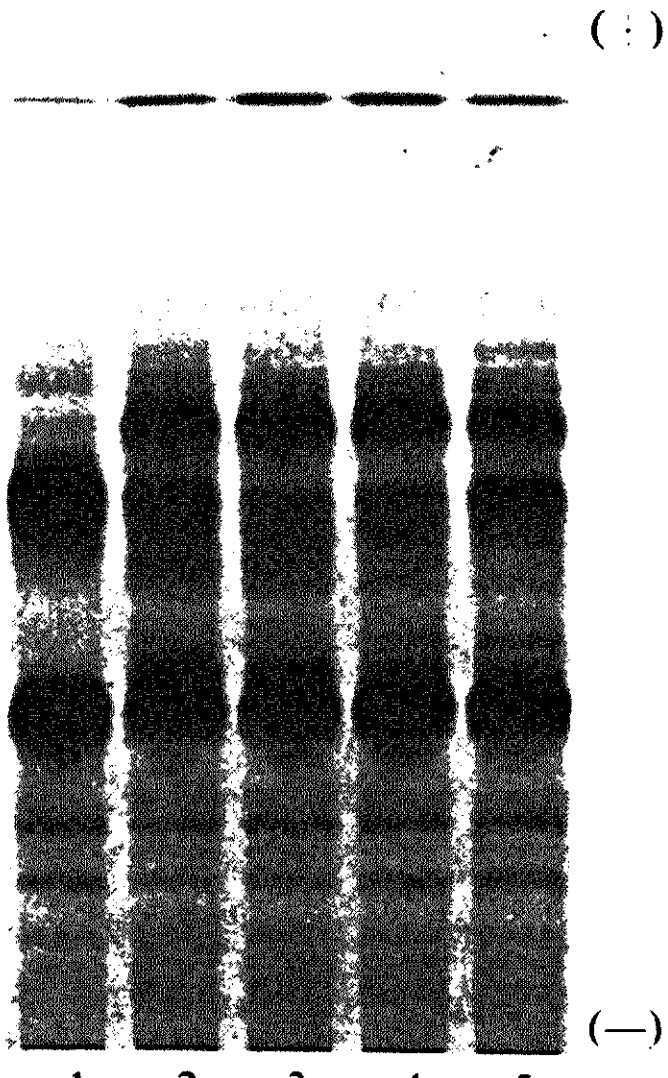

Fig. 9. Patterns of protein degradation in the cheeses with rennet of Fig. 7B. Slot 1: 'curd', as a blank. Slots 2 to 5 inclusive: patterns of cheeses containing 0, 2, 4 and 8 % NaCl in the moisture, respectively.

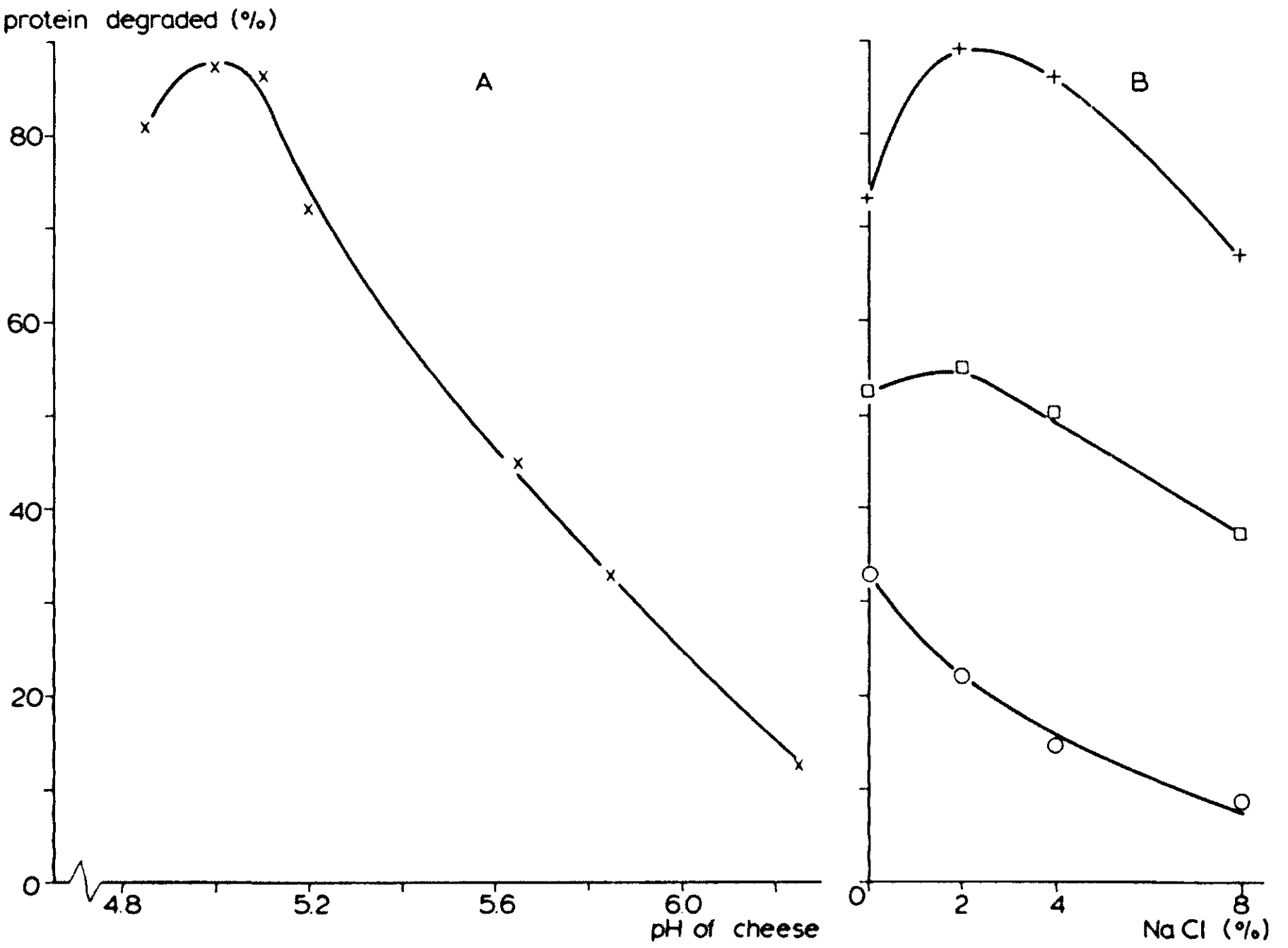

Fig. 10. Protein breakdown in the cheeses shown in Figs 8 (A) and 9 (B), as measured by densitometry. (×——×) and (+——+): α_{s1} casein; (○——○): β casein; (□——□): α_{s1} + β casein.

batch of 'curd' and stored for 28 days at 13 °C, are shown in Fig. 11. Visual observation of the gel already indicated that α_{s1} casein had become very strongly and increasingly degraded with increasing acidities of the cheese (Slots 2 - 8). Densitometric calculations revealed that more than 90 % of this casein had been broken down at pH values below 5.6. Since at a very low pH the action of milk protease against α_{s1} casein is very small when compared with that of rennet and, moreover, decreases with increasing high acidities (4), it could be deduced that rennet had shown its maximum activity against this casein at pH 4.9 to 5.0.

When related to the formation of bands in front of the β casein, rennet had been very weakly active against this casein. Electrophoretic patterns suggested that maximum breakdown had occurred at pH 5.4 - 5.6. However, pH values between 5.6 and 6.1 have not been tested. Moreover, β-I casein might have been degraded by milk protease, particularly at a higher pH.

Densitometric calculations indicated a decreasing breakdown of β casein with increasing acidity of the cheese. At the lower pH values the degradation

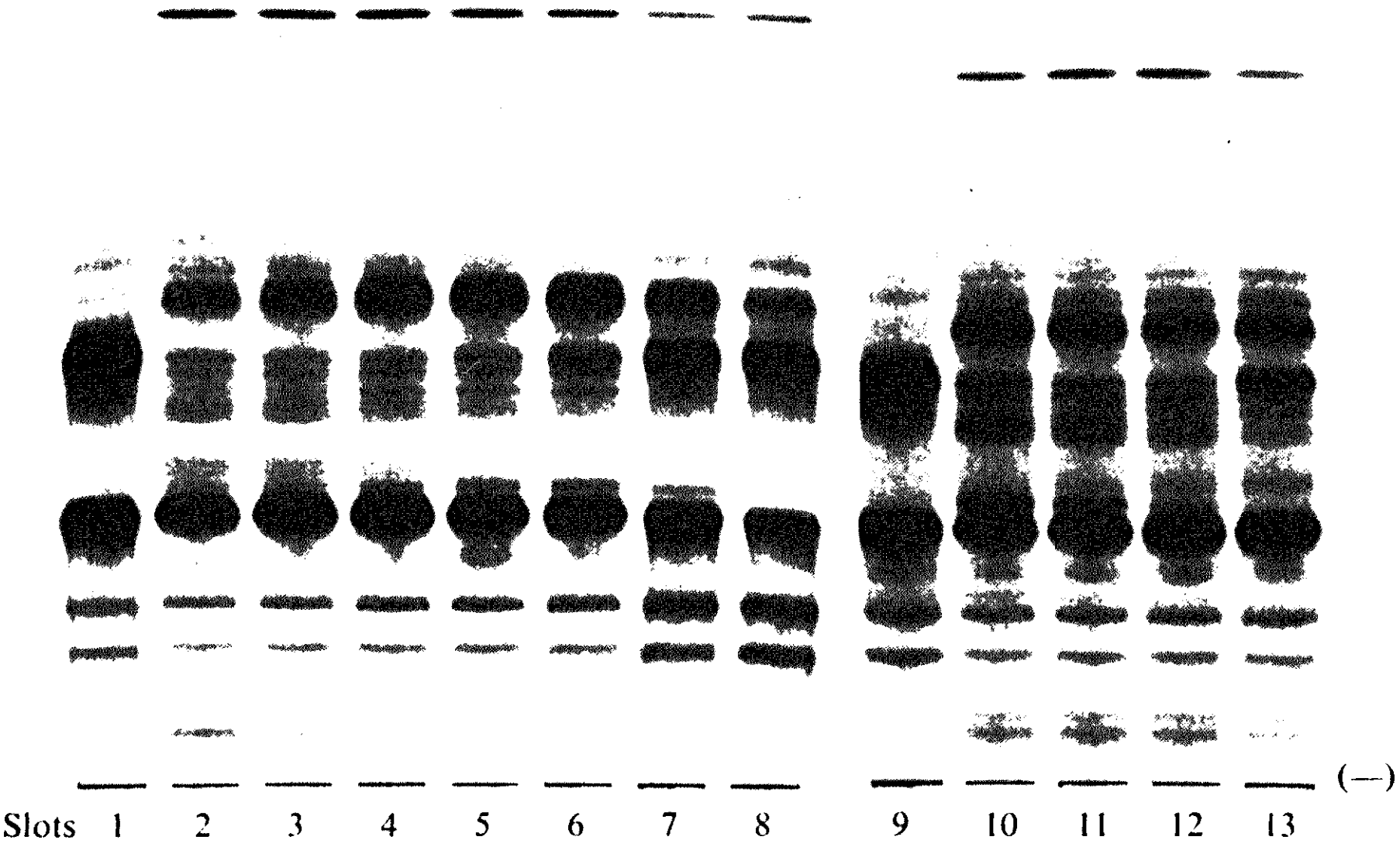

Fig. 11. Patterns of protein degradation by rennet and milk protease in cheeses made with 'curd', stored for 28 days at 13 °C under various conditions. Slots 1 and 9: 'curd', as a blank. Slots 2 to 8 inclusive: cheeses containing 4 % NaCl in the moisture, with pH values of 4.9, 5.0, 5.2, 5.4, 5.6, 6.1 and 6.3, respectively. Slots 10 to 13 inclusive: cheeses of pH 5.0, containing 0, 2, 4 and 8 % NaCl in the moisture, respectively.

had remained very weak. With respect to the total protein breakdown, it thus could be concluded that rennet action had been at a maximum at a pH of about 5. In accordance with that, the formation by rennet of soluble nitrogenous compounds was found to be maximal at that pH value.

Both methods used to follow protein degradation indicated the existence of a minimum in the total breakdown of protein. At the pH values 5.6, 6.1 and 6.3 the proportions of protein degraded were found to be 50, 45 and 55 %, whereas the values of % SN/TN amounted to 11.5, 9.9 and 12.4, respectively. The factors responsible for this minimum have already been reported in Section 3.2.

The influence of various NaCl concentrations on the degradation of protein (Fig. 11, Slots 10-13) corresponded to that observed with the cheeses of the previous experiment (Fig. 9). The degradation of α_{s1} casein had been stimulated in the presence of 2 and 4 % NaCl (92 % against 83 % in the cheese without NaCl) and had been less, but still very extensive, at 8 % NaCl (78 %). The degradation of β casein had decreased regularly from 34 % at 0 % NaCl to about 10 % at 8 % NaCl. The total breakdown amounted to about 60 % at 0, 2 and 4 % NaCl and 50 % at 8 % NaCl. When estimated

by the amounts of soluble N, rennet had degraded the protein at increasing NaCl concentrations to 10.48, 10.29, 10.01 and 7.63 %, respectively.

After one month of storage, the ratio between the values of % SN/TN and the percentage of protein degraded as measured by densitometry amounted to 1 : 4 to 1 : 6 under the various conditions of test.

4 Discussion

The proteolytic activity of rennet is greatly influenced by the nature of the substrate (3, 7, 8, 9, 10, 11, 12, 13). This factor, the different methods used for the estimation of protein degradation and, in all probability, differences in the composition of the rennets investigated, all account for the rather confusing data in the literature concerning the activity of rennet at different acidities and salt concentrations. The degradation of isolated α_{s1} and β casein and whole casein in solution has been reported to increase with increasing acidity (13), even down to pH 3.0 (14, 15). In pasteurized milk, rennet was found to be most active at pH 6.3 (16). As indicated by changes in the electrophoretic patterns in polyacrylamide gels, Fox (7) observed that in solutions of sodium caseinate, incubated at 4 and 32 °C, both α_{s1} and β casein were maximally degraded at a pH near to 5.8. Very variable results were found, however, when changes in the amount of non-protein nitrogen soluble in 2 % TCA were taken as the criterion for proteolysis. At 32 °C no optimum was observed down to pH 3.5, whereas at 4 °C a slight optimum was reached at pH 5.2. Fox (7) also observed that the degradation of α_{s1} casein was more pH dependent than that of β casein and that the latter casein, relative to α_{s1} casein, underwent greater proteolysis at lower rather than at higher temperatures. Using quantitative gel electrophoresis, de Jong & de Groot-Mostert (3) studied the breakdown of protein by commercial calf rennet (the same as used in our study) in solutions of sodium paracaseinate at 13 °C. In agreement with the observations of Fox (7), they also found a maximum degradation of α_{s1} and β casein at a pH of about 5.8. However, the degradation of β casein appeared to be more pH dependent than that of α_{s1} casein. At all acidities tested within the range pH 4.8 - 6.6, β casein was more extensively degraded than was α_{s1} casein. The presence of $CaCl_2$ and, in particular, that of $CaCl_2$ and NaCl, greatly influenced the rates of degradation of the different caseins. According to Stadhouders (17), paracasein complex in suspension (10 %) became increasingly degraded at increasing acidities in the absence of NaCl, whereas there was a maximum degradation at pH 5.2 in the presence of 3 % NaCl. In solutions of sodium caseinate and sodium paracaseinate, β casein degradation was found to be significantly reduced by already low

concentrations of NaCl, whereas the degradation of α_{s1} casein was less influenced by salt and maximum in the presence of NaCl concentrations up to about 5 % (3, 9).

Our experiments indicated that, apart from differences in a quantitative sense, rennet degraded the protein in a corresponding manner in the different simulated cheeses under comparable test conditions. In these cheeses, sodium chloride influenced the proteolytic activity of rennet in a manner largely corresponding to that reported in the literature. The degradation of α_{s1} casein was stimulated by NaCl concentrations in the moisture up to about 4 %, and inhibited at higher salt contents, whereas β casein was already considerably less degraded in the presence of 2 % NaCl. The total protein breakdown decreased however with increasing salt contents. With respect to the influence of the pH on the action of rennet, our results differed significantly from those obtained by other workers, the activity of rennet against α_{s1} casein being maximal at a pH of about 5; that against β casein did not become clear. Rennet was predominantly active against α_{s1} casein. Relative to this casein, β casein was only weakly degraded under most of the test conditions. This picture resembled very closely that of a ripening Meshanger cheese (18). For other types of cheese, it has also been reported that rennet degrades α_{s1} casein preferably to β casein (19, 20, 21, 22).

Calcium may effect the susceptibility of α_{s1} and β casein to degradation by rennet (3,8). In the simulated cheeses prepared with 'curd' and with paracasein complex made from low-temperature pasteurized milk, the calcium concentration amounted to 0.6 %. The concentration in the cheeses prepared with milk protease-free complex was 0.85 %. The difference in calcium content and, possibly, in its chemical conformation, may have contributed to the differences in the degrees of proteolysis of cheeses with corresponding pH and NaCl content, as reflected by the amounts of soluble N liberated (Figs 1, 6 and 7).

With respect to the total degradation of protein, the results obtained by the estimation of soluble N were well correlated with those obtained by the electrophoretic method. Discrepant results, observed or described by workers who used other analytical methods (7, 9), were not encountered by us. With cheeses stored for 14 days, the ratio between the results of both methods amounted to 1 : 7 to 1 : 9. After one month of storage this ratio was 1 : 4 to 1 : 6. This difference may be reasonably explained by the fact that in conditions in which intact α_{s1} casein was scarcely present any more or in which it became only slightly further degraded, the pool of soluble nitrogenous substances remained continuously fed by the breakdown of insoluble – to soluble degradation products. As was also found in our previous study on milk prote-

ase activity (4), the results of the present study supported the usefulness of the soluble N fraction obtained by our extraction method as an index of protein degradation in cheese (23).

As could be expected, rennet did not liberate amino acids in any detectable amount.

Protein breakdown in Meshanger cheese is predominantly determined by the activity of rennet. The cheese ripens from the inside to the outside and the weakening of the cheese body was found to be very closely related to the degree of protein degradation (1). Our study established that under the conditions (pH, NaCl content in the moisture) which exist during the greater part of the ripening period of the cheese, rennet degrades α_{s1} casein very extensively whereas β casein is only weakly attacked. Since the degradation of α_{s1} casein has been found to be the main factor responsible for the changes in the consistency of the cheese (24), the characteristic and complicated way of softening of the cheese can now be easily explained. The decrease of the pH progresses most rapidly in the interior of the cheese. This factor, together with the moderate salt content, produces very quickly in the centre of the cheese those conditions which are most favourable for the action of rennet against α_{s1} casein.

In addition to proteolysis, the changes of cheese consistency are influenced by the physical and chemical conditions in the cheese. In a previous report we discussed in particular the contribution of rennet to the ripening of soft cheeses with a surface flora, showing the visual ripening from the outside to the inside (1). The hypothesis was advanced that at low pH values rennet may degrade the protein in a way and to an extent which potentially allows the cheese to become soft, but that the weakening only occurs when the pH has been raised as a result of the de-acidifying activity of the surface flora. The results concerning the degradation of α_{s1} casein in the simulated cheeses strongly supported this conception. At pH 4.8, at which Meshanger cheese shows a firm and short consistency, this casein was degraded almost as much as at, for example, pH 5.2, at which the cheese shows a soft appearance (Figs 3 and 10A).

Acknowledgment

The author owes many thanks to Mrs G. C. van Roekel-Jansen, Miss A. P. Stasse and Mrs A. E. A. de Groot-Mostert for technical assistance.

Samenvatting

A. Noomen, *De activiteit van proteolytische enzymen in kunstkaasjes met een hoog vochtgehalte (Meshanger type). 2. De activiteit van kalfsstremsel*

Met behulp van kunstkaasjes werd een onderzoek verricht naar de proteolytische activiteit van kalfsstremsel in zachte kaas onder verschillende omstandigheden. De kaasjes werden samengesteld met (1) een calciumparacaseïnaat-calciumfosfaat-complex, bereid uit melk waarin melkprotease door een hittebehandeling onwerkzaam was gemaakt, of met (2) een uit laaggepasteuriseerde melk bereid calciumparacaseïnaat-calciumfosfaat-complex, of met (3) stremselvrije kaaswrongel. De twee laatstgenoemde kaasjes bevatten melkprotease. De kaasjes bevatten ongeveer 2,2 ml stremsel per kg eiwit, hetgeen ongeveer 50 % meer was dan de concentratie in een rijpe Meshanger kaas. De eiwitafbraak werd bestudeerd met behulp van kwantitatieve polyacrylamide-gelelectroforese en door bepaling van de gevormde hoeveelheid opgeloste stikstofverbindingen. De met beide methoden verkregen resultaten aangaande de totale eiwitafbraak bleken goed gecorreleerd te zijn.

Afgezien van verschillen in kwantitatieve zin bleek het stremsel in de verschillende kaasjes overeenkomstig werkzaam te zijn, en onder gunstige omstandigheden het eiwit in zeer sterke mate af te breken. Zo werd bijvoorbeeld in kaasjes met pH 5,0 en 4 % NaCl in het vocht 35 tot 50 % van het totale eiwit (65-90 % van het α_{s1}-caseïne) erdoor afgebroken tijdens een bewaring gedurende 14 dagen bij 13 °C. Onder alle omstandigheden werd α_{s1}-caseïne veel sterker afgebroken dan β-caseïne.

Ten opzichte van α_{s1}-caseïne was de activiteit van het stremsel maximaal bij een pH van ca. 5. De resultaten lieten geen conclusie toe aangaande de pH waarbij β-caseïne het sterkst werd afgebroken. De totale eiwitafbraak was het grootst bij een pH van 4,9 tot 5,0.

De afbraak van α_{s1}-caseïne nam toe bij lage zoutgehalten in het vocht, en nam weer af bij concentraties boven de ongeveer 4 % NaCl. De afbraak van β-caseïne verliep maximaal bij afwezigheid van NaCl en werd reeds sterk geremd bij lage zoutconcentraties. Dit kan onze waarnemingen verklaren dat de totale eiwitafbraak verminderde bij een toenemend zoutgehalte.

Onder de getoetste omstandigheden vormde het stremsel geen aminozuren in een meetbare hoeveelheid.

Onder de omstandigheden in een rijpende Meshanger kaas breekt het stremsel α_{s1}-caseïne in zo grote mate af dat de hoofdrijping van de kaas erdoor wordt veroorzaakt. Omdat er ook is vastgesteld dat de in de kaas optredende consistentieveranderingen zeer sterk verband houden met de mate van afbraak van α_{s1}-caseïne, kan de karakteristieke en gecompliceerde, van binnen naar buiten voortschrijdende, vervloeiing van de kaas worden verklaard. Tijdens de rijping zijn de meest gunstige omstandigheden voor de afbraak van α_{s1}-caseïne door het stremsel (pH, zoutgehalte) namelijk het snelst aanwezig in het midden van de kaas. In Meshanger kaas wordt β-caseïne slechts zeer weinig afgebroken.

De resultaten van het onderzoek ondersteunden in sterke mate onze mening dat stremsel even wezenlijk is voor de rijping van andere kaassoorten met een hoog vochtgehalte. Dit betreft zowel kazen zonder een oppervlakteflora zoals Butterkäse en St. Paulin, als kazen met een oppervlakteflora zoals Kernhem, Brie, Camembert en Limburger.

References

1. A. Noomen, *Neth. Milk Dairy J.* 31 (1977) 75-102.

2. M. L. Green, *J. Dairy Res.* 44 (1977) 159-188.
3. L. de Jong & A. E. A. de Groot-Mostert, *Neth. Milk Dairy J.* 31 (1977) 296-313.
4. A. Noomen, *Neth. Milk Dairy J.* 32 (1978) 26-48.
5. G. Kleter & Tj. de Vries, *Neth. Milk Dairy J.* 28 (1974) 212-219.
6. F. M. W. Visser, *Neth. Milk Dairy J.* 30 (1976) 41-54.
7. P. F. Fox, *J. Dairy Sci.* 52 (1969) 1214-1218.
8. P. F. Fox, *J. Dairy Res.* 37 (1970) 173-180.
9. P. F. Fox & B. F. Walley, *J. Dairy Res.* 38 (1971) 165-170.
10. P. F. Fox & J. Guiney, *J. Dairy Res.* 40 (1973) 229-233.
11. J. A. Phelan, J. Guiney & P. F. Fox, *J. Dairy Res.* 40 (1973) 105-112.
12. L. K. Creamer, *N. Z. Jl Dairy Sci. Technol.* 6 (1971) 91.
13. R. A. Ledford, J. H. Chen & K. R. Nath, *J. Dairy Sci.* 51 (1968) 792-794.
14. T. Itoh, *Milchwissenschaft* 27 (1972) 470-473.
15. J. J. Tam & J. R. Whitaker, *J. Dairy Sci.* 55 (1972) 1523-1531.
16. N. P. Melachouris & S. L. Tuckey, *J. Dairy Sci.* 47 (1964) 1-7.
17. J. Stadhouders, *Proc. 16th int. Dairy Congr.* B (1962) 353-361.
18. L. de Jong, *Neth. Milk Dairy J.* 30 (1976) 242-253.
19. M. L. Green & P. M. D. Foster, *J. Dairy Res.* 41 (1974) 269-282.
20. J. C. Gripon, M. J. Desmazeaud, D. le Bars & J. L. Bergère, *Lait* 55 (1975) 502-516.
21. R. B. O'Keeffe, P. F. Fox & C. Daly, *J. Dairy Res.* 43 (1976) 97-107.
22. F. M. W. Visser, *Neth. Milk Dairy J.* 31 (1977) 247-264.
23. A. Noomen, *Neth. Milk Dairy J.* 31 (1977) 163-176.
24. L. de Jong, *Neth. Milk Dairy J.* 31 (1977) 314-327.

Neth. Milk Dairy J. 29 (1975): 153-161

Proteolytic activity of milk protease in raw and pasteurized cow's milk

A. Noomen

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

Received: 15 May 1975

Summary

A sensitive method is described for the estimation of proteolytic activity of milk protease in cow's milk. Samples of aseptically drawn milk with colony counts of less than 30/ml showed considerable proteolysis when incubated anaerobically for 3 or 6 days at 37 °C. Proteolytic activity was increased by 30 – 40 % when milk was pasteurized for 15 s at 72 °C and by 8 – 24 % when a heat treatment for 30 min at 63 °C was applied. Results obtained with the method always correlated very well with those found by PAE analysis. Both α_s- and β-casein were attacked by milk protease; β-casein was degraded two to three times faster than α_s-casein. It is suggested that the contribution of milk protease to protein breakdown in cheese is of much more interest than it is frequently thought to be. Experiments are continuing in this direction.

1 Introduction

Several workers have contributed to the evidence that cow's milk contains a proteolytic enzyme of non-bacterial origin (1–4).

The proteolytic activity of milk protease in cow's milk is generally assumed to be very weak. This may be attributed to the presence of the enzyme in very small amounts or to its occurrence in an inactive form, or to the presence of a protease inhibitor as demonstrated by Kiermeier & Semper (2). However, it must also be taken into consideration that the low values normally found for proteolytic activity can be caused by insufficient sensitivity of the methods used for the estimation of that activity. In this connection, Reimerdes & Klostermeyer (4) recently demonstrated the frequently used method, based on the determination of liberated tyrosine in a given reaction mixture, to be less suitable. This was attributed to the fact that considerable amounts of compounds containing non-aromatic amino acids were not detected. These compounds were formed by the action of milk protease on β-casein, the preferred substrate under the experimental conditions used by these authors.

At the moment, an extensive programme is being carried out at our laboratory concerning the contribution of proteolytic enzymes of bacterial and non-bacterial origin to protein breakdown in cheese. For this purpose, a simple and reliable method has been worked out for the estimation of the degree of ripening of cheese. After some modification, the method could be equally well applied for the determination of proteolysis in milk. It was thought to be of interest to study the proteolytic activity of milk protease in cow's milk with this modified method. Investigations into the possible effect of different heat treatments of the milk on that activity were also involved. With regard to this aspect, two pasteurization methods were chosen, namely the vat pasteurization for 30 min at 63 °C and the flash pasteurization for 15 s at 72 °C, the latter one being the heat treatment normally applied to milk in the Dutch cheese industry.

In this paper the results of these preliminary investigations are reported.

2 Material and methods

2.1 Aseptically drawn milk

On one occasion, samples from individual cows were obtained from the dairy herd of the University. On five other occasions bulk samples were taken from a limited number of cows (usually 3 – 5). All cows belonged to the Friesian breed. Milk was drawn aseptically, according to the methods described by Kleter & de Vries (5).

2.2 Treatment of milk samples

Samples from individual cows were handled without further heat treatment as indicated below. Bulk milk samples were usually divided into three portions. One portion was pasteurized in a laboratory pasteurizer for 15 s at 72 °C, and the second one for 30 min at 63 °C. After pasteurization, the milk was quickly cooled down to room temperature. The third portion of milk was left unheated.

All samples of raw and pasteurized milk were analysed for total colony count and pH. In the raw milk samples total nitrogen (TN) was also estimated, and this value was considered to be the same in the corresponding pasteurized samples. A small quantity of each of the raw milk samples was frozen at —20 °C, later on serving as a blank in gel-electrophoretical experiments.

From a given sample of milk, 10-ml aliquots were transferred to six weighed tubes, provided with aluminium caps and each containing a weighed amount (1 ml) of a solution of thimerosal in water (0.11 %). So, the mixture

contained 100 mg thimerosal per litre as a preservative. Preliminary experiments showed that enzyme activity was not influenced by this concentration. Subsequently, the tubes were weighed again, permitting the weight of added milk and thus the amount of nitrogen present in each tube to be calculated. The contents of each of two tubes were analysed immediately for soluble nitrogen, with the method described in Section 2.4.1. The remaining four tubes were incubated at 37 °C for a given period of time under anaerobic conditions, created by the BBL GasPak system (BBL, Division of Bio Quest, Cockeysville, Maryland 21030, USA). After incubation, the contents of each of two tubes were analysed for total colony count, pH and soluble nitrogen. The remaining two tubes were kept at —20 °C, their contents being used afterwards for the examination of gel-electrophoretical properties.

An aseptic technique was applied throughout the experiments where necessary.

2.3 Estimation of total nitrogen

A weighed amount of 5 ml of milk was gently heated with 10 ml HCl (25 %). After cooling, the solution was transferred quantitatively to a 100-ml volumetric flask and made up to this volume with distilled water. Nitrogen was estimated in 2 ml of the solution by the micro-Kjeldahl method, with HgO and K_2SO_4 as catalysts.

2.4 Estimation of soluble nitrogen in milk samples

2.4.1 Non-incubated samples. A tube containing the sample to be tested was provided with 0.004 ml commercial rennet (1 ml of a freshly prepared 1 : 250 dillution in distilled water), and the contents of the tube were well mixed. Next, the tube was placed in a thermostatically controlled water bath at 30 °C for 30 min. The coagulum formed was transferred quantitatively to a glass beaker, with 0.037 *M* $CaCl_2$ solution as a rinsing agent (5.5 g $CaCl_2 \cdot 2H_2O$/1000 ml water). The mixture was homogenized for 5 min with a Sorvall omni-mixer, after which the homogenate was transferred quantitatively to a 100-ml volumetric flask and made up to this volume with the $CaCl_2$ solution mentioned above. The pH of the suspension, brought to about 30 °C, was adjusted to a final value of about 7.5 by the addition of 1 *N* NaOH. The reaction mixture, showing a heavy precipitate of coagulated protein, was centrifuged for 10 min at 40 000 *g* (25 – 30 °C). The supernatant was filtered and nitrogen was estimated in the filtrate by the micro-Kjeldahl method. Soluble nitrogen (SN) was calculated as in a percentage of the total nitrogen (% SN/TN).

A more detailed description of this method will be given in subsequent reports on cheese ripening.

Table 1. Proteolytic activity of milk protease in individual cow's milk. Incubation 3 days at 37 °C.

Cow No	% SN/TN		Proteolytic activity
	before incubation	after incubation	
1	25.13	35.74	10.61
2	23.10	33.69	10.59
3	22.43	32.70	10.27
4	21.00	28.99	7.99
5	21.25	27.20	5.95
6	20.25	26.09	5.84

2.4.2 Incubated samples. From each tube a quantity of 1 ml was removed for the estimation of total colony count, causing a decrease in the amount of nitrogen present in the tube, and the remaining nitrogen was calculated. After pH measurement the sample was treated further as described in Section 2.4.1.

Proteolytic activity in a sample was expressed as the difference in % SN/TN after and before incubation.

2.5 Polyacrylamide gel electrophoresis (PAE)

Changes of protein in test samples by PAE and densitometry of the gels were studied according to the methods described by de Jong (6).

3 Results and discussion

The results are listed in Tables 1 and 2. Total colony counts and pH values of samples are not recorded. In freshly drawn milk, colony counts never exceeded 30/ml, while in incubated milk they were 0/ml without exception. It can thus be concluded with certainty that proteolysis was not caused by enzymes of bacterial origin, but by the activity of milk protease only.

Before incubation the pH values of raw and pasteurized milk samples were between 6.63 and 6.70, and after incubation these values were found to be lowered by about 0.05 pH unit.

As can be seen from Table 1, samples of individual cow's milk showed a distinct proteolytic activity under experimental conditions used. The activity varied considerably among individual cows, which has also been reported by other authors (1, 2, 7).

Gel-electrophoretic patterns of protein degradation in individual cow's milk are shown in Fig. 1. In accordance with the literature (7, 8), both α_s- and β-casein were attacked by the enzyme. It was established by densitometry

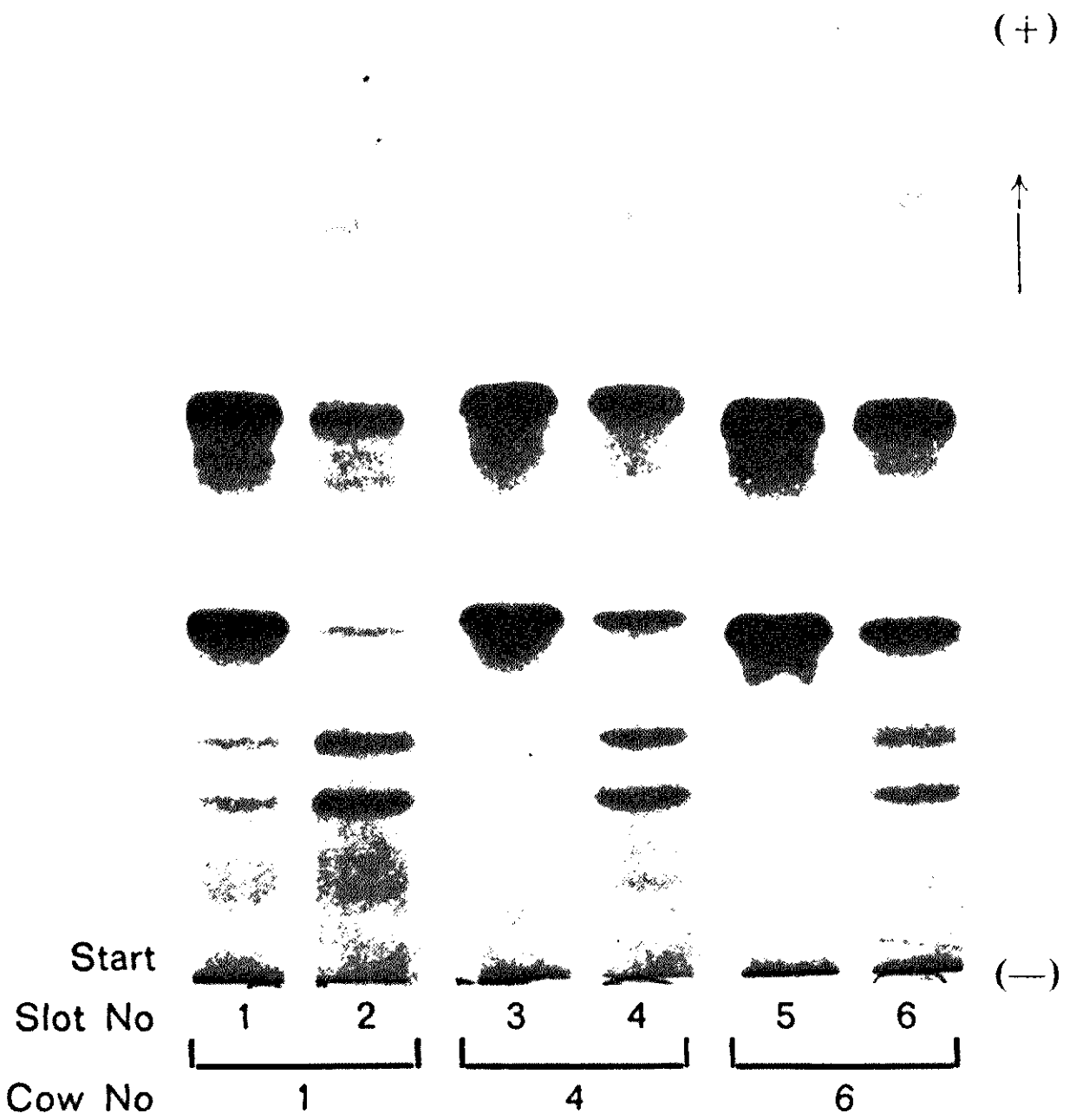

Fig. 1. PAE patterns of protein degradation in milk obtained from individual cows. Slot 1, 3 and 5: non-incubated samples. Slot 2, 4 and 6: samples incubated for 3 days at 37 °C.

of the gels that β-casein was degraded two to three times faster as α_s-casein, among other things resulting in the formation of typical bands below the β-casein band as was also found by Yamauchi & Kaminogawa (7).

With regard to the magnitude of proteolysis, densitometric evaluation of the gels showed the same tendency as was established with the method described in Section 2.4.1.

The results obtained with bulk milk samples are given in Table 2 and Fig. 2. Raw milk samples showed the same behaviour with respect to proteolytic activity and gel-electrophoretic patterns as those obtained from individual cows. In pasteurized samples, proteolytic activity proved to be increased significantly by a pasteurization of the milk for 15 s at 72 °C (30 – 40 %) and, although to a somewhat less extent, by a pasteurization for 30 min at 63 °C (8 – 24 %). As far as we know this phenomenon has not been mentioned earlier in the literature. A clear explanation for this stimulation of enzyme activity by moderate heat treatments cannot be given at the moment. A probable reason might be a partial inactivation of a protease inhibitor, which has been shown to be a natural constituent of cow's milk (2).

Generally, proteolytic activity of milk protease in cow's milk is considered

Table 2. Proteolytic activity of milk protease in bulk cow's milk; raw, pasteurized for 30 min at 63 °C and for 15 s at 72 °C, respectively. Samples were incubated at 37 °C.

Sample No.	Incubation time (days)	% SN/TN						Proteolytic activity		
		raw		30 min 63 °C		15 s 72 °C		raw	30 min 63 °C	15 s 72 °C
		before incubation	after incubation	before incubation	after incubation	before incubation	after incubation			
1	6	22.34	37.00			21.36	41.63	14.66		20.27
2	6	20.43	35.05			17.47	37.14	14.62		19.67
3	3	19.23	26.18	20.32	28.74	19.93	29.68	6.95	8.42	9.75
4	3	20.14	26.46	20.22	27.04	19.92	28.03	6.32	6.82	8.11
5	3	21.67	31.99	20.75	33.50	20.74	34.37	10.32	12.75	13.63

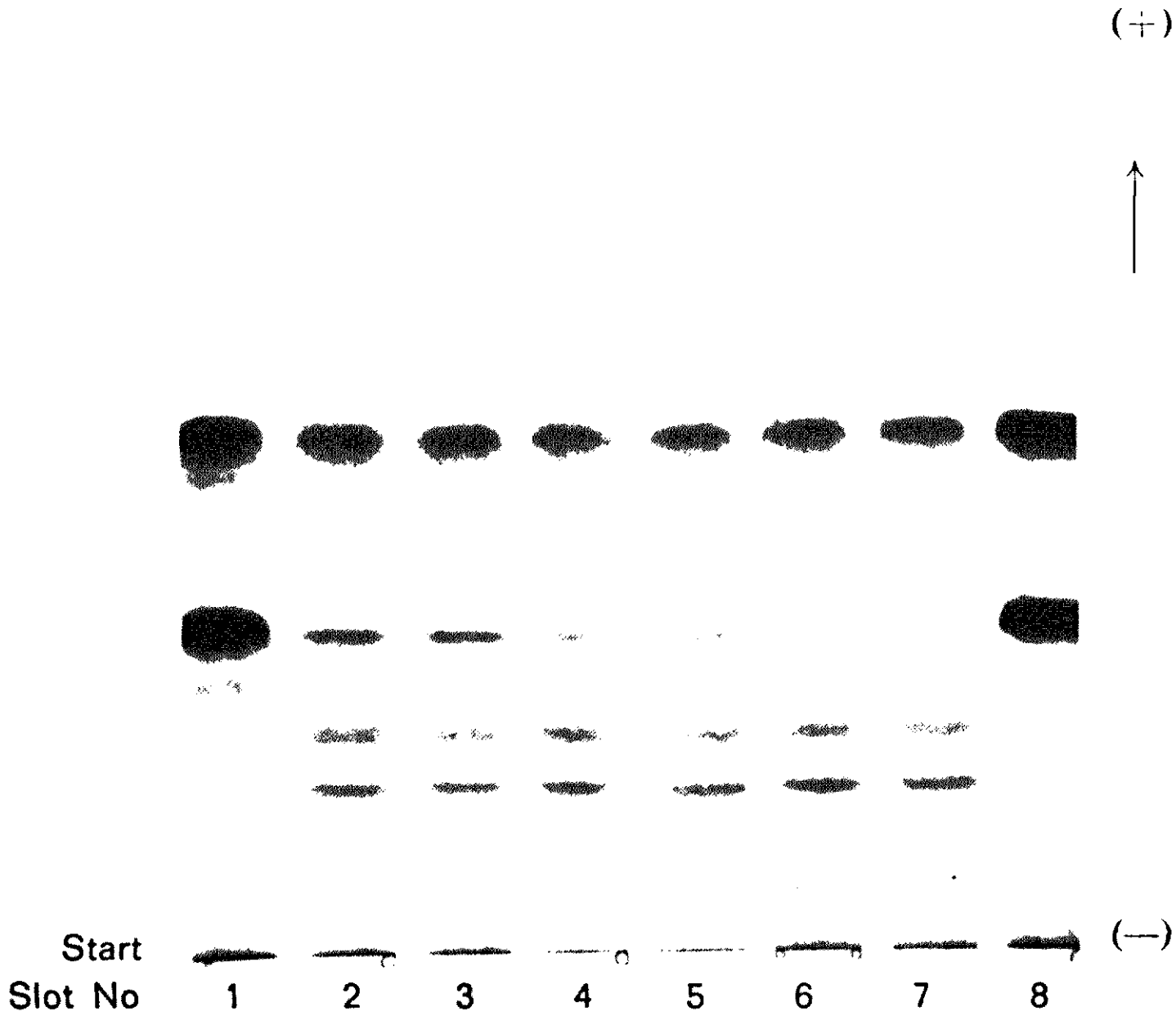

Fig. 2. PAE patterns of protein degradation by milk protease in bulk cow's milk (Experiment 5, Table 2). Samples were incubated for 3 days at 37 °C.
Slot 1 and 8: raw milk, not incubated.
Slot 2 and 3: incubated raw milk.
Slot 4 and 5: incubated milk, heated for 30 min at 63 °C.
Slot 6 and 7: incubated milk, heated for 15 s at 72 °C.

to be very weak. From the results obtained during this investigation, however, it can be concluded that, due to the action of the enzyme, raw milk can show very considerable proteolysis, which can even be increased by pasteurization of the milk. For instance, when incubated raw for six days at 37 °C, proteolytic activity in sample No 1 (Table 2) was found to be 14.66 %. Calculated on the non-soluble N before incubation (77.66 %), about 19 % of this fraction was apparently degraded to soluble N substances under the experimental conditions. With the flash-pasteurized sample this value can be calculated to be about 26 %. This does not necessarily mean that the insoluble fraction, after incubation, consisted of unchanged protein.

Data from other experiments, which will be reported in subsequent papers, clearly established the reliability of the method described in this paper for measuring proteolytic activity in milk. First of all, as far as casein is concerned, only breakdown products of this protein, formed by the action of milk protease and by the action of rennet during a renneting time of 30 min

at 30 °C, are estimated. Theoretically, the application of rennet might distort the true picture of proteolytic activity of milk protease in milk as a result of the possible interaction of enzyme activities, particularly at the end of the incubation period of the milk. If that was the case, one would expect the values for % SN/TN to be influenced by the amount of rennet used. Even a five-fold concentration of rennet (0.02 ml), however, did not change these values. In addition to the degradation products of casein, soluble nitrogen includes N substances occurring in the milk serum and possibly degradation products of these substances. Secondly, proteolytic activities estimated by this method always correlated very well with results obtained by PA electrophoresis. Finally, the method seems to be much more sensitive than methods used up to the present by other workers. On one occasion this was clearly demonstrated with a sample of aseptically drawn raw milk, incubated for 3 days at 37 °C. In this case increases in nitrogen soluble in trichloracetic acid 3 % and 12 %, after renneting of the milk as described in Section 2.4.1, were also taken as measures of proteolytic activity. Activities found with the three methods were 9.36, 2.14 and 1.07 %, respectively.

Preliminary experiments on the role of milk protease in cheese ripening indicated that the contribution of this enzyme to proteolysis in cheese can be of more interest than generally expected. Investigations on this subject are continuing.

Acknowledgment

The author is indebted to Mr T. Zandstra, Mrs A. E. A. de Groot and Mrs G. E. van Roekel, who performed the analyses.

Samenvatting

A. Noomen, *Proteolytische activiteit van melkprotease in rauwe en gepasteuriseerde koemelk*

Een gevoelige methode wordt beschreven om de proteolytische activiteit van melkprotease in koemelk te bepalen. Bij incubatie gedurende 3 of 6 dagen bij 37 °C onder anaerobe omstandigheden vertoonden monsters aseptisch gewonnen melk, met een kiemgetal van minder dan 30/ml, een aanzienlijke proteolyse. Tengevolge van laagpasteurisatie van de melk (15 s, 72 °C) nam de proteolytische activiteit toe met 30 – 40 %, terwijl standpasteurisatie (30 min 63 °C) een toename met 8 – 24 % te zien gaf. De met de methode verkregen resultaten waren steeds zeer goed gecorreleerd met die welke met behulp van PAE werden gevonden. Zowel α_s- als β-caseïne bleken door melkprotease te worden afgebroken; β-caseïne werd twee- tot driemaal zo snel aangetast als α_s-caseine. Er wordt verondersteld dat de bijdrage van melkprotease tot de eiwitafbraak in

kaas van groter belang kan zijn dan algemeen wordt aangenomen. Het onderzoek wordt in deze richting voortgezet.

References

1. W. J. Harper, J. A. Robertson, Jr. & I. A. Gould, *J. Dairy Sci.* 43 (1960) 1850-1851.
2. F. Kiermeier & G. Semper, *Z. LebensmUnters. u. -Forsch.* 3 (1960) 282-307, 373-380, 483-493.
3. J. R. Dulley, *J. Dairy Res.* 39 (1972) 1-9.
4. E. H. Reimerdes & H. Klostermeyer, *Milchwissenschaft* 29 (1974) 517-522.
5. G. Kleter & Tj. de Vries, *Neth. Milk Dairy J.* 28 (1974) 212-219.
6. L. de Jong, *Neth. Milk Dairy J.* 29 (1975) 162-168.
7. K. Yamauchi & S. Kaminogawa, *Agric. biol. Chem.* 36 (1972) 249-254.
8. S. Kaminogawa, H. Mizobuchi, K. Yamauchi, *Agric. biol. Chem.* 36 (1972) 2163-2167.

Discussion and conclusions

1 Regulation of pH in Meshanger cheese

In traditional production of Meshanger cheese the curd was not washed. So the freshly prepared cheese would contain much lactose. However, lactic acid fermentation did not result in an acid and crumbly cheese core, except as a defect. Therefore, in reconstructing the making of the cheese, primary attention was paid to the mechanism of pH regulation.

Several factors are involved in the regulation of pH in the reconstructed cheese:

- lactic acid is formed slowly in the cheese, because of slow acid-producing properties of the starter bacteria, their sensitivity to salt, the amount of starter added and the ripening temperature of the cheese
- the cheese must be salted immediately after pressing
- the moisture content of the cheese must be high, allowing rapid diffusion of salt through the cheese, so that even in the centre the concentration of salt in the moisture phase soon retards growth of starter bacteria
- the final salt concentration in the moisture phase
- the size of the cheese
- the continuously decreasing pH during ripening, which influences the rate of growth of starter bacteria.

The contribution of the surface flora to pH regulation during normal ripening was negligible.

Starter bacteria develop under continuously changing and locally different concentrations of salt in the moisture phase. By diffusion, the initially high concentration at the outside decreases and that in the core increases until the salt is uniformly distributed. Because of the sensitivity of the starter bacteria to salt, lactic acid fermentation proceeds most rapidly at the centre during the first days of ripening. The pH at the centre most quickly reaches values at which growth of starter bacteria is checked. At the end of ripening, pH tends to become uniform (Fig. 1).

2 Extraction method to estimate protein breakdown

In principal, an extraction method to estimate nitrogen compounds soluble

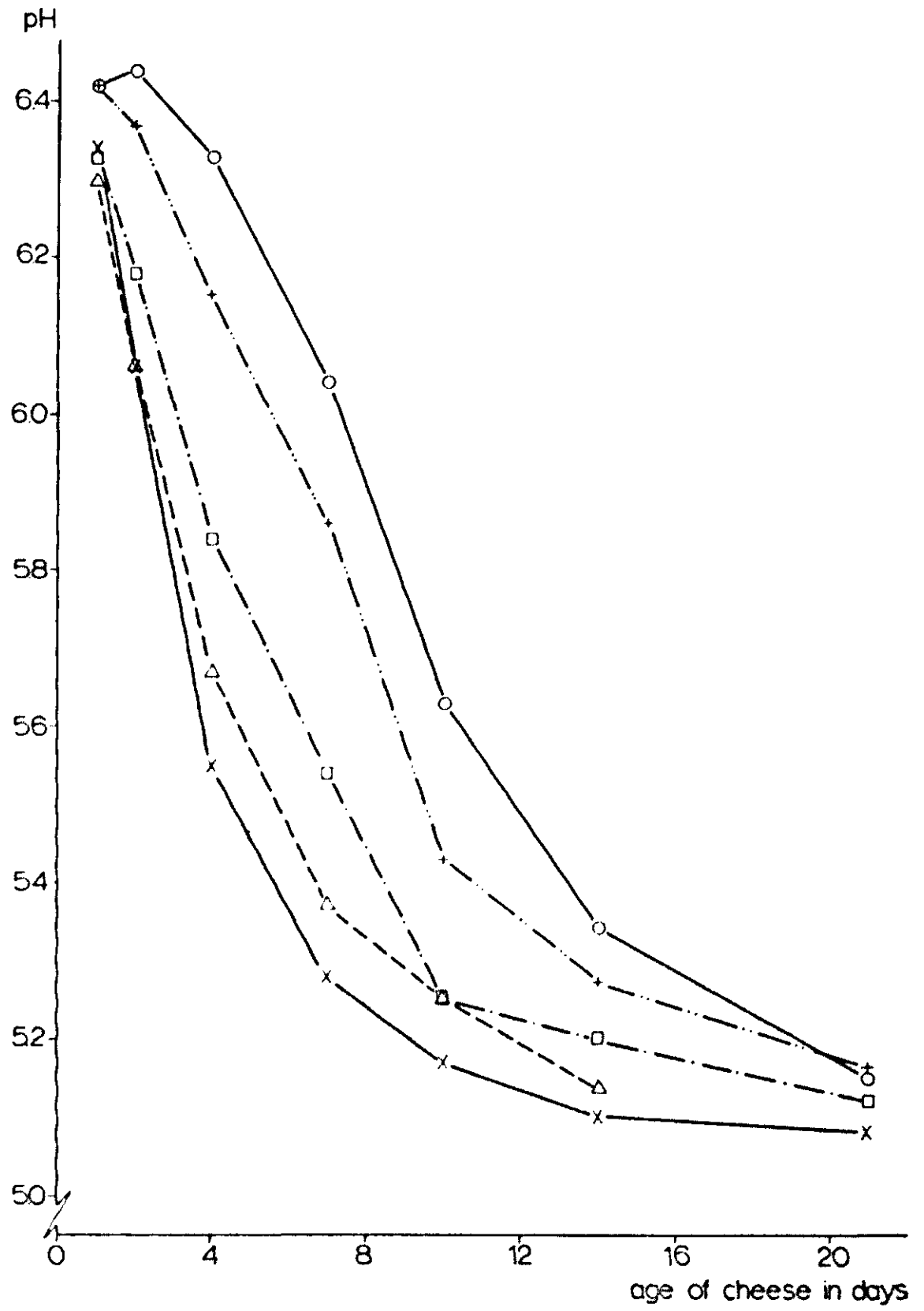

Fig. 1. Development of pH in horizontal layers of Meshanger cheese during its ripening. (○——○): outer 2 mm of the cheese; (+— . . —+), (□— . —□) and (△- - -△): successive layers of each 5 mm of cheese; (×——×): centre of the cheese.

in the moisture phase of cheese requires that the conditions of the extract correspond as closely as possible with those in cheese moisture. So extraction conditions were adapted in the most essential points (pH, temperature, calcium and sodium concentrations). However, the extracts obtained this way were turbid, because of the presence of dispersed intact protein and, to a lesser extent, insoluble products of protein breakdown. The extracts had to be clarified. The method finally chosen was selected because equal amounts of soluble nitrogen compounds are found in a particular cheese at any pH above 7. The method was applicable to all varieties of cheese. Results for total breakdown of protein obtained with the extraction method were well correlated with those of the electrophoretic method.

3 Rennet in the ripening of soft cheese

Protein breakdown in Meshanger cheese was predominantly due to calf ren-

net. Changes in consistency were related to protein breakdown, but also to physical and chemical conditions in the cheese. In soft cheeses with an initially very low pH ripening proceeds inwards, supposedly under the influence of extracellular proteolytic enzymes of the surface flora. These enzymes would enter the cheese by diffusion (1, 2). However, in our laboratory, it was observed that the diffusion of enzymes in cheese, even in Meshanger cheese with its very high content of moisture (60 %), proceeds slowly (3). The tests with simulated soft cheese revealed that calf rennet degrades caseins in cheese with a low pH and a crumbly consistency, as in softer cheese with a higher pH. The physico-chemical conditions apparently determine whether cheese softens or not. The ripening, for instance, of Camembert cheese may therefore be due to regulation of the pH by the surface flora, which also gives the cheese a specific taste and flavour. Protein breakdown in the cheese would merely be due to rennet and proteolytic enzymes of lactic acid bacteria.

4 pH and NaCl in rennet activity

The simulated cheeses, made up from calcium paracaseinate–calcium phosphate complex, water, lactic acid and NaCl or with chemically acidified and salted aseptic cheese curd, proved convenient to study the proteolytic activity of calf rennet at acidities and salt concentrations that may prevail in Meshanger cheese. Electrophoretic patterns of the different cheeses were comparable and also resembled the picture of proteolytic processes in normal Meshanger cheese. Optimum pH for rennet action was close to 5.0 for α_{s1} casein; for β casein it became not clear. Total breakdown was maximum at pH 4.9 - 5.0. These results differed markedly from those observed by other workers in tests under conditions far remote from those in cheese and with other methods of estimation of proteolytic activity (4, 5, 6, 7, 8, 9). Breakdown of α_{s1} casein was stimulated by low concentrations of NaCl and retarded by high concentrations, whereas the breakdown of β casein was retarded considerably, even by low concentrations. Total breakdown of protein decreased with increasing salt content.

During most of the ripening of Meshanger cheese, conditions in the simulated cheeses would favour extensive breakdown by rennet of α_{s1} casein, with which changes in consistency were strongly related (10).

Extrapolation of the results to conditions (pH, NaCl) in different parts of the normal ripening cheese indicated that rennet would act against α_{s1} casein most quickly in the centre of the cheese, so explaining its characteristic way of softening from the centre outwards. Rennet degrades β casein weakly in Meshanger cheese.

5 Activity of milk protease in simulated cheese and milk

As studied with simulated soft cheeses made up with rennet-free cheese curd and a rennet-free calcium paracaseinate–calcium phosphate complex, the contribution of milk protease to protein breakdown in certain soft cheeses may be far from negligible (Fig. 2). It depended on pH, concentration of NaCl in the moisture phase, time and temperature of ripening, and will also be determined by the protease activity of the cheese milk. Activity of alkaline milk protease may be considerable in soft cheeses ripening with a surface flora, and showing a high pH in the outer layers for some weeks of ripening.

The method developed to estimate protein breakdown in milk proved much more sensitive than the tyrosine method and was also superior to the occasionally used method at which protein breakdown by milk protease was estimated by the increase in nitrogen in TCA filtrates (11, 12, 13).

Contrary to the generally assumed weak activity of milk protease in milk, our experiments revealed that activity of milk protease may be considerable in milk stored at 37 °C. The activity varied between milks of different cows. Surprisingly, protein breakdown in milk was stimulated in milk subjected to a vat pasteurization for 30 min at 63 °C and, even more so, in milk pasteurized for 15 s at 72 °C. Perhaps enzyme activity was stimulated. Alternatively, the activity of milk protease as such could be partly destroyed by pasteurization

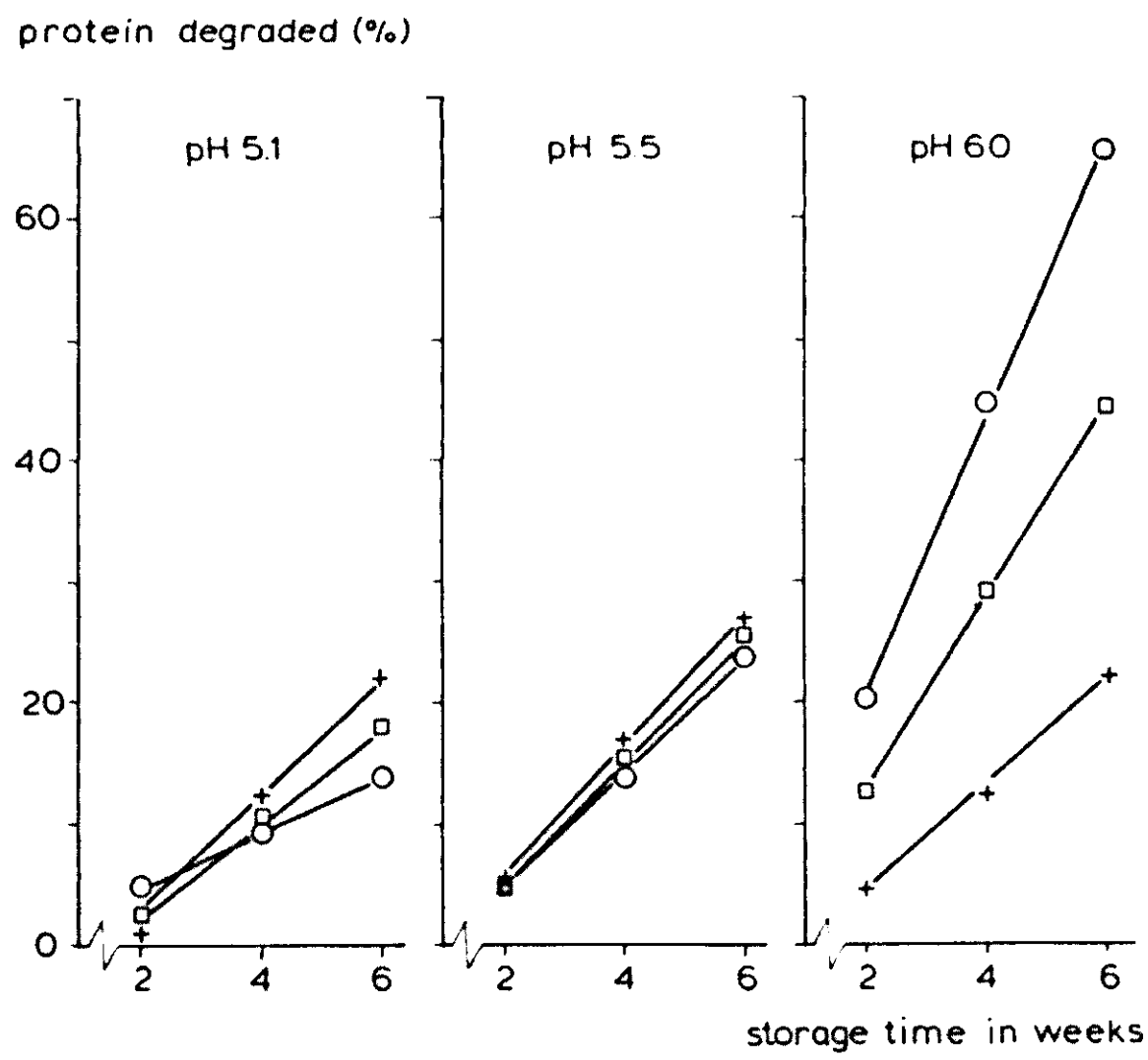

Fig. 2. Densitometric evaluation of protein breakdown by milk protease in simulated soft cheeses, made up with a rennet-free calcium paracaseinate-calcium phosphate complex, depending on the pH and the storage time at 13 °C. The cheeses contained 4% NaCl in the moisture. (+): α_{s1} casein; (○): β casein, and (□): α_{s1} + β casein.

but protease inhibitors in the milk (e.g. 14, 15, 16) could be inactivated even more, so that more protein would be degraded.

References

1. G. Seeler. Mikroskopische Untersuchung des Weiszschimmel – Wachstums in Camembert – Käsen anhand von Mikrotomschnitten. Milchwissenschaft 23 (1968) 661-665.
2. A. M. Knoop & K. H. Peters. Submikroskopische Strukturveränderungen im Camembert – Käse während der Reifung. Milchwissenschaft 26 (1971) 193-198.
3. L. de Jong, A. Noomen & H. Mulder (to be published).
4. J. Stadhouders. The Proteolytic Activity of Rennet and Starter Bacteria in Cheese with Reference to Bitter Flavour. Proc. 16th int. Dairy Congr. B (1962) 353-361.
5. N. P. Melachouris & S. L. Tuckey. Comparison of the proteolysis produced by rennet extract and the pepsin preparation Metroclot during ripening of Cheddar cheese. J. Dairy Sci. 47 (1964) 1-7.
6. P. F. Fox. Influence of Temperature and pH on the Proteolytic Activity of Rennet Extract. J. Dairy Sci. 52 (1969) 1214-1218.
7. J. J. Tam & J. R. Whitaker. Rates and Extents of Hydrolysis of Several Caseins by Pepsin, Rennin, Endothia parasitica Protease and Mucor pusillus Protease. J. Dairy Sci. 55 (1972) 1523-1531.
8. T. Itoh. Comparison of the proteolytic action of rennet extract and pepsin on casein fractions. Milchwissenschaft 27 (1972) 470-473.
9. L. de Jong & Aliza E. A. de Groot-Mostert. The proteolytic action of rennet on different casein substrates under various conditions. Neth. Milk Dairy J. 31 (1977) 296-313.
10. L. de Jong. Protein breakdown in soft cheese and its relation to consistency. 2. The influence of the rennet concentration. Neth. Milk Dairy J. 31 (1977) 314-327.
11. R. C. Warner & E. Polis. On the presence of a Proteolytic Enzyme in Casein. J. Amer. Chem. Soc. 67 (1945) 529-532.
12. V. Moreno & F. V. Kosikowski. Some characteristics and products of β-casein proteolytic activity. Milchwissenschaft 27 (1972) 433-436.
13. E. H. Reimerdes & H. Klostermeyer. Milchproteasen I. Micellenassoziierte Protease – Anreicherung und Hydrolyse von Caseinen. Milchwissenschaft 29 (1974) 517-522.
14. F. Kiermeier & G. Semper. Über das Vorkommen eines proteolytischen Enzyms und eines Trypsin-Inhibitors in Kuhmilch. II. Mitteilung: Zur inhibitorischen Wirkung. Z. Lebensm. Unters. Forsch. 3 (1960) 373-380.
15. E. H. Reimerdes, H. Klostermeyer & E. Sayk. Milch-Proteinasen VII. Fraktionierung von Komponenten des Proteinasen – Inhibitor – Systems der Milch. Milchwissenschaft 31 (1976) 329-334.
16. S. Kaminogawa, F. Sato & K. Yamauchi. Purification and Some Properties of Milk Protease. Agric. Biol. Chem. 35 (1971) 1465-1467.

Summary

The ripening of soft cheese was studied with particular reference to the question which proteolytic enzymes were predominantly involved in the primary degradation of protein and the changes in cheese consistency related to that.

Noordhollandse Meshanger cheese, an extinct Dutch cheese with a high moisture content, served as a model in the investigation. The method of production of the cheese was reconstructed and its ripening was studied. The proteolytic activity of calf rennet and of milk protease under different physical and chemical conditions was studied with preserved simulated soft cheeses, composed with either calcium paracaseinate–calcium phosphate complex, water, lactic acid and NaCl or with chemically acidified and salted cheese curd. Milk protease activity in raw and pasteurized cow's milk was also investigated.

Protein breakdown was estimated by an extraction method, which gives a good estimate of the amount of N compounds soluble in the moisture of cheese, and by quantitative polyacrylamide gel electrophoresis. Results for total breakdown of protein obtained with both methods were well correlated.

The regulation of pH in Meshanger cheese was found to be decisive to the way the cheese body softened from the centre outwards. It was mainly determined by the growth of starter bacteria producing lactic acid slowly, the high moisture content and the salting process of the cheese.

Results for proteolysis in the simulated cheeses were comparable and differed only quantitatively. The picture of proteolytic processes resembled that in normal Meshanger cheese.

Protein breakdown in Meshanger cheese, which was closely related to the development of consistency, was caused by calf rennet. The rennet degraded α_{s1} casein far more than β casein. Its maximum activity against α_{s1} casein was found at a pH near to 5.0; against β casein it became not clear. The total degradation of protein was maximum at pH 4.9 - 5.0.

Concentrations up to about 4 % NaCl in the cheese moisture stimulated the degradation of α_{s1} casein, whereas higher concentrations had an inhibitory effect. The breakdown of β casein by rennet was maximum in the absence of NaCl and was reduced even at low salt contents. The total breakdown of protein decreased with increasing NaCl concentrations. It is postulated that the primary degradation of protein in soft cheeses with an initially very low

pH that show visual ripening from the outside to the inside under the influence of a surface flora, are caused by rennet enzymes and by proteolytic enzymes of the internal flora of lactic acid bacteria. The role of the surface flora is to lower the acidity of the cheese and so make the cheese body soft, and to give the cheese a specific taste and flavour.

The contribution of milk protease to protein breakdown in soft cheese is determined by pH, NaCl concentration in the cheese moisture, ripening temperature and ripening time of the cheese, and by protease activity of the cheese milk used. Results suggested that besides alkaline milk protease an acid protease may be active in cheese, which contrary to the former degrades α_{s1} casein preferably to β casein. The degradation of both caseins was stimulated by low concentrations of NaCl, maximum breakdown being found with 2 % NaCl in the moisture under the conditions tested. At a high pH both α_{s1} and β casein were increasingly degraded at higher temperatures up to 37 °C (the highest temperature tested). At a low pH, the breakdown of α_{s1} casein showed a corresponding behaviour, whereas β casein was no further degraded at temperatures above 20 °C. Milk protease is of minor importance to proteolysis in soft cheeses, except perhaps certain cheeses with a surface flora and with conditions favourable to the activity of alkaline milk protease.

Milk protease may considerably degrade the protein in milk at a favourable temperature (e.g. 37 °C). The proteolytic activity varies between milks of different cows. Compared to that in raw milk, protein breakdown by milk protease is increased in low-temperature pasteurized milk (30 min at 63 °C; 15 s at 72 °C).

Samenvatting

Er werd een onderzoek verricht naar de rijping van weke kaas. De vraag welke enzymen in het bijzonder betrokken zijn bij de primaire afbraak van het eiwit en de daarmee verband houdende consistentieveranderingen stond centraal.

Noordhollandse Meshanger, een in de vergetelheid geraakte kaas met een hoog vochtgehalte, fungeerde als model bij het onderzoek. De bereidingswijze van de kaas werd gereconstrueerd en de rijping ervan werd onderzocht. De proteolytische activiteit van kalfsstremsel en van melkprotease onder verschillende fysische en chemische omstandigheden werd bestudeerd met behulp van geconserveerde kunstkaasjes met een qua samenstelling week zuivel. Deze kaasjes werden samengesteld met calciumparacaseïnaat-calciumfosfaat-complex, water, melkzuur en NaCl, en met chemisch verzuurde en gezouten wrongel. De activiteit van melkprotease in rauwe en gepasteuriseerde koemelk werd eveneens onderzocht.

De eiwitafbraak werd nagegaan met een extractiemethode die een goed beeld geeft van de in kaasvocht opgeloste stikstofverbindingen, en met kwantitatieve polyacrylamidegel-elektroforese. De met beide methoden verkregen resultaten aangaande de totale eiwitafbraak lieten een goede correlatie zien.

De regeling van de pH in Meshanger kaas bleek van doorslaggevend belang te zijn voor het van binnen naar buiten voortschrijdende week worden van de kaas. Deze regeling werd voornamelijk bepaald door de groei van langzaam zuurvormende zuurselbacteriën, het hoge vochtgehalte en het zoutingsproces van de kaas.

De resultaten van de eiwitafbraak in de verschillende kunstkaasjes waren vergelijkbaar en verschilden slechts in kwantitatief opzicht. Het beeld van de proteolytische processen geleek op dat in normale Meshanger.

De eiwitafbraak in Meshanger kaas, waarmee de ontwikkeling van de consistentie sterk verband hield, werd veroorzaakt door kalfsstremsel. Het stremsel brak α_{s1}-caseïne veel meer af dan β-caseïne. Het vertoonde een maximale activiteit ten opzichte van α_{s1} caseïne bij pH ca. 5,0; ten opzichte van β-caseïne werd dit niet duidelijk. De totale eiwitafbraak was het grootst bij pH 4,9 tot 5,0. De afbraak van α_{s1}-caseïne werd gestimuleerd bij lage zoutgehalten in het vocht, terwijl concentraties boven de 4 % NaCl remmend werkten. De afbraak van β-caseïne door het stremsel was het grootst bij afwezigheid van NaCl en nam reeds af bij lage zoutgehalten. De totale afbraak

verminderde bij toenemend zoutgehalte. Er wordt gesteld dat in weke kaassoorten met een aanvankelijk zeer lage pH en die van buiten naar binnen rijpen onder de invloed van een oppervlakteflora, de primaire eiwitafbraak wordt veroorzaakt door stremselenzymen en proteolytische enzymen van de interne flora van melkzuurbacteriën. De rol van de oppervlakteflora bestaat vooral uit het verlagen van de zuurtegraad van de kaas waardoor het zuivel week wordt, en uit het vormen van voor de kaas specifieke smaak- en geurstoffen.

De bijdrage van melkprotease tot de eiwitafbraak in weke kaas wordt bepaald door de pH, het NaCl-gehalte in het kaasvocht, de rijpingstijd en rijpingstemperatuur van de kaas, en de proteaseactiviteit van de gebruikte kaasmelk. De resultaten deden veronderstellen dat naast alkalisch melkprotease een zuur protease in kaas werkzaam kan zijn, dat in tegenstelling tot het eerstgenoemde α_{s1}-caseïne meer afbreekt dan β-caseïne. De afbraak van beide caseïnes werd gestimuleerd bij lage NaCl-gehalten; onder de getoetste omstandigheden werd de grootste afbraak gevonden bij een gehalte van 2 % NaCl in het vocht. Bij een hoge pH werden α_{s1}- en β-caseïne in toenemende mate afgebroken bij hogere temperaturen (de hoogst getoetste temperatuur was 37 °C). Bij een lage pH vertoonde de afbraak van α_{s1}-caseïne eenzelfde beeld, terwijl de afbraak van β-caseïne niet meer toenam bij temperaturen boven 20 °C. Melkprotease is van minder belang voor de proteolyse in weke kaas, misschien met uitzondering van bepaalde kazen met een oppervlakteflora waarin omstandigheden kunnen voorkomen die gunstig zijn voor de activiteit van alkalisch melkprotease.

Melkprotease kan bij gunstige temperaturen (bijvoorbeeld 37 °C) in melk een aanzienlijke afbraak van het eiwit veroorzaken. De proteolytische activiteit varieert tussen melk van verschillende koeien. Vergeleken met rauwe melk neemt de eiwitafbraak door melkprotease toe bij laagpasteurisatie van melk (30 min 63 °C; 15 s, 72 °C).