Proteolysis in cheese can be divided into three phases: proteolysis in milk before cheese manufacture, the enzymatically induced coagulation of the milk, and proteolysis during cheese ripening. Extracellular proteinases from psychrotrophs may cause reduced cheese yields and off-flavors, but the problem does not appear to be significant at populations <10^6 cfu/ml. Proteinases from leucocytes may also reduce yields, but these are less active than bacterial proteinases. Plasmin (indigenous milk proteinase) causes significant hydrolysis of β-casein, especially in late lactation, but because most of this occurs prior to milking, yield losses due to plasmin activity (i.e., via formation of protease peptones) are largely unavoidable.

Coagulation of milk for rennet cheeses is accomplished by the specific cleavage of the casein micelle-stabilizing protein, κ-casein, at the phenylalanine (105)-methionine (106) bond by acid proteinases (rennets). The significance of the primary, secondary, and tertiary structures of κ-casein on the sensitivity of this bond is now established, as are pH, ionic strength, temperature, heat treatment of milk, and environmental and processing variables.

Proteolysis is probably the most important biochemical event during the ripening of most cheese varieties, with a major impact on flavor and texture. Techniques have been developed that permit quantitation of the principal ripening agents to proteolysis, i.e., rennet, milk proteinase, and the proteinases from starter, nonstarter bacteria, and perhaps secondary inocula. The contribution of each of these agents to proteolysis in the principal varieties has been established in general terms. Several techniques have been used to monitor and quantify proteolysis in cheese during ripening; the principal of these are described and evaluated. Standardization of analytical techniques for assessing proteolysis in cheese appears warranted.

INTRODUCTION

Cheese manufacture is essentially a dehydration process in which the fat and casein of milk are concentrated 6- to 10-fold, depending on the variety. With the exception of some minor varieties (e.g., the Norwegian whey cheeses in which thermal evaporation is used), dehydration is traditionally achieved by coagulating the casein enzymatically, isoelectrically, or by a combination of heat and acid; if present, fat is occluded in the coagulum. Ultrafiltration offers the possibility of concentrating the total colloidal phase of milk, but the retentate is usually renneted or acidified to provide characteristic texture. Most, if not all, ripened cheeses are produced by rennet coagulation; proteolysis is a sine qua non in the manufacture of these cheeses and also plays a major role during ripening; many authors think proteolysis is the single most important event during the ripening of most cheese varieties. Some proteolysis also occurs in milk before coagulation. Hence, in this review, proteolysis will be divided into three phases: premanufacture, coagulation, and ripening.
DISCUSSION

Proteolysis in Milk Premanufacture

There are two main causes of proteolysis in cheese milk premanufacture: microbial and indigenous milk proteinases. Psychrotrophic bacteria dominate the microflora of milk cooled on-farm prior to collection, during transportation, and during storage at the factory; storage of raw milk at low temperatures for 4 or 5 d is common in developed dairying countries. Apparently, psychrotrophic bacteria are not significant as far as proteolysis is concerned unless the population exceeds about 10^6 cfu/ml; Higher psychrotroph populations are likely to cause reduced recovery of milk solids as cheese, higher moisture contents, pasty texture, and off-flavors in the cheese (29, 49, 84, 139). Lipases produced by psychrotrophs are probably more important in the development of flavor defects in cheese than are proteinases, possibly because the proteinases are water-soluble and are lost in the whey, whereas the lipases are adsorbed onto the fat globules and are, in fact, concentrated in the cheese.

As it comes from the cow, milk contains many proteinases, the principal one being plasmin, i.e., alkaline milk proteinase, which preferentially hydrolyzes β-casein to γ-caseins and proteose peptones. It also hydrolyzes α2-β-casein rapidly, but the products have not been identified. γ2-Casein is hydrolyzed slowly; λ-casein may be one of the products (1). Plasmin has little effect on κ-casein, which is fortunate in view of the importance of this constituent for milk stability. Andrews and Alichanidis (5) reported that the classical proteose-peptone fraction consists of 38 peptides, 52% of which may originate from β-casein, 29% from α1-casein, 9% from α2-β-casein, and 4% from κ-casein. The γ-caseins normally represent approximately 3% of the casein N in milk (36, 37) but may be as high as 10% in late lactation or in milk from mastitic cows (11, 12, 45). The proteose-peptone fraction represents approximately 3% of total N (199).

Most of the proteose-peptone fraction is lost in acid or rennet whey, and consequently, a reduction in cheese yield can be assumed as a consequence of plasmin action, although definite information on this is lacking. The rennetability of milk and the syneresis properties of the resulting gel deteriorate with advancing lactation, and cheese made from such milk has a high moisture content (16). Donnelly et al. (46) present evidence suggesting that plasmin activity is at least a contributor to such problems, but Pearce et al. (171), using synthetic micelles, claim that up to 50% of the β-casein can be hydrolyzed without adverse effects on the rennetability and cheesemaking properties of casein. Grufferty and Fox (74) also showed that very significant plasmin activity had little effect on the rennet coagulability of milk. Because most of the action of plasmin in milk occurs within the mammary gland, with relatively little after milking (45, 199), not much can be done about it, although there may be characteristic individual cow and breed variability (11, 36, 189, 199). Plasmin activity is probably not particularly important in milk from mixed-calving herds but is very significant in herds with synchronized calving patterns, e.g., in New Zealand, Ireland, and Victoria, Australia.

Proteases from leucocytes are a further potential cause of proteolysis in milk, especially that from cows suffering from clinical or subclinical mastitis. Grieve and Kitchen (69) reported that plasmin in milk was 2 to 8 times more proteolytic than leucocytes added to milk at 10^6/ml. However, leucocyte proteinases are capable of causing proteolysis in milk, which may adversely affect cheese yield and quality (201, 223).

Enzymatic Coagulation of Milk

It has been known in a general way since the pioneering work of Hammersten in the 1880s that the rennet coagulation of milk involves proteolysis with the formation of para-casein and nonprotein nitrogen (NPN). It is now well-known that rennet coagulation as a two-stage process, the first involving the enzymatic formation of para-casein and peptides, the second involving the precipitation of para-casein by Ca^2+ at temperatures >20°C. Both stages, especially the primary phase, are now fairly clearly understood.

Primary Phase of Rennet Action

Alais et al. (3) and Nitschmann and Keller (157) clearly demonstrated that specific prote-
Proteolysis occurs during the primary phase of rennet action, that this proteolysis was complete before the onset of coagulation, that more than one peptide was produced (two to three times as much NPN was soluble in 2% TCA as in 12% TCA), and that \( \alpha \)-casein, rather than \( \beta \)-casein, was the substrate for this specific proteolysis.

With the isolation of \( \kappa \)-casein and the demonstration that it is responsible for micelle stability and that its micelle-stabilizing properties are lost on renneting (240), it became possible to define the primary phase of rennet action precisely. Only \( K \)-casein is hydrolyzed during the primary phase of rennet action (238), the cleavage site being Phe\(_{105}\)-Met\(_{106}\) (41). This particular bond is many times more susceptible to hydrolysis by acid proteinases (most commercial rennets are acid proteinases) than any other in the milk protein system.

The unique sensitivity of the Phe-Met bond has aroused interest. The dipeptide, H-Phe-Met-OH, is not hydrolyzed, nor are tripeptides or tetrapeptides containing a Phe-Met bond. However, this bond is hydrolyzed in the pentapeptide, H-Ser-Leu-Phe-Met-Ala-OMe (85, 86), and reversing the positions of serine and leucine in this pentapeptide to give the correct sequence of \( \kappa \)-casein increases the susceptibility of the Phe-Met bond to hydrolysis by chymosin (200). Both the length of the peptide and the sequence around the sectile bond are important determinants of enzyme-substrate interaction. Serine-104 appears to be particularly important (85, 86), and its replacement by Ala in the pentapeptide renders the Phe-Met bond very resistant to hydrolysis by chymosin (185) but not by pepsins (183); even substituting D-Ser for L-Ser markedly reduces the suitability of this peptide as a substrate (183). Extension of the pentapeptide from the N or C-terminal to reproduce the sequence of \( \kappa \)-casein around the Phe-Met bond increases the efficiency with which the Phe-Met bond is hydrolyzed by chymosin (235, 236). Taking the pentapeptide H-Ser-Phe-Met-Ala-Ile-OH, i.e., the sequence 104 to 108 of \( \kappa \)-casein, as a standard, studies at pH 4.7 showed that extending the peptide toward the C-terminal by three residues to give Ser\(_{104}\)-Lys\(_{111}\) causes a 6-fold increase in the catalytic ratio, \( K_{cat}/K_m \), whereas addition of Leu\(_{103}\) to the pentapeptide increases the ratio 600-fold (Table 1). Addition of His\(_{102}\) and Pro\(_{101}\) (i.e., Pro\(_{101}\)-Ile\(_{108}\)) increases \( K_{cat}/K_m \) a further 5-fold. Although Jolles et al. (98) reported that the tryptic peptide His\(_{98}\)-Lys\(_{111}\) of \( \kappa \)-casein (His\(_{100}\) mistakenly omitted) was not hydrolyzed by chymosin, Hill and Hocking (87) found that the sequence His\(_{98}\)-Glu\(_{129}\) of \( \kappa \)-casein includes all the residues necessary to render the Phe-Met bond as susceptible to hydrolysis by chymosin as it is in intact \( \kappa \)-casein. In fact, the peptide representing the sequence His\(_{98}\)-Lys\(_{111}\) (or Lys\(_{112}\)) includes all the necessary determinants (234). At pH 6.6, the peptide His\(_{98}\)-Lys\(_{111}\) is hydrolyzed about 66,000 faster than the parent pentapeptide, with a \( K_{cat}/K_m \) of \( \sim 2 \mu M^{-1} s^{-1} \), which is very close to that for intact \( \kappa \)-casein. \( \kappa \)-Casein and the peptide His\(_{98}\)-Lys\(_{111}\) are readily hydrolyzed at pH 6.6 (and at pH 4.7), but these small peptides are not hydrolyzed at pH 6.6. Thus, the sequence His\(_{98}\)-Lys\(_{111}\) appears to contain the necessary determinants for rapid cleavage of the Phe-Met bond by chymosin and presumably by other acid proteinases.

The Phe\(_{105}\)-Met\(_{106}\) bond of \( \kappa \)-casein is the only Phe-Met in the milk protein system; however, these two residues are not intrinsically essential. There are numerous Phe residues in all milk proteins and a substantial number of Met residues. Replacement of Phe by Phe(NO\(_2\)) reduces the catalytic ratio by a factor of about 3 whereas substitution of cyclohexylamine for Phe reduces the ratio almost 50-fold (236). Oxidation of Met\(_{106}\) reduces catalytic constant (\( K_{cat} \)/Michaelis constant (\( K_m \)) 10-fold but substitution of Ile for Met increases this ratio about 3-fold. Neither porcine or human \( \kappa \)-casesins possess a Phe-Met bond (both have a Phe-Ile bond at this position (17, 28, 53)), yet both are readily hydrolyzed by calf chymosin, although more slowly than bovine \( \kappa \)-casein; in contrast, porcine milk is coagulated more effectively than bovine milk by porcine chymosin (56), indicating that unidentified subtle structural changes influence chymosin action. Thus, the sequence around the Phe-Met bond, rather than the bond itself, contains the important determinants of hydrolysis. The particularly important residues are Ser\(_{104}\); the hydrophobic residues Leu\(_{103}\) and Ile\(_{108}\), at least one of the three histidines (residues 98, 100, or 102, as indicated by the inhibitory effect of photooxida-
TABLE 1. Kinetic parameters for hydrolysis of \( \kappa \)-casein peptides by chymosin at pH 4.7.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>( K_{m} )</th>
<th>( K_{m} )</th>
<th>( K_{cat}/K_{m} )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(s(^{-1}))</td>
<td>(mM)</td>
<td>(s(^{-1})-mM(^{-1}))</td>
</tr>
<tr>
<td>S.F.M.A.I.</td>
<td>104-108</td>
<td>.33</td>
<td>8.5</td>
<td>.038</td>
</tr>
<tr>
<td>S.F.M.A.I.P.</td>
<td>104-109</td>
<td>1.05</td>
<td>9.2</td>
<td>.114</td>
</tr>
<tr>
<td>S.F.M.A.I.P.P.</td>
<td>104-110</td>
<td>1.57</td>
<td>6.8</td>
<td>.231</td>
</tr>
<tr>
<td>S.F.M.A.I.P.P.K.</td>
<td>104-111</td>
<td>.75</td>
<td>3.2</td>
<td>.239</td>
</tr>
<tr>
<td>L.S.F.M.A.I.</td>
<td>103-108</td>
<td>18.3</td>
<td>.85</td>
<td>21.6</td>
</tr>
<tr>
<td>L.S.F.M.A.I.P.</td>
<td>103-109</td>
<td>38.1</td>
<td>.69</td>
<td>55.1</td>
</tr>
<tr>
<td>L.S.F.M.A.I.P.P.</td>
<td>103-110</td>
<td>43.3</td>
<td>.41</td>
<td>105.1</td>
</tr>
<tr>
<td>L.S.F.M.A.I.P.P.K.</td>
<td>103-111</td>
<td>33.6</td>
<td>.43</td>
<td>78.3</td>
</tr>
<tr>
<td>L.S.F.M.A.I.P.P.K.K.</td>
<td>103-112</td>
<td>30.2</td>
<td>.46</td>
<td>65.3</td>
</tr>
<tr>
<td>H.L.S.F.M.A.I.</td>
<td>102-108</td>
<td>16.0</td>
<td>.52</td>
<td>30.8</td>
</tr>
<tr>
<td>P.H.L.S.F.M.A.I.</td>
<td>101-108</td>
<td>33.5</td>
<td>.34</td>
<td>100.2</td>
</tr>
<tr>
<td>H.P.H.P.H.L.S.F.M.A.I.P.P.K.</td>
<td>98-111</td>
<td>66.2</td>
<td>.026</td>
<td>2509</td>
</tr>
<tr>
<td></td>
<td>98-111(^1)</td>
<td>46.2(^1)</td>
<td>.029(^1)</td>
<td>1621(^1)</td>
</tr>
</tbody>
</table>

\( \kappa \)-Casein\(^2\)

| L.S.F. (NO\(_2\))Nle A. L. OMe | 2-20     | .001-0.05   | 200-2000       |
| L.S.F. (NO\(_2\))Nle A. L. OMe | 12       | .95        | 12.7           |

\(^1\)pH 6.6.

\(^2\)pH 6.4.

...
thermore, the common rennets have markedly different specific activities on synthetic \( \kappa \)-casein-related peptides (40, 141, 192).

The subject of rennet substitutes will not be reviewed here; literature reviews are extensive (48, 65, 154, 175, 197, 209).

Factors Affecting Hydrolysis of \( \kappa \)-Casein

**pH.** The pH optimum for chymosin and bovine pepsin on the small peptides used in the preceding section is approximately 4.7 (40, 85, 183), but Visser et al. (230) found the optimum on the peptide His\(_{98}\)Lys\(_{111/112}\) to be 5.3 to 5.5. The pH optimum for the hydrolysis of insulin and acid-denatured hemoglobin by chymosin is 4.0 and 3.5, respectively (54, 55). Isolated \( \kappa \)-casein is optimally hydrolyzed at pH 5.2 to 5.5 and at slightly lower values in Na caseinate (92). Von Hooydonk et al. (220) found the pH optimum for the first stage of rennet action in milk to be -6.0 at both 4 and 30°C.

**Organic Acids.** Polyprotic acids, especially EDTA, citrate, and phytate, prevent or inhibit the rennet coagulation of milk, undoubtedly due to the chelation of Ca\(^{2+}\). Several fatty acids also retard or prevent rennet coagulation (97), probably via Ca\(^{2+}\) chelation, although the primary phase of rennet action was not investigated. However, Kato et al. (103) reported that EDTA and orthophosphate stimulate the primary phase of rennet action. Sodium dodecyl sulfate inhibits the primary action of rennet, possibly by binding to \( \kappa \)-casein (27).

**Ionic Strength.** Increasing ionic strength (.01 to .11) reduces the rate of hydrolysis of His\(_{98}\)Lys\(_{111/112}\), the effect becoming more marked as the reaction pH is increased but independent of ion type (229, 234). The influence of ionic strength on the primary phase of rennet coagulation was discussed above (2, 73, 78, 169, 170). Kato et al. (97) also reported that NaCl, as well as CaCl\(_2\) and MgCl\(_2\), all at 1 mM, stimulated the hydrolysis of \( \kappa \)-casein in isolated form and in sodium caseinate.

**Temperature.** The optimum temperature for the coagulation of milk by calf rennet at pH 6.6 is near 45°C; presumably, the optimum for the hydrolysis of \( \kappa \)-casein is about this value. The temperature coefficient (Q\(_{10}\)) for the hydrolysis of \( \kappa \)-casein in solutions of Na caseinate is 1.8, activation energy, E\(_a\), is approximately 10,000 cal/mol and activation entropy, \( \Delta S \), is approximately 39 cal/degree per mol (156). Generally, similar values (free energy of activation [\( \Delta F^* \)] = 15,800 cal/mol; energy of activation [E\(_a\)] = 7000 cal/mol; enthalpy of activation [\( \Delta H^* \)] = 6300 cal/mol; entropy of activation [\( \Delta S^* \)] = -31 cal/degree per mol) were reported by Garnier (62) for the hydrolysis of isolated \( \kappa \)-casein by chymosin.

**Degree of Glosyoslation.** There are several reports [see (44, 237)] that the efficiency of \( \kappa \)-casein as a substrate for chymosin decreases with the degree of glycosylation. The most detailed study of this effect is that of Vreeman et al. (237), who showed that at pH 6.6, \( K_{cat} \) decreased from approximately 43 s\(^{-1}\) for carbohydrate-free \( \kappa \)-casein (B-1) to approximately 25 s\(^{-1}\) for \( \kappa \)-casein B-7 (i.e., that containing 6 mol N-acetyl neuraminic acid/mol). However, \( K_m \) decreased from approximately 9 \( \mu \)M for B-1 to 3.5 \( \mu \)M for B-7; \( K_{cat}/K_m \) was optimal for B-5 (3 mol N-acetyl neuraminic acid [NANA]/mol). Polymerization (aggregation) markedly increased \( K_m \) with little effect on \( K_{cat} \).

**Other Proteins.** Mikawa et al. (145) reported that \( \alpha_\text{S} \)- and \( \beta \)-caseins inhibited the hydrolysis of \( \kappa \)-casein when mixed with it in the ratio of 2:1. Apparently, \( \alpha_\text{S} \)-, \( \beta \)-, and \( \kappa \)-caseins interfere with the aggregation of para-\( \kappa \)-casein (120). Whey proteins are reported (23) to have no effect on \( K_m \) and maximum velocity (V\(_m\)) for chymosin on micellar casein.

**Heat Treatment of Milk.** It has long been recognized that heat treatment >65°C adversely affects the rennet coagulation of milk if the exposure is of sufficient duration; if heat treatment is very severe (>90°C for 10 min), the milk fails to coagulate on renneting. It is now known that although changes in salt equilibrium are involved, the principal factor responsible for the increased rennet coagulation time of heated milk is intermolecular disulfide bond formation between \( \kappa \)-casein and \( \beta \)-lactoglobulin or \( \alpha \)-lactalbumin (88, 101, 148, 198, 203, 204, 241, 242).

Current information on the influence of heat treatment on the primary phase of rennet action is inconclusive: although most authors [e.g., (88, 148, 206, 207, 221, 241, 242)] have shown that the primary phase of rennet action is inhibited in heated milk, Marshall (140) found little difference in the rate or extent of formation of
p-κ-casein in milk heated at 85°C for 30 min compared with raw milk, although there was significant inhibition in milk heated at 75°C for 30 min. It is generally agreed that the secondary (nonenzymatic) phase of rennet coagulation is adversely affected by severe heat treatments. The strength of rennet gels is also adversely affected by heating. The adverse affects of heating can be reversed by acidification before or after heating or by addition of CaCl₂ (9, 140, 206, 221).

Secondary (Nonenzymatic) Phase of Coagulation

Proteolysis of κ-casein reduces the zeta potential and steric stabilization of the casein micelles. When approximately 85% of the ω-casein has been hydrolyzed, the casein micelles begin to aggregate. The secondary (nonenzymatic) phase of coagulation will not be reviewed here [for reviews, (34, 35, 67, 239)].

Proteolysis During Ripening

Some cheese varieties, especially acid-coagulated cheeses, are consumed fresh, but the majority of rennet-coagulated cheeses are ripened (matured) for periods ranging from 4 wk to more than 2 yr; the duration of ripening is more or less inversely proportional to the moisture content of the cheese. During ripening, a multitude of chemical and biochemical changes occur in which the principal constituents of the cheese—proteins, lipids, and residual lactose—are degraded to primary products and later to secondary products. Among the principal compounds that have been isolated from several cheese varieties are: peptides, amino acids, amines, acids, thiols, thioesters (from proteins), fatty acids, methyl ketones, lactones, and esters (from lipids), organic acids, especially lactic acid but also acetic and propionic acids, carbon dioxide, esters, and alcohols (from lactose). In the right combinations, these compounds are responsible for the characteristic flavor of various cheeses.

Significance of Proteolysis

Proteolysis contributes to cheese ripening in at least four ways: 1) a direct contribution to flavor via, e.g., amino acids and peptides, some of which may cause off-flavors such as bitterness, [see (133, 134, 233)] or indirectly via catabolism of amino acids to amines, acids, thiols, thioesters, etc. [see (82, 116)], 2) greater release of sapid compounds during mastication (138), 3) changes in pH via the formation of NH₃, 4) changes in texture from breakdown of the protein network, increase in pH and greater water binding by the newly formed amino and carboxyl groups (121). Although the ripening of some varieties (e.g., Blue, Romano, Parmesan) is dominated by the consequences of lipolysis, proteolysis is more or less important in all varieties. In the case of Cheddar and Dutch-type cheeses, and probably other varieties, many authors think proteolysis is the major biochemical event during ripening. A high correlation exists between the intensity of Cheddar cheese flavor and the concentration of free amino acids (7, 8). Attempts have been made [e.g., (174, 195)] to develop proteolytic indices of cheese maturity; although indices of proteolysis correlate well with age and maturity, they fail to detect off-flavors and should therefore be regarded as complementary to organoleptic assessment of quality (7, 8).

Proteolysis in cheese is a popular research subject and a considerable amount of information is now available on the level and type of proteolysis in the principal cheese groups [reviews include (22, 42, 64, 116, 182)]. This review is not intended to be comprehensive but rather to complement earlier ones.

Proteolytic Agents in Cheese

Four, and possibly five agents are involved in the ripening of cheese: 1) rennet or rennet substitute (i.e., chymosin, pepsin, or microbial proteinases), 2) indigenous milk enzymes, which are particularly important in raw milk cheeses but are also important in pasteurized milk cheese, especially those subjected to high cook temperatures, 3) starter bacteria and their enzymes, which are released after the cells have lysed, 4) enzymes from secondary starters (e.g., propionic acid bacteria, Brevibacterium linens, yeasts and molds, such as Penicillium roqueforti and Penicillium candidum) are of major importance in some varieties, 5) nonstarter bacteria (e.g., organisms that either survive pasteurization of the cheese milk or gain access to

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the pasteurized milk or curd during manufacture; on death, these cells lyze and release enzymes. The contribution of enzymes from nonstarter bacteria to cheese quality is controversial; there is a commonly held view that Lactobacilli (in the case of Cheddar and Dutch cheeses), Pediococci, and Micrococci probably have negative effects on cheese quality, although they almost certainly contribute to the intensity of cheese flavor.

There has been interest for 30 yr in devising model systems that would permit quantitation of the contribution of each of these five agents to the primary aspects of cheese ripenings (i.e., proteolysis, lipolysis, and glycolysis) and to the secondary reactions. In order to do this, rather interesting techniques and systems have been devised that, with a little effort and luck, permit a good estimation of the contribution of each of the principal agents to cheese ripening.

Nonstarter bacteria can be excluded by an aseptic milking technique and the use of aseptic vats. If cows are carefully selected, milk essentially free of microorganisms, and certainly free of those capable of growth in milk, can be obtained (104, 107). No microorganisms were detected in such milk after pasteurization at 72°C for 15 s (107). However, heat treatment of 83°C for 15 s is required to give a 10⁶ reduction in the population of some nonstarter lactic acid bacteria (216), indicating the importance of low initial counts. Contamination of the milk can be avoided by using an aseptic vat system, at least five of which have been developed and used successfully (26, 105, 122, 137, 173, 226). Antibiotics are usually included, and are probably necessary, especially for starter-free cheese; starter bacteria produce a range of antibiotics, which very effectively inhibit the growth of nonstarter bacteria.

The principal function of starter, i.e., to produce acid, can be replaced by the use of either a preformed acid or an acidogen, usually glucono-δ-lactone (GDL) (136). When added to milk, GDL hydrolyzes to gluconic acid, reducing the pH at a much faster rate than in biologically acidified cheese (164). A lower than normal pH at draining causes greater retention of chymosin in the curd (31, 89) and the solution of colloidal calcium phosphate from the micelles which disintegrate too soon and become susceptible to proteolysis (164). The pH profile of biologically acidified Cheddar cheese can be simulated closely by incremental additions of lactic acid and GDL to milk and curd, respectively (164).

Production of a coagulant-free cheese is rather complicated; obviously, a coagulant is necessary to produce a rennet cheese, but a number of techniques have been developed to inactivate the rennet after hydrolysis of κ-casein: 1) porcine pepsin may be used as coagulant; this enzyme is more sensitive to high pH than is chymosin and is rapidly denatured at pH 7. O'Keefe et al. (162) used this approach to produce coagulant-free curd; after cutting the renneted milk gel, the pH of the curd-whey mixture was increased to about 7, and the temperature increased rapidly to 40°C to inactivate the pepsin. 2) An alternative approach (224) is to use calcium-depleted milk, which does not coagulate when renneted and can be heated at 72°C for 20 s to inactivate the rennet. The heated, renneted milk is cooled to <15°C, CaCl₂ added, and the temperature raised >25°C by dielectric heating under quiescent conditions. We have tried this system but have experienced extensive syneresis during dielectric heating. 3) Piglet chymosin hydrolyzes bovine κ-casein quite effectively but appears to be incapable of hydrolyzing α₂- or β-casein in cheese curd; the potential of this approach has been demonstrated in small-scale experiments (151). 4) Immobilized rennets, which would hydrolyze κ-casein without being incorporated in the curd, would appear to offer an ideal method for producing rennet-free curd. Although there are numerous reports that immobilized rennets can coagulate milk, it appears likely that some enzyme leaches from the support and that coagulation is in fact caused by soluble enzyme that had been immobilized (21, 35, 58). This approach is, therefore, unsuitable for the preparation of rennet-free cheese.

Direct assessment of the contribution of indigenous milk proteinase, plasmin, may necessitate inactivation of this enzyme which is heat-stable and survives even UHT sterilization. It could probably be inhibited by employing soybean trypsin inhibitor, but I am not aware of any studies in which this approach has been evaluated.
Assessment of Proteolysis in Cheese

Several approaches have been adopted to monitor quantitatively proteolysis in cheese during ripening: 1) solubility of peptides in various solvents or precipitants, 2) liberation of reactive functional groups, 3) various forms of chromatography, and 4) various forms of electrophoresis. The literature has been reviewed by Grappin et al. (64) and Rank et al. (182), and only a summary and an update are given here.

Solubility

Several protein solvents and precipitants have been used to extract or fractionate cheese nitrogen, including: water; buffers near pH 4.5; 5% NaCl; 2, 5, 10, or 12% TCA; 5% phosphotungstic acid; 2.5% sulphosalicylic acid; picric acid; ethanol; ethanol-acetone; chloroform-methanol.

Extraction Methods

Homogenization of cheese in water, probably the simplest way of preparing a soluble N fraction, has been used by several investigators [e.g., (18, 47, 79, 111, 165, 186, 208)]. These authors used different procedures, which should be standardized for comparable results; an attempt to do this was made by Kuchroo and Fox (111). The approximate pH of the water extract from internal bacterially ripened cheeses (e.g., Dutch, Swiss, Cheddar) is 5.2 and there is very little difference between the levels of N soluble in water or in pH 4.6 buffers (111). However, in the case of cheeses in which the pH increases markedly during ripening (e.g., Camembert, Brie, Blue), the water-soluble N is much higher than the pH 4.6-soluble N. Some investigators [e.g., (71, 95, 218, 219)] dissolved cheese in citrate buffer, pH 7, and then adjusted the pH of the dispersion to pH 4.4 to 4.6 with HCl. This method gives somewhat higher values for pH 4.6-soluble N than extraction methods; it may also be easier to standardize, although more difficult to perform. O'Keeffe et al. (165) adjusted the pH of the water extract of cheese to pH 9.0, and after holding at this pH for 1 h, adjusted the pH to 4.6 with HCl; the principal objective was to inactivate rennet but some dissociation of peptides was also achieved.

Some workers have endeavored to maintain the ionic composition of the aqueous phase of cheese when preparing soluble N. Thus, Dahlberg and Kosikowski (33) extracted cheese with a solution containing 14.4 g acetic acid, 34 g sodium acetate, 3 H2O, 11.75 g NaCl, and 2.23 g CaCl2 (anhydrous)/L. The pH of this solution is 5.5 (109). Kuchroo and Fox (111) reported that soluble N obtained by this method was approximately 30% that of water soluble N. Noomen (158) and Visser (225) extracted soluble N with a solution containing .137 mol CaCl2 and .684 mol NaCl/L and adjusted to pH 5.1.

Extraction of soluble N with 5% NaCl was used by Chakravorty et al. (24) and Gupta et al. (77); both groups found very high values for brine-soluble N, and this was confirmed by Reville and Fox (188), who reported that 5% NaCl solubilized 92% of the total N in a 10-wk-old Cheddar. A 5% NaCl extract might provide a good starting point for further fractionation but such does not appear to have been reported. Presumably, the inclusion of CaCl2 in the NaCl brine (33, 158, 225) greatly increases the selectivity of this method; indeed, Kuchroo and Fox (112) found that only 60% of the water-soluble N of Cheddar cheese was soluble in .1 M CaCl2.

Several other approaches have been used to prepare water-soluble extracts of cheese. For example, McGugan et al. (138) centrifuged cheese (presumably grated) at 30,000 × g for 25 min (temperature not stated), mainly to separate the fat and aqueous phases. The latter was extracted repeatedly with a mixture of methanol, methylene chloride, and water. A water-soluble fraction was prepared by diluting the solvent layer, obtained on centrifugation, with water. This method was also used by Aston and Creamer (6) who showed by HPLC that the water-soluble fraction was not markedly different from that prepared by a simpler direct water extraction procedure. Mabbitt (135) prepared a "soluble" fraction from cheese by pressing finely ground cheese, mixed with sand, at 1 ton/in2; the expressed liquid was centrifuged and filtered through a Seitz filter.

Harwalkar and Elliott (81) extracted freeze-dried, blended cheese with 2:1 chloroform-methanol. Addition of water to the extract created a biphasic system with lipid in the lower
chloroform layer. Removal of methanol, by vacuum evaporation, from the aqueous methanol layer caused precipitation of peptide material that was not characterized. The supernatant was very bitter and astringent. The astringent factor could be isoelectrically precipitated at pH 6 to 7, and both soluble and insoluble fractions were further purified by gel filtration. The astringent fraction was further purified and partially characterized by Harwalkar (80). Chloroform extraction of an aqueous extract of cheese had been used earlier by Raadsveld (179) to concentrate bitter peptides from Gouda cheese, and chloroform-methanol (2:1) was used by Visser et al. (233) to isolate bitter peptides from casein hydrolyzates. Rank et al. (182) report that more N is consistently extracted by chloroform-methanol than by direct water extraction, presumably due to better extraction of hydrophobic peptides; comparison of the peptide profile of chloroform-methanol and water extracts has not been made.

-Butanol has been used (149) to extract bitter peptides from casein hydrolyzates but I am not aware of its use in cheese.

Fractionation of Extracts

Various protein precipitants have been used to fractionate the water-soluble or pH 4.6-soluble extracts. A fraction called proteose-N was prepared by Chakravorty et al. (24) by removing albumins and globulins from a citrate-HCl extract of cheese by heat denaturation and filtration.

Several protein precipitants, especially TCA (2 to 12%), have been used to precipitate medium and small peptides in the water- or pH 4.5-soluble extract of cheese. One of the principal disadvantages of TCA is the need to remove it before studying the TCA-soluble fraction, e.g., by chromatography or electrophoresis. Dialysis is usually unsuitable because many of the small peptides and free amino acids will be lost; the sample may be freed of TCA by repeated ether extraction, gel filtration, or ion exchange chromatography.

We prefer to use 70% ethanol instead of 12% TCA; both reagents give approximately the same degree of precipitation, although not necessarily of the same peptides, and the ethanol may be readily removed by evaporation (112). Another advantage of ethanol, in our experience, is that it gives very clean fractionation of peptides between supernatant and precipitate; with TCA, many peptides appear to partition between soluble and insoluble fractions.

Precipitation and extraction of cheese or its water-soluble fraction with ethanol has, in fact, been used in several studies. Thus, Ismail and Hansen (95) extracted the free amino acids from a pH 4.6 filtrate of a trisodium citrate solution of cheese by means of 80% ethanol for analysis using an autoanalyzer. Poznanski et al. (178) fractionated TCA-soluble N, after extraction of the TCA with ether, by making to 90% ethanol or 75% acetone. Gonashvily (63) fractionated the water-insoluble fraction of cheese by making it to 65% ethanol while Aston and Creamer (6) fractionated water-soluble extract with methanol (33%).

More discriminating precipitants include phosphotungstic acid (PTA)-H2SO4, 5% sulfosalicylic acid (SSA), or 1% picric acid. Picric acid is supposed to be the most discriminating protein precipitant and is reported to precipitate all nitrogenous compounds except amino acids. This does not appear to have been demonstrated for cheese, although Reville and Fox (188) found that picric acid extracts contained the lowest level of soluble N for cheeses ranging from 2 to 18 mo; however, the extracts were not studied further. Salji and Kroger (193) also used picric acid to fractionate cheese N for amino acid analysis. Picric acid interferes with the determination of soluble N by spectrophotometric or Kjeldahl methods.

Formation of amino acid N is commonly used as an index of ripening and precipitation of water extracts with a mixture of PTA-H2SO4 has been widely used too prepare an amino N fraction (71, 106, 160, 208, 225). The precision and accuracy of this procedure were investigated by Jarrett et al. (96), who found that lysine and arginine were not soluble in this reagent, whereas certain peptides with MW <600 might be soluble. The PTA-soluble N was a good index of cheese maturity (96). In our experience, precipitation is not quite so clear-cut; some free amino acids partition between the PTA supernatant and precipitate, and some adjustment for this is necessary. The PTA-soluble peptides have not been characterized.

Sulfosalicylic acid (2.5%) has also been used (181, 186) to prepare cheese samples for amino
acid analysis, but Kuchroo and Fox (111) found this reagent precipitated only 10% of the water-soluble N. Hickey et al. (83) extracted the free amino acids from cheese by macerating the sample (10 g) with 33 ml .15 M Ba(OH)$_2$ and 33 ml .14 M ZnSO$_4$; however, no information is given on how thoroughly the amino acids were extracted or whether the extracts also contained peptides (the amino acids were analyzed by autoanalyzer). I am not aware of other studies in which these reagents were used. Other methods used to prepare fractions for amino acid analysis include heating a water extract (79); an 80% ethanol-soluble fraction of WSN (18), an 12% TCA-soluble fraction of WSN (16).

Kuchroo and Fox (112) assessed equilibrium dialysis as a means of partitioning medium from small peptides; resolution appeared to be adequate, but the procedure is very slow, requiring 3 or 4 d. Ultrafiltration through a 10,000-dal membrane is currently used in our laboratory to fractionate water-soluble peptides; the retentate contains all the peptides detectable by gel electrophoresis. However, potential problems include the rejection of hydrophobic peptides by UF membranes, aggregation, and hence, rejection, of small peptides in the absence of dissociating solvents; neither of these aspects has been studied to date. Neither has the use of a sequence of membranes with a range of porosities to achieve greater fractionation been investigated. Obvious advantages of UF are that it is possible to work on a large scale and no solvents or precipitants are used, both of which facilitate taste panel work. Our laboratory has found that the UF permeate is fully soluble in 12% TCA, but the retentate may be partitioned into two clearly different fractions by making to 2% TCA (Figure 1). Aston and Creamer (6) used UF of the water-soluble fraction through a 1000-dal membrane prior to resolution of the permeate by HPLC. Diafiltration using a 500-dal membrane has been used to remove very small molecules from a bitter hydrophobic peptide preparation (228, 233).

**Formation of Reactive Groups**

Proteolysis may be assessed by monitoring the liberation of amino or carboxyl groups. The former have been monitored in cheese by reaction with trinitrobenzene sulphonic acid (114, 194), fluorescamine (31), or ninhydrin (168) with very satisfactory results. These reagents can be applied to cheese or fractions thereof; when applied to cheese, they have the obvious advantage of measuring a direct consequence of proteolysis (e.g., formation of amino groups) and are not dependent on an indirect effect (e.g., solubility in some particular solvent). The formol titration, which was used by Vakaleris et al. (218), also falls into this category as do dye binding methods (110, 193). Formation of ammoniacal N has been used in a few studies [e.g., (168)].

**Measurement of the tyrosine content of alcohol-, TCA-, or water-soluble extracts is a well-established traditional method of assessing proteolysis. This can be done by measuring absorbance at 280 nm or more specifically by using the Folin-Ciocalteau reagent. Absorbance of UV light has been widely used to monitor proteolysis in cheese; for example, Vakaleris and Price (219) measured soluble tyrosine and tryptophan in cheese by absorbance at 270 and 290 nm and fitted the data to a set of quadratic equations; the Folin reagent had been used less widely [e.g., (194, 205)]. These methods are clearly easier and faster than the determination of soluble N.**

**Chromatography**

Various forms of chromatography have been used to fractionate cheese N or, more usually, fractions thereof.

**Paper and Thin-Layer Chromatography.** Many early workers on free amino acids in cheese used paper chromatography [e.g., (16, 18, 108, 130)]. Thin-layer chromatography could be used for the same purpose but has been used more commonly for the resolution of small peptides [e.g., (47, 232, 233)].

**Gel Permeation Chromatography.** Probably the first application of gel filtration to cheese ripening was by Lindqvist (125), who fractionated Svecia cheese, dissolved in EDTA and defatted by centrifugation, on Sephadex G25; seven peaks were resolved, the major one being eluted at the void volume. Since then, gel filtration on various types of Sephadex has been used widely (6, 47, 57, 66, 71, 72, 81, 90, 126,
Foster and Green (57) attempted to develop a quantitative method for monitoring proteolysis in cheese based on chromatography of a solution of defatted cheese in .1 M Tris-citrate buffer, pH 8.6, containing 1 mmol Na₂ EDTA and 6 mol urea/L in Sephadex G-100. However, the method did not resolve α₁ and β-caseins and their larger breakdown products or para-κ-casein. The method was used by Green and Foster (66) to monitor proteolysis in cheeses made with calf rennet or pig pepsin. In our own experience, gel permeation chromatography is too slow to be suitable for large numbers of samples and the use of buffers containing 6 M urea causes very slow flow rates due to high viscosity. Kuchroo and Fox (113) concluded that gel filtration was an effective method for resolving the water soluble fraction, although homogeneous fractions were not obtained, probably due to aggregation of peptides. Amino acids can be resolved by chromatography on Sephadex G-15, presumably as a result of interaction with the gel matrix (6).

Peptides were isolated on a column of Cu-Sephadex by Ney (155) but the Cu complexes of amino acids and peptides have been resolved on Sephadex QAE-A25 (177).

Cellulose Derivatives. Although DEAE cellulose is widely used to fractionate milk (and other) proteins, it has received very limited application to the fractionation of cheese peptides. Creamer and Richardson (32) isolated α₁-I casein from cheese by chromatography on DEAE cellulose and Kuchroo and Fox (113) obtained good resolution of 70% ethanol-soluble and insoluble fractions of the water-soluble N on this medium. The technique is quite effective for the 2% TCA soluble and insoluble fractions of the UF retentate of the water soluble N on this medium. The technique is quite effective for the 2% TCA soluble and insoluble fractions of the UF retentate of the water soluble N. A major attraction of chromatography on DEAE is the possibility of fractionating relatively large samples compared with most other chromatographic methods. Mulvihill and Fox (152) fractionated the peptides produced form α₁-casein by chymosin on phosphocellulose, but this medium does not appear to have been applied to cheese.

Hydrophobic Chromatography. Kuchroo and Fox (113) fractionated the water-soluble N into two well-resolved fractions by hydrophobic chromatography on phenyl or octyl Sepharose CL-4B and Visser et al. (233) isolated bitter peptides by chromatography on Sephadex LH20.

High Performance Liquid Chromatography. As yet, HPLC has been applied by only a few authors to the fractionation of cheese peptides, but very promising results have been obtained (6, 25, 99, 174). Other studies on the use of HPLC or fast performance liquid chromatography (FPLC) to fractionate milk proteins or peptides include references (10, 13, 14, 19, 20, 93, 222, 237). A long-standing problem in the study of cheese ripening has been the availability of an easy quantitative method for the large water-insoluble peptides in cheese, e.g., residual α₁- and β-caseins. HPLC would appear to be suitable for this purpose but I am not aware of studies on this. Ramos et al. (181) resolved the amino acids in the sulfosalicylic acid-soluble fraction of cheese by HPLC.

Ion Exchange Chromatography. Huber and Klostermeyer (90) purified the peptides in a fraction of water-soluble N obtained by gel filtration by chromatography on Dowex 50 W × 2. Mabbutt (135) fractionated the amino acids in a cheese extract by chromatography on Dowex 50 using a manual method and autoanalyzers have been used in quite a few studies (e.g., 83, 95, 193, 232) for analysis of free amino acids in cheese.

Silica Gel. Chromatography on silica gel G, with n-propanol/water (7:3, vol/vol) as eluent, was used by Visser et al. (232) to fractionate bitter peptides from rennet-treated casein.

Electrophoresis. Electrophoresis is probably the most widely used technique for monitoring primary proteolysis in cheese. The first study appears to be that of Lindqvist et al. (131), who used paper electrophoresis to study proteolysis in several cheese varieties. This was followed by a series of studies from the same laboratory on proteolysis in cheese and model systems using free boundary electrophoresis [see (127, 128, 129)].

Electrophoresis in polyacrylamide, first applied to cheese by Melachouris and Tuckey (144) and Ledford et al. (123), has become the most widely used electrophoretic technique, although electrophoresis in starch gels is preferred by some investigators. The literature on the application of electrophoresis to the study of proteolysis in cheese has been re-
Figure 1. Fractionation scheme for cheese nitrogen. DEAE = Diethylaminoethyl, FPLC = fast protein liquid chromatography.

viewed by Shalabi and Fox (202). They also compared the efficiency of various protocols, buffers and staining procedures and recommend the stacking gel system of Andrews (4), using polyacrylamide gels containing 6 M urea. The direct staining protocol of Blakesley and Boezi (15) using amino black or PAGE Blue gave very good results. The PAGE method of Andrews (4) also resolves water soluble peptides and UF retentate very effectively but the peptides in UF permeate do not fix on polyacrylamide gels. The PAGE method has been criticized (57) as being nonquantitative, but good quantitative results have been obtained using it (31, 38, 212). Isoelectric focusing has been used by a few investigators (215) as has high voltage paper electrophoresis (72, 81, 90, 112, 232). In our experience, the latter has limited value for fractionation of peptides, at least as a sole technique (112).

Overall Fractionation Scheme

Over the past few years we have been trying to devise a scheme that would fractionate cheese N into individual, homogeneous peptides and amino acids. The system we presently use is shown in Figure 1. A mixture of two parts of water and one part of cheese is homogenized in a stomacher. After holding at 40°C for approximately 30 min, the homogenate is centrifuged and the supernatant filtered. For quantitative results, the pellet should be reextracted as we have described. As mentioned earlier, if the pH of the cheese is high, it may be necessary to adjust the pH of the filtrate to 4.6. The water-soluble fraction is ultrafiltered through a 10,000-dal membrane. The permeate, which contains mainly amino acids and some peptides, may be fractionated on Sephadex G-10, normally into four or five peaks, the first of which contains a single peptide that consists of about six amino acids; the other peaks contain only free amino acids. It is planned to assess the application of HPLC for the fractionation of UF permeate.

The UF retentate is fractionated by 2% TCA. Both soluble and insoluble fractions are further resolved by chromatography on DEAE cellulose. Some of the fractions appear almost homogeneous on PAGE. It is planned to subject these fractions to HPLC. Obviously, other techniques, e.g., fractionation with ethanol, acetone, chloroform, PTA, SSA, gel filtration, FPLC, prepartive gel electrophoresis, or TLC could probably by used with effect to resolve the peptides in UF permeate and retentate.

Proteolytic Systems in Cheese

Rennet. Only about 6% of the rennet added to cheese milk is retained in the curd. The proportion of chymosin retained increases markedly as the pH at draining decreases, but the retention of microbial rennets appears to be independent of pH (31, 89). According to Green and Foster (66), little active porcine pepsin remains in Cheddar cheese, but O'Keeffe et al. (163) found significant activity. All the principal commercial rennets are acid proteinases that show specificity for peptide bonds to which hydrophobic residues supply the carboxyl group; all show generally similar specificities on the B-chain of insulin (65). The commercial fungal rennets (ie., from Mucor) are less active on αs1-casein than chymosin, and their specificities are distinctly different (175).

The proteolytic specificity of chymosins and pepsins on individual caseins in solution and in cheese have been established, as have the influence of pH and NaCl thereon; the literature has
been reviewed by Grappin et al. (64). There are several chymosin-susceptible bonds in both α_{s1}- and β-caseins (172) but only a few of these are hydrolyzed in cheese. In all varieties, α_{s1}-casein is the principal target for proteolysis in cheeses made with commercial rennets; in fact rennets are selected, *inter alia*, on the basis of low activity of β-casein. The most susceptible bond is Phe_{23}-Phe_{24} or Phe_{24}→Val_{25}, hydrolysis of which yields α_{s1}-I. This primary cleavage appears to be responsible for the softening of the cheese body during the early stages of ripening (30, 39). α_{s1}-I-Casein is further hydrolyzed, probably to α_{s1}-V (f29/33-169) or α_{s1}-VII; further work is required to establish the structure of these secondary products more precisely. Obviously, the situation is complicated by the activity of indigenous milk and starter and non-starter proteinases (discussed later in this paper).

Although three or four bonds in β-casein in solution are readily hydrolyzed by chymosin and other rennets (19, 231), these are not hydrolyzed in cheese by chymosin or pepsins, possibly due to intermolecular hydrophobic interactions between the hydrophobic C-terminal region of β-casein, which contains the chymosin-susceptible bonds. The NaCl also inhibits the hydrolysis of β-casein by chymosin (61, 212) but even in salt-free cheese, little hydrolysis of β-casein by chymosin occurs (176).

The action of chymosin and other rennets on α_{s2}-casein has received little attention, but it appears to be very resistant in cheese. The PAGE indicates the presence of peptides with electrophoretic mobilities similar to α_{s1}-casein, but these could be peptides that originate from α_{s1}-casein. Further work in this area is required. Para-κ-casein appears to be resistant to chymosin (66). Although the γ-caseins contain the chymosin-susceptible bonds of β-casein, γ-caseins accumulate in cheese during ripening; presumably these bonds are inaccessible in γ-caseins, as they are in β-casein.

Indigenous Milk Proteinases. Plasmin is the principal indigenous proteinase in milk, which also contains an acid proteinase and possibly thrombin and a number of peptidases. Reviews include Humbert and Alais (91) and Grufferty and Fox (75). The action of plasmin on individual caseins was described earlier. In milk, plasmin is associated almost exclusively with the casein micelles and is therefore present in rennet cheese curd. Plasmin dissociates from the micelles as the pH is reduced: Richardson and Elston (190) report that some dissociation occurs even at pH 5.7, but Grufferty and Fox (76) found no dissociation >pH 4.8; the latter workers are probably correct, because the results of Richardson and Elston (190) are based on the plasmin activity of isoelectric casein prepared from preacidified milk whereas Grufferty and Fox (76) measured plasmin activity in ultracentrifugally prepared micelles.

The plasmin activity in Swiss and Cheddar cheeses is equivalent to 6 to 13 and 3 to 4.5 μg/g, respectively (191). The higher pH of the former at draining compared with Cheddar was considered (191) responsible for the difference, but if the results of Grufferty and Fox (76) are correct, then all rennet cheeses should contain all the plasmin in milk. An alternative explanation may be that since plasmin is also dissociated from casein micelles by NaCl (76), some plasmin may be dissociated from Cheddar curd, which is salted before pressing and lost in the press whey while both Swiss and Dutch, which are brine-salted, may retain most of the plasmin in milk. This hypothesis requires experimental verification. Considerable amounts of γ-caseins accumulate in both Dutch and Swiss cheeses, indicating plasmin activity. The pH of most cheese varieties is unfavorable to the action of plasmin, the pH optimum of which is ~ 7.5; however, plasmin may be more important in cheese varieties in which the pH increases markedly during ripening. Plasmin is probably significant in Swiss and other high cook varieties in which the coagulant is denatured (143). Although direct experimental evidences is lacking, the general view is that plasmin does not have an indispensable role in cheese ripening [see (159)], more definitive studies via direct experimentation are necessary.

The specificity of acid milk proteinase is generally similar to that of chymosin, with a preference for α_{s1}-casein (100). Acid proteinase is also associated with the casein micelles, but there appears to be no information on the effect of pH or other factors on the association. Although research on rennet-free cheeses indicates the formation of α_{s1}-I-casein (227), suggesting proteolysis by acid milk proteinase, this enzyme is unlikely to make a significant contri-
bution in most cheese varieties under normal circumstances due to the predominant action of the coagulant on $\alpha_{s1}$-casein. A peptide with electrophoretic mobility similar to $\alpha_{s1}$-I casein is formed in Swiss cheese (210), suggesting that acid milk proteinase may be responsible, although it may be formed by the action of microbial proteinases. Definitive research on the contribution of acid milk proteinase to cheese ripening is lacking.

There is no information on the possible contribution of thrombin and milk peptidases to cheese ripening; it is not even known whether they accompany the curd or are lost in the whey during cheese making. Their low concentrations and activities in milk would suggest that even if they accompany the curd, their contribution to proteolysis during ripening is probably not very significant.

**Starter Proteinases.** Although the lactic acid bacteria used as cheese starters are only weakly proteolytic, they contain a range of proteinases and peptidases that together are theoretically capable of hydrolyzing caseins completely to amino acids (150). This view is supported by the fact that most of these bacteria are capable of extensive growth in milk, which contains free amino acids and small peptides sufficient to support the growth of these bacteria to only about 25% of the population achieved in milk; proteinase-negative (Prt-) variants attain only 5 to 25% of the maximum cell density reached by the parent strain [see (211, 213)].

Early literature on the proteolytic systems of starter bacteria and their activities on caseins was reviewed by Castberg and Morris (22). Considering the generally assumed importance of starter enzymes in cheese ripening, it is surprising that so little definitive information on the characteristics of the enzymes and their role is available, although progress has been made in recent years. The literature has been comprehensively reviewed (115, 118, 211, 213). There are very few reports [e.g., (94)] on the microbial proteinases actually present in cheese. Work on several varieties of cheese made with a controlled microflora indicates that starter proteinases/peptidases are primarily responsible for the formation of small peptides and free amino acids, [i.e., for TCA soluble N (43, 71, 106, 163, 165, 225, 227)]. Likewise, these studies indicate that starter proteinases contribute little to the formation of larger peptides, i.e., pH 4.6 or water-soluble N. However, mesophilic streptococci produce endopeptidase capable of hydrolyzing intact caseins, especially $\beta$-casein; apparently only relatively few strains are capable of hydrolyzing $\alpha_{s1}$-casein. The inability of starter proteinases to hydrolyze $\alpha_{s1}$-casein is probably not significant since this protein is rapidly hydrolyzed by chymosin and other rennets. In Dutch and Cheddar cheeses, the concentration of $\beta$-casein decreases slowly during ripening with the formation of little of $\beta$-I (suggesting the lack of chymosin activity) or of $\gamma$-caseins (indicating little plasmin activity). Although Visser and de Groot-Mostert (227) report that the rates of hydrolysis of $\alpha_{s1}$ and $\beta$-caseins are similar in normal aseptic and aseptic starter-free Gouda cheese, it appears reasonable to suggest that the slow hydrolysis of $\beta$-casein is due to starter or other bacterial proteinase(s) in commercial cheese. There is little information to date on the specificity of the starter proteinases on casein and the products of their action in cheese have not been identified. The cell-wall proteinase of *Strep. lactis* NCDO763, purified and characterized by Monnet et al. (147), has been reported (146) to cleave five bonds in $\beta$-casein, i.e., Ser-Gln (166-167), Glu-Lys (175-176), Gln-Arg (182-183), Tyr-Gln (193-194) and Ile-Ile (207-208). These bonds are in the very hydrophobic region of $\beta$-casein, the region in solution cleaved by chymosin. Because chymosin does not hydrolyze $\beta$-casein in cheese, possibly because of intermolecular hydrophobic interactions (as already discussed), it will be interesting to establish whether the starter proteinases are able to hydrolyze this region of $\beta$-casein in cheese.

Starter bacteria attain maximum numbers in Cheddar and Dutch cheeses at or shortly after the end of manufacture, and viable numbers decline quickly thereafter (142, 226). It is generally assumed that the cells lyse after death, releasing the intracellular enzymes that diffuse into the surrounding environment. Electron photomicrographs show that the bacterial cells remain largely intact, presumably due to the gel-like structure in which they are embedded and the high solute concentration in the aqueous environment (217). This suggests that intracellular enzymes, e.g., peptidases, may not have access to substrate. An intracellular dipeptidase
was extracted from cheese, suggesting that the cells had lysed and that intracellular enzymes were free (117, 119). However, it is claimed (213) that the extracting solution would have caused lysis of the cells. Recent studies (51, 52) indicate that certain strains/mutants of *Strep. lactis* and *Strep. cremoris* in broth or skim milk cultures lyse very rapidly on reaching the cook temperature (38 to 40°C); however, these experiments need to be extended to cheese.

It is claimed in a recent report (167) that cheese made using a Prt- starter contained significantly higher concentrations of soluble N [pH 4.6 soluble; (219)] than cheese made using normal Prt+ starter; it is suggested that the higher concentrations of soluble N may be due to greater carryover of soluble N from the medium used to propagate the Prt+ starter. However, the concentrations of soluble N increased at similar rates during ripening, suggesting that starter proteinases play no significant role in proteolysis. This is in general agreement with the results on aseptic starter-free cheese. Use of a more discriminating criterion of soluble N, e.g., phosphotungstic acid, may have given interesting results. Addition of Prt- mutants (in addition to normal starter) has been proposed (8, 50, 68) as a means of accelerating ripening and shown to be effective, presumably as a result of intracellular peptides.

All investigators (43, 71, 106, 163, 165, 225, 227) agree that only very low concentrations of very small peptides and free amino acids are formed in aseptic starter-free cheese. Use of a more discriminating criterion of soluble N, e.g., phosphotungstic acid, may have given interesting results. Addition of Prt- mutants (in addition to normal starter) has been proposed (8, 50, 68) as a means of accelerating ripening and shown to be effective, presumably as a result of intracellular proteinases. Early proteolysis in blue cheeses is due to coagulant with the rapid hydrolysis of αs1-casein to αs1-1; presumably, starter proteinases also contribute. However, following mold growth and sporulation, the fungal enzymes dominate proteolysis in these cheeses, in which very extensive proteolysis occurs [see (70)].

Extensive proteolysis also occurs in surface mold-ripened cheeses. It was generally assumed that the extracellular proteinases secreted by *P. caseicolum* were responsible for this proteolysis, but it has been demonstrated that these proteinases diffuse only a very small distance from the surface. Most proteolysis within the cheese is due to coagulant, probably with a contribution from plasmin, especially when the pH rises during ripening [see (70)]. However, peptides produced by the action of the fungal proteinases have been identified in the core of Camembert cheese, presumably having been produced at the surface from which they diffuse into the cheese. Probably the most conspicuous result of fungal action is the increase of pH from the surface to the center. This is due initially to the oxidation of lactic acid, which migrates from the center to the surface, and, after lactic acid has been consumed, to the production on NH3 at the surface, which diffuses inward. As a consequence of the high surface pH, calcium phosphate precipitates at the surface, causing a concentration gradient, and consequently diffusion, of calcium phosphate from the center to the surface. Proteolysis and related events in Camembert-type cheeses have been reviewed by Gripon (70), Law (116), Lenoir (124), and Karahadian and Lindsay (102).

*Brevibacterium linens.* This strongly proteolytic organism colonizes the surface of smear-ripened cheeses and undoubtedly contributes to proteolysis and amino acid catabolism in such cheese. However, its proteinases are only superficially characterized. Reviews include those by Law (116) and Reps (187).

*Geotricum Candidium* and Other Yeasts. Yeasts of various species inhabit the surface of several smear and surface mold cheeses. Most yeasts are considered to be weakly or non-proteolytic. Some information on the contribution of yeast to cheese ripening is included by Gripon (70) and Reps (187).

**Extent of Proteolysis in Cheese.** The extent of proteolysis in cheese varies from very exten-
sive, (Blue) to relatively limited (Emmental, Romano, Parmesan). No attempt will be made here to review the extent and type of proteolysis in individual varieties; the interested reader is referred to Fox (59).

Accelerated Ripening. Cheese ripening is a slow, expensive, and not fully controllable process. Consequently, there is considerable industrial and academic interest in accelerating the process. Potential techniques that have been investigated include elevated ripening temperatures, increased starter numbers, Prt \textsuperscript{-} mutants (to augment normal starter), attenuated starter (to augment normal starter), and exogenous proteinase (a neutral bacterial proteinase being most effective). The literature has been reviewed by Fox (60).

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