THE EFFECT OF PROCESS PARAMETERS ON THE FLAVOR AND PROTEIN DEGRADATION IN QUICK RIPENED BLUE CHEESE

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ABSTRACT

THE EFFECT OF PROCESS PARAMETERS ON THE FLAVOR AND PROTEIN DEGRADATION IN QUICK RIPENED BLUE CHEESE

By

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The effect of various process parameters on the flavor and protein degradation in quick ripened (QR) Blue cheese was studied. It was observed that lowering the curing room temperature to 52 F (11 C) from the recommended 62 F (17 C) produced a cheese of higher quality that could be replicated from batch to batch. Salt was added using applications of 1/4, 1/4, and 1/2 of the total salt on days 7, 8, 9 or days 1, 9, 10 respectively to obtain a final salt concentration of 4%. The appearance of blue pigmentation in the cheese was used as an index for salting. Cooking the curd, following cutting, to 100 F (37 C) and holding at this temperature for one hour improved drainage of whey and reduced losses of fine curd particles. A Blue cheese of poor quality resulted when starter cultures, pasteurization or homogenization were deleted in manufacture. The addition of commercial lipases

did not improve the flavor of QR Blue cheese. A satisfactory QR cheese was produced from the white mutant of P. roqueforti.

As the pH values increased there was a corresponding increase in non protein nitrogen (NPN) during ripening of QR Blue cheese. The values for pH and NPN at the end of ripening were 5.9 and 45% respectively. Polyacrylamide gel electrophoresis demonstrated that QR Blue cheese ripening could be classified as non-specific since α_s- and β-casein disappeared from gels at approximately the same time.

Peptide mapping of QR Blue cheese and Blue cheese ripened by conventional methods showed that peptides in a molecular weight (MW) range of 700 to 5000 were similar in both cheeses. The complete MW profile of QR Blue cheese after 11 days of ripening showed approximately 25% of the protein existed with a MW of less than 700, 58% in the range of 700 to 5000 and 17% had a MW greater than 5000.

Free amino acids were quantitated in QR cheese and variations were noted in cheeses made in an identical manner. The amino acid content increased to 28% of the total protein after ripening 39 days. This compared to 16% after 11 days. The amino acids that were present in high concentrations in whole casein were generally present in high concentrations in QR Blue cheese.

The presence of tyramine was identified by mass spectrometry and quantitated by GLC. QR Blue cheese

increased in tyramine throughout ripening and had approximately 825 ug tyramine per gram of cheese after 39 days. Conventionally ripened (90 days) Blue cheese contained 40 ug tyramine per gram cheese at the time of sampling.

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INTRODUCTION

Blue mold cheeses have been steadily increasing in popularity and, concurrently, food products flavored with Blue mold cheeses have been in greater demand. The production costs for Blue cheese have generally been greater than for most other varities of cured cheese. The high labor requirements in manufacturing and curing Blue cheese have stimulated the development of mechanized procedures for hooping curds and shortened methods for manufacturing and curing. These innovations have included direct acidification, production of Blue cheese flavor constituents by fermentation and the "quick ripening" of loose curd Blue cheese.

The increasing demand for Blue cheese and for convenience foods by the consumer provides a potentially large market for Blue cheese which could be used for preparation of salad dressing and chip dips.

This project was undertaken to evaluate the effect of processing variables on the flavor of "quick ripening" Blue cheese and to follow protein degradation during ripening to better understand the nature of the proteolytic enzymes involved during ripening.

LITERATURE REVIEW

The term "Blue" as applied to cheese refers to the blue or blue green veining which permeates the curd of certain mold-ripened cheese. Cheeses which are classified as "Blue" are ripened by the mold Penicillium roqueforti; however, such cheese may take on one of many names depending on the country of origin (Scott, 1968). In North America, the term Blue is used to describe such cheese manufactured within this hemisphere.

The sharp, peppery flavor of Blue-veined or Blue mold cheeses has intrigued researchers for years. The flavor of Blue-veined cheese can originate from several sources. The most significant flavor compounds are produced by the metabolism of microorganisms and by the action of natural milk enzymes during the manufacture and curing of cheese. The complexity of the system is evidenced by such work as Day and Anderson (1965) where approximately 100 compounds were identified in the volatile fraction of Blue cheese. Aspects of the flavor and manufacture of Blue-veined cheese were reviewed by Bakalor (1962).

Flavor Components of Blue Cheese and Their Origin

Free Fatty Acids

The lipid components of the milk from which Blue cheese is made are probably the single most important source of the flavor associated with Blue cheese. Morris, Jezeski, Combs and Kuramoto (1955) reported that the pungent, peppery flavor of Blue cheese was due to the presence of butyric, caproic, caprylic and capric acids and their oxidative products. They reported finding an average of 0.77 mg butyric acid/g of Blue cheese made from pasteurized, homogenized milk. The average caproic and higher acid value (average molecular weight 228) was 24.93 mg/g of cheese. Sjöström and Willart (1959) found 1.22, 0.92, and 26.8 mg/g of acetic, butyric, and caproic and higher (average molecular weight 200) acids respectively in Blue cheese. The C_2-$C_{18:3}$ fatty acids in domestic Blue and imported Roquefort was quantitated by Anderson and Day (1965). Considerable variation in the fatty acid content of the cheeses was noted. A difference was also evident in the flavor and aroma of the cheeses. By calculating the mole percent of the free acids present in the cheeses, Anderson and Day (1965) demonstrated that octanoic and decanoic acids were proportionately more abundant than butyric acid in Roquefort cheese. The entire quantity of fatty acids reported probably did not exist as free acids in the cheese. Cheeses with a pH range of 5.3 to 5.6 may

have approximately 75 to 85 percent of their free fatty acids in the salt form, assuming an average pKa of 4.8 for the major acids. Salts of long chain fatty acids were soaps and as such possessed definite flavor properties. The odor resulting from the short chain acids would be reduced if these volatile acids were in the salt form.

Harper (1959) cautioned that even though fatty acids contribute directly to the flavor of Blue cheese, there must be a balance and blend to achieve the most desired flavor.

Glyceride Hydrolysis

The enzymes involved in the process of fat hydrolysis in cheese may originate from true milk lipases, microorganisms from the milk, and from the interior or the surface of the cheese (Stadhouders & Mulder, 1957).

The presence of more than one natural milk lipase system has been demonstrated by Tarassuk and Frankel (1957), Gaffney, Harper and Gould (1966), and Downey and Andrews (1969). These lipases have been shown by Schwartz, Gould and Harper (1956), and Chandon and Shahani (1963) to have the ability to hydrolyze milk fat within a pH range of 5.0 and 10. However, those lipases with a pH optimum of 7 and above could not continue to have a high activity in normal Blue-veined cheese (Bakalor, 1962).

When mixed triglycerides are hydrolyzed, there is an obvious preference for the molecules containing short

chain acids (Jensen, 1964). Milk lipase preferentially releases fatty acids from the primary positions of a triglyceride. Since butyrate appears to be predominantly a primary ester, this explains the apparent release of butyrate during lipolysis. Harwalker and Calbert (1961) reported that lauric and higher acids were the major acids released at all stages of lipolysis, but as lipolysis progressed the ratio of these acids to butyric decreased. Very little change was observed in the mole percentages of the other acid fractions.

Spores of Penicillium roqueforti are used to inoculate the curd in the manufacture of Blue-veined cheese. Penicillium roqueforti produces a water soluble lipase which hydrolyzes milk fat to produce the free fatty acids characterizing the sharp flavor of Roquefort cheese (Currie, 1914). Several workers have characterized two lipases associated with P. roqueforti; however, there has been some controversy as to the optimum pH of the isolated lipases. Morris and Jezeski (1953) characterized two lipases with pH optimum of 6.5 to 6.8 and 7.0 to 7.2. Reports by Imamura (1960a) and Imamura and Kataoka (1963b, 1966), indicated two types of lipase with an optimum pH of 6.5 and 7.5 respectively. Using butter oil as a substrate, Eitenmiller, Vakil and Shahani (1970) show the P. roqueforti lipase to be active over a pH range of 7.5 to 9.0. These authors (1970) suggested that differences in the pH optimum observed in their work, and in that of

other workers, may be due to use of different substrates, different assay conditions and different strains of mold.

Penicillium lipase was found by Shipe (1951) to hydrolyze tributyrin, tricaproin, tricaprylin, and tripropionin in this order of decreasing rates. Imamura and Kataoka (1963a), and Wilcox, Nelson and Wood (1955) reported that P. roqueforti lipase preferentially hydrolyze short chain fatty acids from butter fat.

Different strains of P. roqueforti show certain differences in their lipolytic properties (Thibodeau & Macy, 1942). The lipolytic properties of nine strains of P. roqueforti were examined by Prokš, Doležálek and Pech (1956). Chromatographic analyses showed differences in the ability of the nine strains to hydrolyze butyric, caproic and caprylic acids from butter fat. The results of Niki, Yoshioka, and Ahiko (1966) demonstrated that high lipolytic strains accumulate lower fatty acids rapidly, subsequently decomposing them into methyl ketones and other metabolic products. Strains with moderate proteolytic activity and strong lipolytic activity are the most suitable for the purpose of Blue cheese production. Therefore, proper strain selection is necessary for the production of a Blue cheese.

The pH and temperature of the system are important because they may influence the activity of the enzyme systems involved in ripening (Morris & Jezeski, 1953). Coulter, Combs, and George (1938b) observed a rapid

reduction to pH 4.7 during the first day of ripening. After three months, the pH had increased to 6.5, then decreased slowly to 5.7 at nine months. The marked rise in pH from about 2 weeks to 10 weeks was due to continuing proteolysis (Currie, 1914; Morris, Combs, & Coulter, 1951). Morris (1963) noted a tendency for the rate of rise of pH to decrease as the pH approached 6.5. The lipase systems in Blue cheese were active in the pH range occurring throughout curing. According to Morris et al. (1963), the free fatty acids increased in concentration during ripening, thus indicated lipase activity throughout the ripening period.

The temperature optima for the two mold lipases was found to be between 30-35 C by Shipe (1951) and 30-32 C by Morris and Jezeski (1953). Morris (1969) concluded, in a study of the effect of ripening temperature on the properties of Blue-veined cheese, that those cheeses ripened at 37 F (3 C) scored better than those ripened at 50 F (10 C) or 60 F (16 C) in relation to mold color, body, and flavor. Higher temperatures produced more rapid breakdown of fat and protein, but were found to be detrimental to the flavor, body, and color of the cheese.

Factors other than pH and temperature may also influence the microorganisms and enzymes present in a ripening cheese. Gould (1941) found NaCl inhibited milk lipase in raw homogenized milk and cream. Levels of 5 to 8 percent NaCl were sufficient to inhibit lipolysis almost

completely. Most organisms cannot survive salt concentrations greater than about 6 percent, and essentially none survive a concentration of 10 percent (Morris, 1969). P. roqueforti can grow at salt concentrations up to about 16 percent brine. A 10 percent brine is the concentration maintained in Blue-veined cheeses. Therefore, about a 4 percent salt concentration in a cheese of 40-44 percent moisture will provide the proper brine concentration to discourage contaminating molds, yeast and bacteria from growing in Blue-veined cheeses.

Another important factor involved in maintaining proper mold growth is the necessity of having exchange of carbon dioxide, produced by the mold, with oxygen from the air during ripening. Studies by Golding (1940a,b) revealed that mold would grow in a low (4.2 percent) oxygen concentration, but growth was inhibited by a large concentration of carbon dioxide. Low concentrations of carbon dioxide favored mold growth.

In a study of the hydrolysis of milk fat by microbial lipases, Khan, Chandon, Dill, and Shahani (1966) noted that free fatty acid composition in Blue cheese suggested that the lipase of P. roqueforti, as well as some other lipase, may be involved in the flavor development. Fryer and Reiter (1967) demonstrated that lactic streptococci and lactobacilli had lipolytic activity. Devoyod and Sponem (1970) isolated 81 strains of yeasts during the manufacture and ripening of Blue cheese.

Acids From Sources Other Than Milk Triglycerides

Some of the acids identified in Blue cheese do not occur in milk triglycerides or are present in very low concentrations (Anderson, 1966). Acids such as valeric (Thomasow, 1947), isovaleric and heptanoic (Coffman, Smith, & Andrews, 1960), and acetic (Simonart & Mayaudon, 1956) are in this classification. Many microorganisms have the ability to carry out glycolysis, which provides for the formation of lactic acid and its metabolities, acetic and propionic acid. Intermediate stages of the citric acid cycle provide succinic, pyruvic, fumaric, glyoxalic and alpha-ketoglutaric acid. Free amino acids are decomposed by trans- and deamination to form alpha-keto butyric, p-hydroxyphenyl pyruvic, p-hydroxybenzoic and other keto acids. Keto acids in Blue cheese can also be formed through citric acid fermentation and beta-oxidation (Harper, 1959). The value to aroma and taste of all these acids differed, but they may have contributed overall to the flavor of Blue-veined cheese.

Methyl Ketones

The contribution of methyl ketones to the flavor and aroma of Roquefort cheese, and the involvement of *P. roqueforti* in the production of methyl ketones, was indicated by Störkle (1924). Patton (1950) isolated 2-pentanone, 2-heptanone, and 2-nonanone from Blue cheese and concluded, as did Hammer and Bryant (1937), that these

methyl ketones, particularly 2-heptanone, have an aroma typical of Blue cheese. Prokš, Doležálek, and Pech (1959) found a direct relationship between the amount of methyl ketone present and the typical flavor of Roquefort-type cheeses. Acetone and 2-pentanone were among the neutral carbonyl compounds identified by Bassett and Harper (1958) in Blue cheese. The presence of 2-heptanone and 2-pentanone were noted by Scarpellino and Kosikowski (1961) in a concentrate of the volatile organic compounds in Blue cheese. Gas chromatographic and mass spectral identification of the components of Blue cheese aroma, by Day and Anderson (1965), confirmed the presence of C_3, C_5, C_6, C_7, C_8, C_9, C_{10}, C_{11}, and C_{13} methyl ketones in Blue cheese volatiles.

The ketone content of a well ripened Blue cheese was determined by Nesbitt (1953) to be about 60 to 80 ppm. Quantitation of the methyl ketones in Blue-veined cheeses, by Anderson and Day (1965), indicated a large variation in the quantity of ketones in different cheeses, with 2-heptanone being the predominant ketone in all samples. Although the cheeses varied in their respective methyl ketone contents, all the cheeses were judged as having an acceptable flavor.

Störkle (1924) attempted to determine the origin of the methyl ketones, 2-nonanone and 2-heptanone, that had been distilled from Roquefort cheese. A mechanism, advanced for the formation of these ketones, was the oxidation of the fatty acid to the beta-keto acid followed

by decarboxylation of the beta-keto acid. Girolami and Knight (1955) confirmed much of the earlier work by showing that resting cells from surface grown cultures of P. roqueforti formed a methyl ketone from individual fatty acids. There are conflicting reports on the ability of the vegetative cells of molds to produce methyl ketones. Störkle (1924) and Stokoe (1928) showed that when single fatty acids are used as substrates, certain mold mycelia produced methyl ketones. Gehrig and Knight (1958, 1963) demonstrated that the ability to form methyl ketones from fatty acids resided in the mold spore. The capacity to form methyl ketones disappeared rapidly and progressively as the spores germinated. The slow rate of formation of 2-heptanone from octanoic acid by washed spores suspensions of P. roqueforti was shown by Lawrence (1965) to be markedly increased by the addition of the same specific amino acids and sugars that have been shown to stimulate mold germination.

The fact that only methyl ketones of one fewer carbon atoms are produced from fatty acids, has been confirmed by Gehrig and Knight (1963) with P. roqueforti using [1-^{14}C]-octanoate as substrate. The 2-heptanone synthesized was unlabelled and $^{14}CO_2$ was produced. Lawrence (1966) found that the 2-heptanone formed from [2-^{14}C]-octanoate was radioactive and that some radioactive carbon dioxide was also present. Therefore, ketone formation and beta-oxidation proceed simultaneously. Gehrig and Knight

(1963) found that when one micromole of labeled sodium octanoate was used as a substrate for P. roqueforti, most of the radioactivity was present as carbon dioxide and no ketone was produced. At higher concentration (20 micromoles), part of the molecule appeared as 2-heptanone and part as carbon dioxide. Neither Gehrig and Knight (1963) nor Lawrence (1966) was able to discover any of the intermediate compounds of the beta-oxidation of octanoic acid. Beta-keto acids which are intermediates of fatty acid oxidation have been isolated from Blue cheese by Basset and Harper (1958). Lawrence (1963) established that a complete range of methyl ketones with odd numbers of carbon atoms from C_3 to C_{15} were formed during the heat treatment of butterfat. Van der Ven, Bergemann, and Schogt (1963) isolated from butterfat six even-numbered carbon (C_6 to C_{16}) beta-keto acids thus supporting the contention of Wong, Patton, and Forss (1958) that beta-keto esters are the precursors of methyl ketones liberated in butterfat upon heating in the presence of moisture. The amount of methyl ketones of intermediate chain length found in the fat phase of cheese eliminates the natural ketone precursor of milk fat as the direct and only source of ketones in cheese (Schwartz & Parks, 1963). These authors (1963) indicated that possibly the methyl ketone precursors in milk are metabolized by microorganisms and therefore make a small contribution to final methyl ketone levels.

Results of work by Anderson and Day (1966) indicate that the quantity of each methyl ketone produced by the action of P. roqueforti in Blue cheese does not depend directly upon the amount of available fatty acid precursor.

Lawrence (1966) demonstrated the effect of environmental parameters on the rate of beta-oxidation. A marked decrease in beta-oxidation of free fatty acids to methyl ketones was shown at pH values above 7.0. The rate of formation of 2-heptanone exhibited a maximum at 27 C and decreased rapidly with increasing temperatures. Gehrig and Knight (1963) showed the optimum temperature for methyl ketone formation to be 25-30 C and that no ketones were produced under anaerobic conditions. Lawrence (1966) demonstrated that aeration increased ketone production.

Other Flavor Compounds

Secondary and tertiary products found in Blue cheese are beta-keto acids, aldehydes, ketones, acetylmethyl carbinol, diacetyl, primary and secondary alcohols and esters. Of these, the secondary alcohols are probably the most important contributors to flavor. Secondary alcohols were recognized by Stokoe (1928) as being one of the products that resulted from the action of P. roqueforti on coconut oil. Results appeared to indicate that secondary alcohols were intermediate products in oxidation of fatty acids to methyl ketones. If not intermediate products, they must have been produced by reduction of ketones. The

alcohols, 2-pentanol, 2-heptanol, and 2-nonanol, were isolated from Blue cheese by Jackson and Hussong (1958). The secondary alcohols were not observed to appear until considerable amounts of methyl ketones were produced, which suggested that the alochols were formed as a result of the reduction of the corresponding methyl ketone analog. Day and Anderson (1965) identified 2-propanol, 2-pentanol, 2-heptanol, 2-octanol, and 2-nonanol as components of Blue cheese aroma; however, the alcohols were found in much lower concentrations than the methyl ketones.

Acetaldehyde, propionaldehyde, and isobutyraldehyde were identified in Blue cheese volatiles by Nawar and Fagerson (1962). Sato et al. (1966) and Niki et al. (1966), found varying amounts of acetaldehyde in Blue cheese and noted its development during ripening. The breakdown of citrate by starter organisms and P. roqueforti probably accounts for much of the acetaldehyde present (Morgan & Anderson, 1956). Aldehydes such as 3-methylbutanal may be formed from amino acids via oxidative deamination and decarboxylation.

Ethanol (Bavisotto, Rock, & Lesniewski, 1960) acetylmethylcarbinol and diacetyl (Bassett & Harper, 1958) have been documented in Blue cheese. Diacetyl was believed to originate from the metabolism of the lactic acid cocci found in the starter. Methanol, ethanol, n-pentanol, 2- and 3-methylbutanol, and 2-phenylethanol were identified in Blue cheese by Day and Anderson (1965).

Prescott and Dunn (1959) indicated that small amounts of ethanol are produced by species of Pencillium. Anderson (1966) postulates that 2- and 3-methylbutanol and 2-phenylethanol are formed from amino acids via oxidative deamination and decarboxylation with subsequent reduction of the aldehyde analog.

Prior to the investigation of Day and Anderson (1965) esters had not been identified in Blue cheese. The methyl esters of the even-numbered carbon fatty acids acetic through decanoic were also present. Other esters positively identified in Blue cheese were ethyl formate, isopropyl hexanoate and decanoate, pentyl hexanoate and 3-methylbutyl butanoate. Blue cheese had a large quantity of free fatty acids available for esterification with the alcohols present in Blue cheese. The esters may play an important role in the overall flavor profile of Blue cheese (Anderson, 1966).

Contribution to Blue Cheese Flavor by Microorganisms Other Than P. Roqueforti

The surface microflora of Blue cheese consists primarily of bacteria, yeasts and molds (Morris et al., 1951). Parmelee and Nelson (1949a) noted that the addition of a culture of Candida lipolytica to milk for cheese making improved the flavor score of Blue cheese. The microflora of Roquefort cheese was studied by Maxa and Jicinský (1956). A yeast Torulopsis sphaerica was present

in the ripening cheese and was believed to improve the flavor and body of the cheese. The yeast exhibited slight proteolytic and lipolytic tendencies and formed small amounts of ethanol, allowing for the production of esters of fatty acids. Yeasts isolated from Blue cheese by Kanauchi, Yoshioka, and Hamamoto (1962a) included Torulopsis sphaerica, T. candida, Candida pseudoliopicialis and Debaromyces hensenii. The same authors (1962a,b) isolated numerous strains of Micrococcaceae and Brevibacteriaceae from the surface slime of Blue-veined cheese. The influence of selected organisms on ketone-alcohol interconversions was examined by Anderson and Day (1966). Mycoderma, Geotrichum and Torulopsis cultures actively interconverted 2-pentanone and 2-pentanol, and acetone and 2-propanol. Streptococci had no effect on any substrate. Anderson and Day (1966) concluded that yeasts associated with Blue cheese ripening could influence formation of secondary alcohols, and also produce certain esters and alcohols.

Protein Degradation in Blue Cheese

Protein degradation varies with the variety of cheese. Primary protein hydrolysis to amino acids occurs to some extent in all cheese varieties (Harper & Kristofferson, 1956). In certain types, such as Limburger and Blue, proteolysis is much more extensive than in types such as Swiss. Little information is available on the

role of proteolysis and its effect on Blue cheese flavor. P. roqueforti has active proteolytic enzymes. Imamura and Tsugo (1953) studied the changes of milk proteins by P. roqueforti and found that although albumin was most readily decomposed of all the milk proteins, the changes in casein were of greater significance. The large quantities of amino acids released impart a brothy taste which constitutes an important background on which the typical Blue cheese flavor is superimposed. Thibodeau and Macy (1942) indicate that in order to obtain a good quality Blue cheese, hydrolysis of fat must be properly coordinated with the partial hydrolysis of protein by the proteolytic enzymes of P. roqueforti, and that attempts to hasten the ripening of Blue cheese by adding lipase should also be balanced by adding protease at the same time.

Variation of Proteolysis in Relation to Strain

Funder (1949) found a wide variation in the proteolytic properties of various strains of P. roqueforti. The difference in enzymatic properties was such that extremely different results were obtained in cheese making experiments. Often the difference between morphologically similar strains was greater than between two widely different species of Penicillium. This author (1949) suggests that more attention should be focused on the physiological variation of the molds, their formation of mycelium and the reaction products formed during ripening of the cheese.

Proks et al. (1956) also noted a large difference in the proteolytic activity of nine strains of P. roqueforti isolated from good Roquefort cheese. The strains varied in quantity and also in the type of amino acids released when incubated with casein.

Salvadori, Bianchi, and Cavailli (1962a) divided 19 strains of P. roqueforti into three groups according to amino acid patterns obtained by two dimensional chromatography of casein hydrolysates. Based on proteolytic activities, these authors established criteria for choosing a mold for use in the dairy industry. In a related series of experiments, Salvadori et al. (1962b) studied three strains of P. roqueforti displaying weak, intermediate and strong proteolytic activity. It was shown that histidine and methionine increased the proteolytic activity of all three strains.

Niki et al. (1966) observed that strains of P. roqueforti possessing low lipolytic activity exhibited high proteolytic activity and vice versa. The most suitable strain for manufacturing Blue cheese was found to be one which had moderate proteolytic and strong lipolytic activity.

Characteristics of P. roqueforti Protease

Naylor (1930) surface cultured P. roqueforti and checked the protease activity of the filtrate by incubating with casein for 48 hours at 30 C. Activity was based on

recovery of casein precipitated from solution as determined by the Kjeldahl method. The optimum pH for protease activity was 5.3 at 30 C. Thibodeau and Macy (1942) found the optimum pH to be in the range of 5.8 to 6.3 for the protease of P. roqueforti. There was some controversy as to the true nature of the protease at this time but it was thought to be trypsin-like. Thibodeau and Macy (1942) stated that the enzyme was not of the pepsin type and only one protease existed in the mycelium. These authors found that when the fungus was surface cultured the protease was released into the culture medium only when autolysis was occurring in the mycelium. This led to the postulation that lipases and proteases are difficult to obtain in solution, possibly because they are absorbed on the walls of the cells and are liberated only when the mycelial tissue is disrupted.

Nishikawa (1958) reported the pH optimum for proteolytic activity of P. roqueforti protease to be 5.5 to 6.0 when cultured at 40 C and assayed in the presence of 0.6 percent casein sol. The enzyme was stable in the pH range of 5.0 to 6.0, but lower on either side of these limits. Maximum activity was observed at 40 C but at 45 C the enzyme was partially inactivated. Niki et al. (1966) found that P. roqueforti produced an extracellular protease when grown on rennet whey at pH 4.0. In addition, an intracellular protease was also recovered from the mycelium when disrupted. Both proteases were found in varying

quantities in different strains of P. roqueforti. One strain, designated BP-13, had the highest amount of proteolytic activity. Both the intracellular and extracellular protease of the BP-13 strain had a pH optimum of 5.5 with the latter protease having a much narrower range of activity. Modler, Brunner, and Stine (1974a) compared the extracellular proteolytic activity of three strains of P. roqueforti, ATCC 10110, ATCC 6987, and BP-13, and found BP-13 to be the most proteolytic. The extracellular protease isolated by these authors had a pH optimum of 3.0 and 5.5 for bovine serum albumin and casein. Maximum stability to pH occurred in the range of 3.0 to 6.0. The optimum temperature for proteolytic activity was 46 C with thermal inactivation taking place at temperatures above 48 C. Modler et al. (1974b) indicated that the BP-13 extracellular protease was a nonspecific proteolytic enzyme that extensively hydrolyzed α and β casein but K-casein appeared to be unaffected after 20 hours incubation. The enzyme appeared to involve aspartic or glutamic acid residues at the active site.

Matoe and Castin (1970) studied the proteolysis of a 1 percent solution of Hammarsten casein by a preparation of proteolytic enzymes obtained by isopropanol extraction of a dried culture of P. roqueforti. Proteolysis was found to be greatest at pH 5.5 and 25 C. Some of the free amino acids released from casein by this enzyme

included arginine, serine, aspartic acid, valine and norvaline.

Formation and Degradation of Amino Acids

During the ripening period some of the casein is broken down into lower compounds such as polypeptides, oligopeptides, and amino acids. The process, however, does not end here, since amino acids may decompose further into ammonia and carbon dioxide, or may be transformed into carbonyl compounds, amines or other amino acids. The quantity of free amino acids accumulated during the ripening period is thus the dual result of a liberation of amino acids from the casein and a transformation of the amino acids already liberated (Ismail & Hansen, 1972).

Amino acids contribute to the background flavor of cheese but whether amino acids add to a characteristic flavor is problematic (Harper & Kristofferson, 1956). In Cheddar cheese, Harper (1959) has shown a relationship between concentrations of free amino acids and the characteristic flavor. Clemens (1954) could find no relationship between free amino acids, soluble nitrogen and pH in ripening cheese. The characteristic properties of a cheese type were found, by Moeller-Madsen (1959), not to be dependent upon the amino acid distribution. Further evidence of the role of free amino acids in cheese flavor has been obtained by several workers reporting individual findings on the effect of amino acids on the flavor of a

blank base (Harper, 1959; Silverman & Kosikowski, 1953). These authors found that free amino acids added in concentration found in good Cheddar cheese resulted in a flavor that resembled cheese. A Netherlands patent was obtained by Unilever (1966), for the manufacture of a product having a matured cheese flavor. The flavor of the product was improved by adding glutamine, glycine and lysine or methionine. Day (1967) stated that the large quantities of amino acids released by the proteolytic enzymes in mold ripened cheeses impart a taste which constitutes an important background on which the typical flavor is superimposed.

It would appear that the total quantity of free amino acids in cheese depends more on the ripening period than on the type of cheese (Ismail & Hansen, 1972). These authors found that cheeses requiring prolonged ripening periods have the highest content of free amino acids. Antila and Antila (1968) investigated 13 different types of Finnish cheese and found analogously that hard cheeses, having a long ripening period, have the highest content of free amino acids. Also, these authors discovered great variations in the quantity of free amino acids in individual types of cheese. Ismail and Hansen (1972) found Danablue, a Danish blue-veined cheese, to have a greater quantity of free amino acids than Samsoe, Camembert, or Havarti. This was in agreement with Hanni (1967) who, for blue-veined cheeses, states a liberation of amino nitrogen of up to

38 percent of the total nitrogen. The accumulation of individual amino acids increases during the entire ripening period; however, the rate of accumulation may vary, resulting in changes in the relative distribution of the amino acids. During ripening, amino acids are not always liberated in the same proportion as that present in the casein. All cheeses, investigated by Ismail and Hansen (1972), contain more valine, leucine and phenylalanine and less serine, proline, tyrosine, histidine, tryptophan and arginine than could be expected in proportion to the quantities in which they enter into the composition of casein. These authors (1972) found Danablue to be characterized by high contents of aspartic and low contents of asparagine, glutamine and leucine.

Amino acids, after proteolytic decomposition, enter into the acid balance of cheese in different manners (Schormüller, 1968). Acids such as alpha-ketoglutaric, alpha-ketobutyric and pyruvic are formed. Malic and oxaloacetic acid may be formed as amino acid decomposition products by deamination of aspartic acid. Various amines such as tyramine, cadaverine, putrescine, and histamine have been identified from normal ripened cheese (Day, 1967). Sen (1969) quantitated the tyramine content of various Blue-veined cheeses and found from 27-520 micrograms per gram of cheese. The presence of tyramine in Cheddar cheese was noted by Dacre (1953) to have little if any connection with the substances responsible for

typical cheddar flavor. Amino acids and amines also act as precursors of a number of aldehydes reported in Cheddar cheese and are produced probably by transamination and decarboxylation reactions. The Strecker degradation of various amino acids to corresponding aldehydes of one less carbon atom also contributes to the flavor of cheese (Dwivedi, 1973). Although the reaction is nonenzymatic, the proteolytic activity of cheese would exert an influence on the conversion of the amino acids to aldehydes.

Proteolytic Activity of Starter Organisms and Rennet

Investigations into proteolysis in cheese by starter organisms are complicated by the presence of rennet and the results are confused due to the lack of control of the bacterial flora. Consequently, only a few studies have been made on this topic. Sherwood (1935) noted little difference in either the rate or the amount of proteolysis by strains of Streoptococcus lactis and believed acid production to be the only role of the starter. To investigate the action of rennet and lactic acid bacteria in Cheddar cheese, Yamamoto and Yoshitake (1962) made starter cheeses and cheeses in which the starter was replaced by lactic acid. Fifteen amino acids were detected after one month in the cheese with starter but only three amino acids were found in the cheese without the starter, implying that the amino acids were formed by the starter and not the

rennet. The proteolytic activity of rennet and the lactic acid bacteria were also studied in milk by Jespersen (1966) under conditions that occur in cheesemaking. It was reported that proteolysis by lactic acid bacteria was accelerated by the presence of rennet. Imamura (1960b) suggested that starter organisms such as Streptococcus lactis, Str. cremoris and Lactobacillus bulgaricus had little proteolytic activity but stimulated the growth of P. roqueforti in milk protein systems.

Investigations into the optimum conditions for proteolysis by starter cultures were generally undertaken to determine whether the proteinases are active under the conditions prevailing in cheese. Optimal pH for the activity of the intracellular proteases assumed to be liberated into the cheese upon autolysis of the cells (Van der Zant & Nelson, 1953, 1954; Vadehra & Boyd, 1963) and the extracellular proteases (Williamson, Tove, & Speck, 1964) was in the range of pH 6 to 8.5 with only slight or no activity being exhibited at pH 5.0.

Itoh and Thomasow (1971) found that rennin was most stable between pH 5.5 and 6.2 and that it was destroyed at an increasingly rapid rate as the pH was raised from 6.2 to neutrality. Tauber and Kleiner (1934) showed that rennin could be digested by proteolytic enzymes. Changes in electrophoretic patterns of α and β caseins during hydrolysis with crystalline rennin were studied by Lindqvist and Storgords (1959). Their patterns indicated that hydrolysis

of α casein over a period of 25 days followed a stepwise degradation while hydrolysis of β casein resulted in a profusion of degradation products. Non-protein nitrogen (soluble in 2 percent trichloracetic acid) was liberated three times faster from α than from β casein. Melachouris and Tuckey (1964) found that when the pH of cheese milk was adjusted from 6.5 to 5.3, rennet extract had maximum proteolysis at pH 6.3. Imamura (1960b) stated that rennet extracts contributed little proteolysis to the total degradation of milk proteins in the presence of cell extracts of P. roqueforti.

Improving the Flavor and/or Shortening the Ripening Time of Blue Cheese

The findings of Currie (1914), that the flavor of Roquefort cheese was partly due to the accumulation of free fatty acids during ripening, led Lane and Hammer (1937) to investigate the effect of homogenization of cheese milk on subsequent flavor development. Cheese made from homogenized milk was faster-ripening, comparatively light-colored and softer-bodied than cheese made from unhomogenized raw milk. Pasteurized, homogenized milk showed a more rapid development of volatile acid and a more typical flavor than cheese made from unhomogenized raw milk. Another important characteristic of homogenized milk cheese was that mold growth was more luxuriant. To assist flavor production, Babel (1953) devised a method of separating the cream from skimmilk and homogenizing the cream. The homogenized

cream was added back to the skim and the mixture pasteurized. Homogenization of the cream only enabled acclerated ripening and early production of methyl ketones. When homogenized cream was recombined with skimmilk and the mixture pasteurized, Schchedushnov (1961, 1962) found that the ripening process was accelerated, and the quality of Roquefort-type cheese improved. The largest amount of free fatty acids was observed in raw homogenized milk, followed in order by pasteurized, homogenized and raw milk (Morris et al., 1955). Peters and Nelson (1961) demonstrated that ripened Blue cheese made from homogenized milk, both raw and pasteurized, was higher in quality than similarly ripened cheese made from raw or pasteurized nonhomogenized milk. Harper and Gould (1959) found measurable lipolysis in milk used for cheese manufacture when the time interval between homogenization and pasteurization was 30 seconds. Milk held at 120 F (49 C) for 5 minutes between homogenization and pasteurization resulted in a cheese milk with a dinstinct rancid flavor.

Since the natural milk lipases are partially or completely inactivated by pasteurization, attempts have been made to stimulate or accelerate lipolysis by the addition of certain agents to the cheese or cheese milk. By the addition of steapsin to the cheese milk or curd, Coulter and Combs (1939) obtained the same degree of flavor within 5 months as in normal cheese ripened 12 months. The cheese was critized as being bitter, however.

The addition of low molecular weight fatty acids by Parmelee and Nelson (1949b) to pasteurized, homogenized milk improved the flavor of Blue cheese made from such milk. Parmelee and Nelson (1947) observed that the addition of cultures of the lipolytic organisms Achromobacter lipolyticum, Alcaligenes lipolyticus, and Pseudomonas fragi to milk did not increase fat hydrolysis and flavor development in Blue cheese. Cultures of Candida lipolytica added to pasteurized milk improved the flavor of Blue cheese. The need for strain selection when using C. lipolytica was stressed by Wilcox et al. (1955) after discovering that different strains differed markedly in their ability to improve the flavor of Blue cheese. P. roqueforti enzymes were demonstrated to produce a fine quality Blue cheese which was marketable in 5 months as compared to 10 months for control cheeses (Thibodeau & Macy, 1942). Parmelee and Nelson (1949b) prepared a mold-enzyme preparation by adding mold spores to an emulsified butterfat medium and allowing the preparation to grow for 7 days. Addition of the mold-enzyme preparation to pasteurized, homogenized milk improved the flavor of Blue cheese made from such milk. The effective use of this preparation required careful control. Kosikowski and Mocquot (1958) indicated that such lipolytic agents as pancreatin, erepsin, steapsin, mulberry juice lipase, and chicken stomach lipase caused undesirable rancid and bitter flavors to develop in the cheeses to which they were added.

Various methods of reducing the labor requirements necessary for Blue cheese manufacture have been researched (Graham & Rowland, 1959, Janzen, 1964). Processes have been developed whereby draining, hooping, and inoculation of Blue cheese curd is done mechanically.

Research has been conducted by Kondrup and Hedrick (1963) on methods for decreasing the time needed to ripen Blue cheese. A process for the quick-ripening of loose curd Blue cheese has been patented by Hedrick, Kondrup, and Williamson (1968). Pasteurized milk and normal manufacturing procedures were followed until after the curd was cut and drained. The curd was placed on a fine mesh, stainless steel screen at a depth of 3 to 4 inches. The product was moved to a curing room where salt was added and the curd stirred periodically. After 10 days the cheese had developed sufficient flavor. Judges rated the flavor and body of the Blue cheese cured by the short method as equal to or better than the controls which were cured according to common commercial practice (Kondrup et al., 1964).

Direct acidification procedures were developed by Shehata (1966) to produce a Blue cheese curd similar in body and texture to curd made by traditional methods. This procedure involved acidifying milk with hydrochloric acid to pH 5.6, adding rennet and 0.5 percent lactic starter culture to control undesirable fermentations and aid in flavor development of cheese during ripening. Shehata

(1967) incorporated a commercial lipase preparation into the milk to be used for making Blue cheese by the direct acidification method. Blue cheese made by this method had good flavor and mold growth after ripening for 4 to 6 months.

Knight, Mohr, and Frazier (1950) produced a mutant strain of P. roqueforti with white pigmenting spores. The mutant, produced by ultraviolet irradiation, retained lipolytic and proteolytic activity and could be used to produce cheese without the characteristic blue-mold color. Such cheese could be employed in the manufacture of dressing where lack of color is desired or to produce a cheese acceptable to consumers who dislike the appearance of the blue mold. Nelson (1970) reported that submerged fermentation of white mutants of P. roqueforti in a culture medium composed of homogenized milk plus lipolyzed milk fat produced, after 24 to 72 hours of fermentation, a ketone concentration of 7 to 15 times that found in Blue cheese. This technique was based on the earlier work of Gehrig and Knight (1958, 1963) and Girolami and Knight (1955) and produces a ketone profile very similar to that found in Blue cheese as judged organoleptically and gas chromatographically.

Using qualitative and quantitative data obtained from previous studies (Anderson & Day, 1966; Day & Anderson, 1965), synthetic Blue cheese flavor was compounded by Anderson and Day (1966). The mixture contained methyl

ketones, secondary alcohols and the C_2-C_8 fatty acids found in Blue cheese. The fatty acids were present at two-thirds the level found in Blue cheese samples, because this level prevented a bitter, soapy flavor and provided for a more predominant ketone flavor. The addition of 2-phenylethanol, ethyl butyrate, methyl hexanoate and methyl octanoate gave a desirable character to the mixture. The synthetic mixture did not duplicate the flavor and aroma of a high quality Blue cheese, possibly because there was no free amino acids or other proteolysis products present and a complete complement of the compounds identified in Blue cheese was not present. A possible suggested use for the synthetic flavor mixture was as an additive to some type of short cured Blue cheese that could be produced at a lower cost than conventionally cured Blue cheese.

Patton (1951) developed a Blue cheese flavor concentrate by extracting volatile flavor components from Blue cheese using vegetable oil as a solvent. The extraction was then incorporated into a salad dressing mix. Lukas (1973) devised a procedure for growing P. roqueforti aerobically to obtain a strong Blue cheese flavor. Ney et al. (1972) patented a method for the manufacture of food products having a Blue cheese flavor. Amino acids and methyl ketones were the primary flavor components in this process. Utilizing a continuous fermentation, with a mycelial culture of P. roqueforti, Dwivedi and Kinsella (1974) demonstrated the production of carbonyls from

lipolyzed milk fat. The major carbonyls produced were 2-pentanone, 2-heptanone, 2-nonanone, and 2-undecanone. Unilever (1966) obtained a Netherlands patent for the manufacture of a product having a matured cheese flavor. The flavor of the product was improved by adding glutamine, glycine and lysine or methionine. Litchfield (1971) showed that factors affecting submerged culture fermentations were substrate, pretreatment, nitrogen source, trace elements, aeration, agitation, pH, flavor additives and temperature.

The possibility of using pancreatin to accelerate the ripening of cheese and provide a pronounced flavor and improved consistency to cheese was demonstrated by Arsenova and Chebotarev (1972).

Kasik and Luksas (1972) patented a procedure for producing cheese flavor by culturing mixtures of certain Bacilli and Streptococci in an aqueous medium containing protein and carbohydrate. A patent was issued to Guilloteau (1972) for the production of pasteurized, soft, solubilized, skimmilk Blue cheese. Streptococcus thermophilus and Lactobacillus bulgaricus were used in the process. Improved mold distribution and therefore a more complete ripening through the use of a gas producing culture of Streptococcus diacetilactis added to cheese milk was the subject of another patent (Williamson & Purko 1972). Lundstedt (1973) patented a procedure for the preparation of Blue cheese from soybean milk which had been fortified with butterfat and nonfat milk solids. The organisms described

in the patent were P. roqueforti and S. diacetilactis. The cheese was prepared in the loose curd form and required approximately two weeks to obtain a desired flavor profile.

Blakely (1970) prepared a quick ripened filled Blue cheese. By using coconut oil and following the quick ripening procedure developed by Kondrup et al. (1964), a high percentage of particular fatty acids and methy ketones was obtained. The flavor was described as not completely typical of Blue cheese. Wang, Harper, and Kristofferson (1971) reported that in the ripening of filled cheese, the most important factor in obtaining a typical cheese was the free fatty acid formation while the amino acid content was not influenced to a large extent.

EXPERIMENTAL PROCEDURES

Manufacture of Quick Ripened Blue Cheese

Quick-ripened Blue cheese was made according to the method of Hedrick et al. (1968) using pilot laboratory equipment described by Kondrup and Hedrick (1963). Raw milk containing 3.5 percent fat was preheated to 130 F (54 C) and immediately homogenized at 2000 p.s.i. on the first stage and 500 p.s.i. on the second stage. The milk was pasteurized at 143 F (62 C) for 30 min and cooled to 84 F (29 C).

A frozen cheese culture was mixed into the milk at the rate of 17 g/20 gal milk. After the titratable acidity had risen 0.02 percent, 10 ml of decolorizer (chlorophyll in alkaline solution diluted with water), 24 g of dry mold ("Midwest" Blue Mold Powder) and 10 ml of single strength rennet per 20 gal milk were thoroughly mixed into the contents of the vat. One hour later the curd was cut into 3/8 in cubes. The contents of the vat were agitated 30 min after cutting and again immediately following a second 30 min period. The whey was drained promptly and the curd placed in the ripening room on a stainless steel mesh screen to a depth of 3 to 4 in. The

room was controlled to 62 F ± 1 (17 C) and a relative humidity of 95 percent or more. Matting was inhibited by stirring the curd and breaking up any of the large lumps twice during the first 24 hr and once each day for the next 6 days. Salt was usually added on the fourth, fifth, and sixth days by the same stirring action.

The salting rate was one pound for each 22 pounds of Blue cheese curd. However, only one-fourth of the salt was added on the fourth day. One fourth and one-half of the salt were added on the fifth and sixth days, respectively. Salt content of ripened cheese was approximately 4 percent.

After ripening 10 days the cheese usually had a flavor characteristic of Blue cheese and was packaged in polyethylene bags and immediately frozen at -5 F (-21 C) and held at this temperature for further analysis.

Since part of the objective of this research was to further refine and standardize the above procedure, many adaptations to the original method were attempted. The modifications dealt with direct acidification, temperature of curing, salting times and amounts, homogenization, pasteurization, additional lipase, cooking the curd after cutting, and the use of a white mutant of P. roqueforti. All batches of cheese were made in duplicate. The procedure of Hedrick et al. (1968) described above was followed with the exceptions of the following variables.

I PASTEURIZED, UNHOMOGENIZED

Curing room temperature 62 F (17 C) Time--7 days

1/4, 1/4, and 1/2 of the total salt used were added on ripening days 4, 5, and 6 respectively.

Cheese milk was pasteurized at 114 F (62 C) for 30 min; milk was not homogenized.

II HOMOGENIZED RAW MILK

Curing room temperature 62 F (17 C) Time--7 days

1/4, 1/4, and 1/2 of the total salt used were added on ripening days 4, 5, and 6 respectively.

Cheese milk was preheated to 130 F (54 C) and homogenized; milk was not pasteurized.

III HOMOGENIZED--ONE HOUR DELAY BEFORE PASTEURIZATION

Curing room temperature 62 F (17C) Time--7 days

1/4, 1/4, and 1/2 of the total salt used were added on ripening days 4, 5, and 6 respectively.

Cheese milk was preheated to 130 F (54 C), homogenized, and allowed to stand for one hour before pasteurization.

IV LACTIC ACID DIRECT ACIDIFICATION

Curing temperature 62 F (17C) Time--7 days

1/4, 1/4, and 1/2 of the total salt used were added on ripening days 4, 5, and 6 respectively.

Lactic acid (2N) was added to a pH of 5.7. Rennet, decolorizer and mold powder were then added.

V LACTIC ACID AND STARTER--DIRECT ACIDIFICATION

Curing temperature 62 F (17 C) Time--7 days

1/4, 1/4, and 1/2 of the total salt used were added on ripening days 4, 5, and 6 respectively.

Lactic acid (2N) was added to a pH of 5.7. Starter culture, rennet, decolorizer and mold powder were then added.

VI HYDROCHLORIC ACID DIRECT ACIDIFICATION

Curing temperature 62 F (17 C) Time--7 days

1/4, 1/4, and 1/2 of the total salt used were added on ripening days 4, 5, and 6 respectively.

HCL (2N) was added until the pH was lowered to 5.2. Rennet, decolorizer and mold powder were then added.

VII BENZOYL PEROXIDE DECOLORIZER

Curing room temperature 62 F (17 C) Time--7 days

1/4, 1/4, and 1/2 of the total salt used were added on ripening days 4, 5, and 6 respectively.

Benzoyl peroxide used as a decolorizer rather than chlorphyll in an alkaline solution.

VIII SALTING DAYS 0,4,6

Curing temperature 62 F (17 C) Time--8 days

1/2, 1/4, and 1/4 of the total salt used were added on ripening days 0, 4, and 6.

IX SALTING DAYS 1,2,3

Curing temperature 62 F (17 C) Time--7 days

1/4, 1/4, and 1/2 of the total salt used were added on ripening days 1, 2, and 3.

X SALTING DAYS 1,4,5

Curing temperature 62 F (17 C) Time--7 days

1/4, 1/4, and 1/2 of the total salt used were added on ripening days 1, 4, and 5.

XI SALTING DAYS 2,4,5,6

Curing temperature 62 F (17 C) Time--8 days

1/4, 1/4, 1/4, and 1/4 of the total salt used were added on ripening days 2, 4, 5, and 6.

XII TEMPERATURE OF CURING 48 F (9 C) (#1) (salted 4,5,6)

Curing temperature 48 F (9 C) Time--14 days

1/4, 1/4, and 1/2 of the total salt used were added on ripening days 4, 5, and 6 respectively.

XIII TEMPERATURE OF CURING 84 F (9 C) (#2) (salted 11,12,13)

Curing temperature 48 F (9 C) Time--14 days

1/4, 1/4, and 1/2 of the total salt used were added on ripening days 11, 12, and 13 respectively.

XIV TEMPERATURE OF CURING 57 F (14 C)

Curing temperature 57 F (14 C) Time--9 days

1/4, 1/4, and 1/2 of the total salt used were added on ripening days 5, 6, and 7 respectively.

XV TEMPERATURE ON CURING 72 F (22 C)

Curing temperature 72 F (22 C) Time--7 days

1/4, 1/4, and 1/2 of the total salt used were added on ripening days 4, 5, and 6 respectively.

XVI CHEESE CURD COOKED AFTER CUTTING

Curing temperature 62 F (17 C) Time--7 days

1/4, 1/4, and 1/2 of the total salt used were added on ripening days 4, 5, and 6 respectively.

After the curd was cut no agitation was permitted for at least 15 min. The temperature of the curd was then raised from 84 F (29 C) to 100 F (38 C) in one hr.

XVII LIPASE (ITALASE) ADDED TO CHEESE MILK

Curing temperature 62 F (17 C) Time--7 days

1/4, 1/4, and 1/2 of the total salt used were added on ripening days 4, 5, and 6 respectively.

The commercial enzyme preparation "Italase" was added to cheese milk following pasteurization of the milk.

Based upon the information obtained from the above trails, the procedure of Kondrup and Hedrick (1963) for the making of quick ripened Blue cheese was modified and the following alternative methods were suggested:

SUGGESTED STANDARD PROCEDURE #1

Curing temperature 52 F (11 C) Time--11 days

1/4, 1/4, and 1/2 of the total salt used were added on ripening days 7, 8, and 9 respectively.

Curd was cooked after cutting, to a temperature of 100 F (38 C). Samples of cheese made by this procedure were also stored for four weeks at 40 F (4 C) to determine what effect such additional storage would have on the overall quality of the cheese.

SUGGESTED STANDARD PROCEDURE 32

Curing temperature 52 F (11 C) Time--11 days

1/4, 1/4, and 1/2 of the total salt used were added on ripening days 1, 9, and 10 respectively.

Curd was cooked after cutting to a temperature of 100 F (38 C).

Cheese was also made with a white mutant of P. roqueforti obtained from Midwest Mold Company. The following procedure was used for two lots of cheese made with this mold.

WHITE MOLD CHEESE #1

Curing temperature 52 F (11 C) Time--10 days

1/4, 1/4, and 1/2 of the total salt used were added on ripening days 5, 6, and 7 respectively.

Curd was cooked, after cutting, to a temperature of 100 F (38 C).

WHITE MOLD CHEESE #2

Curing temperature 52 F (11 C) Time--10 days

1/4, 1/4, and 1/4 of the total salt used were added on ripening days 1, 6, and 7 respectively.

Curd was cooked, after cutting, to a temperature of 100 F (38 C).

Preparation of White Mold Powder

A slant of the white mutant of P. roqueforti was obtained from Midwest Mold Company. The white mold was propagated by the method of Hussong and Hammer (1935). The method consisted of cutting whole wheat bread into approximately 1/2 in cubes and transferring them to wide mouth bottles. The bottles were then sterilized at 15 p.s.i. steam for 30 min. After cooling, the bread was inoculated and allowed to incubate at 21 C until spore formation had occurred. The time required for spore formation was about 11 days. After incubation the material was pulverized in a Waring blender. The finished powder was placed in glass jars, sealed and stored in a refrigerator at 5 C.

Hammersten Casein

The method of Dunn (1949) was followed for the preparation of Hammarsten casein. Sodium caseinate was prepared by precipitating casein from freshly separated warm (30 C) skimmilk (0.04 percent butterfat) at pH 4.6, with 1N hydrochloric acid. After washing the precipitate

with copious amounts of distilled water, the protein was redissolved by adding 1N sodium hydroxide to bring the pH of the suspension back to 7.0. The precipitation and washing process were repeated. The final casein precipitate was suspended in 95 percent ethanol. Following a series of ethanol and ether washes to remove moisture and fat, the purified product was held at room temperature for 8 hr to remove the ether and attain moisture equilibrium. The final product contained 6.84 percent moisture as determined by the vacuum oven method (A. O. A. C., 1960). Hammarsten casein was used in all assays for non-protein nitrogen (NPN).

Thin-Layer Plates

Silica gel G thin-layer plates were prepared by mixing 55 g of silica gel G with 110 ml of deionized water. The slurried silica gel was applied to 20 x 20 cm glass plates as a layer 500 microns thick using a Desage Brinkman thin-layer spreader. The plates were allowed to air dry for 2 hr and activated at 100 C for 30 min.

ANALYTICAL PROCEDURES

pH

Ten grams of cheese were slurried with 40 ml of deionized distrilled water in a Waring semi-micro blender for 2 min. The slurry was immediately transferred to a glass beaker and pH determined with a Leeds and Northrup (model 7421) pH meter.

Spore Counting

A Petroff-Hausser Bacteria Counter was used as an estimate of spore count/ml. This was necessary so that equal amounts of spores of the white mutant mold powder and blue mold powder could be used in the manufacture of cheese.

Kjeldahl

Total nitrogen was determined using the Kjeldahl method (McKenzie, 1970). This consisted of digesting a sample containing, preferably, 0.2-1.0 mg nitrogen, with 1.5 g of powdered potassium sulfate; 1.5 ml sulfuric acid and 0.5 ml of mercuric sulfate solution (appendix). After digestion the flasks were allowed to cool for a few minutes and each digest was diluted with a few ml of ammonia-free water. The digest flask were transferred to a micro

Kjeldahl distillation apparatus, 10 ml of sodium hydroxide-sodium thiosulfate solution (appendix) were added and the mixture steam distilled into 5 ml of boric acid indicator solution (appendix) contained in a 50 ml Erlenmeyer flask. Distillation was continued until about 40 ml had been distilled. The condenser was rinsed with a few ml of ammonia-free water, the boric acid solution was lowered from the tip until a further 5 ml had been distilled and then distillation was stopped. The contents of the flasks were titrated with potassium biiodate solution (appendix) to a gray-lilac endpoint.

Recoveries of nitrogen were checked with DL tryptophan which had been desiccated over phosphorus pentoxide for one month.

Standardized Assay Procedure for Non-Protein Nitrogen

The Lowry modification of the Folin procedure was used for determining non-protein nitrogen (NPN) (McDonald & Chen, 1965). This modified procedure incorporated a pre-treatment with alkaline copper sulfate. Substances that gave a positive biuret reaction also produced color with the Folin reagent. Without the copper treatment, only substances containing tyrosine and tryptophan produced color when the Folin-Ciocalteau reagent was added, according to Herriott (1941).

To measure NPN, 1.000 g cheese was slurried with 50 ml of 4.5 M urea in a Waring semi-micro blender for

2 min. The slurry was then heated to 75 C and held at this temperature with magnetic stirring for 1 hr. Ten ml of this sol were mixed with an equal amount of 24 percent TCA. Blank determinations were made by adding 10 ml 24 percent TCA to 10 ml 4.5 urea. After standing 30 min the sol was filtered through Whatman no. 44 filter paper. One-half ml of filtrate was then mixed with 5 ml of alkaline copper sulfate-sodium tartrate solution. The solution was allowed to stand 10 min at room temperature. One-half ml of 1N Folin-Ciocalteau reagent was added, and the solution was mixed within 2 sec on a Fisher mini-shaker. The pH of the solution was 9.9 to 10.1 which was the optimum for color development according to McDonald and Chen (1965). The color was developed at room temperature for 1 hr. Absorbance was read at 600 or 750 nm depending on color intensity.

Casein Standard Curves

Two standard curves were prepared from Hammarsten casein using the Lowry-Folin procedure for color development. From these two curves it was determined that absorbances in the range of 0.0 to 0.19 (0 to 50 ug casein) and 0.16 to 0.45 (50 to 200 ug casein) would be read at 750 and 600 nm respectively when determining NPN. The two standard curves for casein were prepared by peptizing Hammarsten casein in 0.03 M citrate buffer and selecting appropriate alliquots to cover the range desired. To 0.5 ml of the casein sol, 2.0 ml of alkaline copper sulfate-sodium

tartrate solution (appendix) were added with the alkali being 0.2 N sodium hydroxide in 2 percent sodium bicarbonate. Color was developed as described in the "assay procedure."

Polyacrylamide Gel Electrophoresis

All electrophoretic analyses were made using a Bio-Rad Model 400 power supply.

Alkaline gels were prepared and protein samples electrophoresed basically by the method of Thompson, Kiddy, Johnston and Weinberg (1964), as altered by Ledford, O'Sullivan, and Nath (1966) for cheese, using disc gel apparatus instead of a vertical electrophoresis cell. The procedure consisted of a pH 9.1-9.3 buffer (appendix) and a 7.0 percent Cyanogum in 4.5 M urea gel stock (appendix). To 50 ml of the gel stock was added 0.1 g ammonium persulfate. The resulting solution was pipetted into gel tubes to a depth of 9.5 mm and a small amount of water was added to the top of the gel to prevent a gel meniscus and this assures a stable interface. The solution gelled in about 20 min and was allowed to age 45 min before the water was removed with the aid of a syringe. Fifteen ul of the sample solution was then layered on the polymerized gel. The remainder of the gel tube was filled by carefully layering gel solution above the sample, followed by small amounts of water to the top of the gel. The addition of the top gel was necessary so that components that normally

had a net positive charge at pH 9.1 could be stained and observed.

Current was applied to the gel as follows: 100 V (50-55 mA) for the first 15 min; 150 V (60 mA) for the next 15 min, followed by slowly increasing the voltage to 250 but never exceeding 70 mA. The current drops with time and after electrophoresis for 2 1/2 hr the final reading was 25-30 mA. The gel was removed, dyed for 3 min with Amido Black staining solution (appendix), excess dye washed out with 7 percent acetic acid, and the gel decolorized in 7 percent acetic acid using a Bio-Rod Model 170 diffusion destainer.

Samples for gel electrophoresis were prepared by slurrying one gram of cheese with 40 ml of distilled water in a Waring micro-blender for 2 min. The slurry was washed from the blender with 10 ml of 7 M urea. Samples were warmed to 37 C to effect a layering of fat, thus facilitating removal of the aqueous phase for electrophoresis. To demonstrate k-casein, 0.4 ml of 2-mercaptoethanol were added to samples 45 min prior to electrophoresis. Fifteen ul of the resulting samples were used for electrophoresis.

Amino Acids

Amino acid analyses were performed employing the Beckman 120 C automatic amino acid analyzer (Moore, Spakman, & Stein, 1958).

The samples were prepared by weighing accurately 10 g of cheese and grinding with 100 ml H_2O in a Waring micro-blender for 2 min. The slurry was removed and heated to 75 C with magnetic stirring. After the sample had cooled to 25 C, the majority of proteins were precipitated by mixing equal amounts of the slurried cheese and 24 percent TCA. The resulting sol was allowed to stand 30 min and was then centrifuged at 2000 rpm for 10 min. An aliquot of supernatant was then added to absolute ethanol at the rate of 1 to 4 respectively to remove the majority of remaining peptides (Kosikowski, 1951). The sol stood undisturbed for 12 hrs before filtering through Whatman no. 42 filter paper. An aliquot of the supernatant was evaporated to dryness using a 25 ml pear-shaped flask and a rotary evaporator before being diluted with buffer and analyzed.

Ninhydrin Assay

The presence of small peptides in samples used for amino acid analysis made it impossible to accurately assess recoveries. Therefore, in order to check recoveries, a ninhydrin assay (Clark, 1964) was used.

The ninhydrin assay consisted of adding ninhydrin solution (appendix) to the protein solution and boiling in a water bath for 20 min. After cooling, 8 ml of 50 percent aqueous n-propanol were added and, after standing 10 min

to develop full color, optical densities were read at 570 nm against a blank.

Glycine was used to prepare a standard curve. The cheese samples were prepared in an identical manner to those for the amino acid analysis. Acid hydrolysis was performed by adding 5 ml of 6 N HCl to the dried sample and the mixture frozen in a dry ice-ethanol bath. The ampoules were evacuated, allowed to melt under vacuum to remove gases, refrozen, sealed and placed in an oil bath maintained at 110 C. After 22 hr the ampoules were removed, allowed to cool to room temperature, opened and ninhydrin assays performed.

Ninhydrin values were obtained on the samples both before and after acid hydrolysis. By comparing these values to the recovery values obtained from amino acid analysis, it was possible to assess the recoveries of amino acid analysis.

Gel Filtration Ghromotography

Gel filtration chromotography using Sephadex G-25 and G-10 was used in this study. An excess of boiled buffer (0.02 M sodium chloride) was added to rehydrate the Sephadex. A uniform suspension of gel was made by carefully stirring the Sephadex into the hot buffer with a glass rod. When cool, the excess buffer was decanted off and another solution of boiled buffer was added to the Sephadex. This procedure was repeated at least three

times. After rehydration was complete, a slurry of the Sephadex gel was made by mixing an equal volume of buffer and swelled Sephadex. The slurry was deaerated under vacuum and carefully poured into a Sephadex chromotographic column. After about 5 to 10 min, the column outlet was opened to allow complete packing of the gel bed.

The column was equilbrated with two column volumes of 0.02 M sodium chloride buffer prior to use. The void volume of the columns was determined using Blue Dextran 2000. The eluant coming from the column was monitored using an Isco UV column monitor at 280 nm.

Peptide Mapping and Molecular Weight Distributions

Samples for peptide mapping were prepared by slurrying 20 g of cheese with 100 ml H_2O in a Waring micro-blender for 2 min.

The resulting sol was heated to 38.7 C and held at this temperature with magnetic stirring for 30 min and was then filtered through Whatman no. 42 filter paper.

In order to obtain peptides in the molecular weight range of 700-5000, gel filtration chromotography was used. Five ml of the supernatant from filtration was placed on a Sephadex G-10 column and the void volume peak (42 ml) collected and concentrated to 2 ml on a rotary evaporator. This concentrate was applied to the top of a G-25 column and the fractionation range (ca. 200 ml) collected and

concentrated to 5 ml. This concentrate was then used for peptide mapping.

Gel filtration was also used for obtaining molecular weight estimations of protein degradation in Blue cheeses. Total nitrogen was determined by the Kjeldahl procedure on samples obtained during the preparation. By determining total protein content of the whole cheese, the supernatant after filtering, the G-10 void volume, the G-25 void volume and fractionation range, it was possible to obtain the percentage of protein with molecular weights of less than 700, 700-5000, and greater than 5000.

Two dimensional peptide mapping was used as an analytical technique to determine if differences existed between cheese samples. Silica gel G thin-layer plates were spotted with 10.0 ul of a peptide solution and dried thoroughly. Ascending chromatography was then performed using a buffer of butanol-acetic acid-water (4:1:5). When the solvent front approached the top of the plates, they were taken out and air dried.

After ascending chromatography in the first dimension, the thin-layer plates were placed in another thin-layer solvent tank containing ethanol-ammonia-water (8:1:1). When the solvent front approached the top of the plates they were taken out, dried and sprayed with ninhydrin (appendix) to detect the peptide spots.

Tryamine

The method of Blackwell, Marley, and Ryle (1965) was used to isolate tyramine. This method consisted of grinding 10 g of sample to a paste with warm water, the volume adjusted to 40 ml, boiled for 5 min to destroy enzymes, centrifuged to deposit protein, and cooled to solidify the supernatant. The cloudy aqueous phase was filtered through Whatman no. 42 filter paper, and 15 ml sample of the filtrate shaken with 1 g of Amberlite IRC 50 (weak acid cation exchanger). The ion exchange resin was used as a device for concentrating the basic compounds and removing non-basic compounds from the sample (Awapura, Davis, & Graham, 1960). The column used for ion exchange was 10 cm long and one was poured for each cheese sample. After the suspension had been shaken for 30 min, it was poured on to the resin column and after the sample had run through, the column was washed with 50 ml of water. The absorbed basic amino compounds were eluted with 30 ml 4 M acetic acid, the eluate dried in pear-shaped flasks on a rotary evaporator at 40 C, and the dried fraction dissolved in 0.5 ml of methanol.

Tryamine was tentatively identified by ascending thin-layer chromatography using thin-layer Silica gel G thin-layer plates in a carrier solvent of butanol-acetic acid-water (4:1:5). After the thin-layer plates had been spotted and allowed to migrate near the top of the plates, they were removed, air dried, sprayed with ninhydrin

(appendix), and R_f values of the unknown sample compared to those of a tyramine standard. Also, tyramine gives a purple color reaction (Sen, 1969) with ninhydrin which aided in the tentative identification.

Gas chromatographic analysis were performed using a Beckman GC-5 dual column gas chromatograph equipped with flame ionization detectors and a stainless steel column (6 ft x 1/8 in od) packed with 3 percent SE-30 on 80-100 mesh Gas Chrom Q. The chromatograph was operated isothermally at 135 C with gas flow rates of 45.0, 18.0, and 300 ml/min for nitrogen (carrier gas), hydrogen and compressed air respectively. Temperatures of the detector and injection port were 240 C and 185 C respectively.

Mass spectra were obtained using a combined gas chromatograph-mass spectrometer LKB 9000 equipped with a glass column (6 ft x 1/8 in od) of 3 percent SP-2100 on 100-120 mesh Supelcoport, with an ionizing electron energy of 70 eV; the flash heater was set 20 C above the GLC column temperature (140 C), molecular separator at 240 C and the ion source at 290 C. The carrier gas (nitrogen) flow rate was 24 ml/min. The spectra were reported as bar-graphs by means of an on-line data acquisition and processing program (Sweeley, 1970).

The quantitation of tyramine was accomplished in the following way. Preparative ascending thin-layer chromatography was used to isolate tyramine. This procedure was similar to the procedure for identification

of tyramine described above. However, instead of spotting the plates, Cordis disposable microapplicators were used to apply 150 ul of the tyramine preparation across the entire width of the plate. The plates were then placed in the migration tanks and ascending chromatography conducted until the solvent neared the top of the plate. After air drying, location of tyramine was accomplished by spraying narrow strips (1.5 cm wide) on the plate edges in the direction of migration with ninhydrin spray. A ruler was used to mark off the area containing the tyramine streak and the Silica gel layer was scraped from this area. The tyramine was eluted from the Silica gel with 20 ml methanol and then stirred magnetically for 12 hr. The silica gel was removed from the sample by filtering through a sintered glass filter. The sample was then dried in pear-shaped flasks on a rotary evaporator at 40 C. The sample was rehydrated with 0.5 ml methanol before gas chromotography analysis to obtain quantitation.

A standard curve was prepared using a tyramine standard subjected to identical treatment as the sample.

RESULTS AND DISCUSSION

Protein Degradation in Quick Ripened Loose Curd Blue Cheese

It was observed in this study that several manufacturing parameters had common effects on pH, non-protein nitrogen (NPN) and polyacrylamide gel electrophoresis (PAGE). Due to these common effects on the results, and the great number of modifications that were attempted, the results and discussion were arranged in the following manner. A general description of pH, NPN, and PAGE is presented followed by a discussion of each modification.

Changes in pH During Ripening

The pH of a cheese was considered important by Morris and Jezeski (1953) because pH could influence the activity of enzyme systems involved in ripening. Therefore, the changes in pH during ripening of quick ripened (QR) Blue cheese were determined.

A comminuted, representative sample of cheese was analyzed each day of the ripening period for changes in pH. The measurements were an average of two pH values obtained from duplicate batches of cheese.

Fresh curd exhibited a pH value of approximately 5.5 prior to being placed in the curing room. The hydrogen ion concentration reached a maximum at about 4.8 on the second or third day of ripening. This was followed by a rapid increase in pH to 5.7-6.2 which generally coincided with the first visible signs of mold growth and occurred on the third, fourth, and fifty days of ripening. Wide differences existed on these days depending on treatments such as salting times and ripening temperatures. The pH then stabilized or in some cases decreased slightly to approximately 6.0 for the remainder of ripening.

The maximum acidity in the QR Blue cheese analyzed was not reached until approximately 48 to 72 hr after manufacture. Since only 1 percent starter was utilized, this may account for the slow acid development during the initial stages of ripening. Maximum acidity is generally reached within 24 hr in Blue cheese manufacture (Foster et al., 1961). Most procedures utilized 2-4 percent starter. However, Coulter, Combs, and George (1938a) and Thibodeau and Macy (1942) used 2-3 percent starter and found pH values reached a minimum about 24 hr after the manufacturing process had been initiated. Slower acid development was noted by Morris and Jezeski (1964) when 0.75 percent starter was used with a maximum acidity being attained on the seventh day of ripening. The early increase in acidity was attributed to the production of lactic acid by Streptococcus lactis-type starter cultures.

In QR Blue cheese the minimum pH attained was followed by a brief gradual increase before a rapid increase in pH was noted. Foster et al. (1961) suggested lactic acid destruction as a reason for the initial gradual decrease in acidity in normally cured Blue cheese.

The rapid increase in pH was attributed to proteolysis by the protease enzymes associated with P. roqueforti. A continuing proteolysis and a simultaneous increase in pH as Blue cheese ripened was observed by Lane and Hammer (1938), Morris et al. (1951), and Currie (1914). Data presented by Morris et al. (1963) revealed a progressive increase in liberated tyrosine during ripening indicating proteolysis may have been responsible for the rapid rise in pH.

The final stabilization or slight decrease in pH values was attributed to increasing levels of free fatty acids liberated by lipolysis due to the lipases of the mold. Another factor that could have contributed to this leveling effect was a decrease in proteolysis. Morris and Jezeski (1964), Coulter et al. (1938b), and Morrie et al. (1963) noted a slow decrease in pH to about 5.7 at 4 to 9 months in Blue cheese and attributed this decrease in pH to an accumulation of free fatty acids in the cheese. Imamura and Kataoka (1961) suggested that volatile fatty acids formed by the action of P. roqueforti lipase system were inhibitory to the mold protease system.

Changes in NPN During Ripening

Non-protein nitrogen values were taken as an indication of proteolysis occurring during the ripening of QR Blue cheese. Morris et al. (1963) used tyrosine liberation as an indicator for protein degradation and found a progressive increase during the ripening.

NPN was defined in this study as the percent of total protein soluble after precipitation of a cheese slurry with 2 percent or 12 percent trichloracetic acid (TCA).

The NPN curves obtained were similar in most preparations of Blue cheese. The data were characterized by a gradual increase in NPN in the early part of ripening. This was followed by a rapid increase in the amount of soluble protein liberated from the cheese. The last days of ripening were distinguished by inappreciable increases in NPN. Generally, 45-60 percent of the total protein was present in the soluble form following precipitation with 12 percent TCA.

There were considerable differences in NPN values obtained from fresh curd. This was likely due to the treatment of the cheese milk before manufacturing. Such parameters as the age of milk, the temperature the milk had been subjected to before cheese manufacture and the microbial population of the milk at the time of processing could have conceivably influenced the initial amounts of NPN.

The progressive increase in NPN during the early part of ripening was likely due to proteolysis resulting from the starter culture and rennet. In order to obtain a better concept of their contribution to proteolysis, NPN values were obtained using a 2 percent TCA precipitation. Jesperson (1966) and Itoh (1972) demonstrated that proteolysis due to starter cultures and rennet could contribute substantially to protein degradation in cheeses ripened for an extended period. The early increase in NPN attributed to these two factors usually amounted to approximately 5 percent.

The marked increase in NPN coincided with the rapid increase in pH. These data are in agreement with Currie (1914) and Morris et al. (1951) and supported the contention that the rapid increase in pH was due to proteolysis. Rennet and starter cultures probably contributed little to this sudden increase in soluble protein. It was probably due almost exclusively to the protease system of P. roqueforti. The rapid rise in NPN also coincided with the first appearance of any blue pigmentation.

Toward the end of ripening there were only slight increases in NPN. Several factors could have contributed to this leveling effect. Imamura and Kataoka (1961) found that volatile fatty acids formed by the action of P. roqueforti lipase were inhibitory to the mold protease. Bolcato, Spettoli, and Perutto (1973) discovered that a 3 percent amino acid concentration repressed the

intracellular protease of Penicillium but induced the extracellular proteolytic enzyme. These authors (1973) suggested extracellular proteolysis was controlled by an unidentified catabolite or catabolites which could be derived from almost any carbon source. Another explanation for this phenomena was that NaCl may have inhibited the activity of the proteolytic enzymes. This appeared doubtful, however, since the leveling effect took place before NaCl concentrations reached 4 percent. Nishikawa (1958) observed that a NaCl concentration of 2 percent stimulated the proteolytic enzymes and a 4 percent concencentration only slightly decreased proteolytic activity.

Changes in PAGE During Ripening

Present electrophoretic techniques provided an excellent means for a novel approach to the study of cheese-ripening by detecting important changes in the intact caseins of cheese. In the past, moving boundary electrophoresis has been used (Lindqvist & Storgards, 1959). Paper electrophoresis also has been used (Young & Ashworth, 1960). Neelin, Rose, and Tessier (1962) demonstrated superb separation of casein fractions in starch gel electrophoresis. Peterson (1963) indicated that polyacrylamide gels gave better resolution than paper electrophoresis. This author (1963) further mentioned advantages of polyacrylamide over starch gel electrophoresis in studying milk proteins.

For the reasons mentioned above, PAGE was performed to study the changes that take place in the specific casein components during ripening of QR Blue cheese. However, PAGE was not without problems. Although the identity of alpha-sub-S (α_S), beta (β) and para kappa (para-K) casein zones in the cheese sample gel pattern was reasonably certain, tentative identification of the gamma (γ) casein zone, which probably represents a number of additional minor caseins such as R-, S-, and TS-caseins (Groves, 1969), was based entirely upon its relatively low electrophoretic mobility characteristics (Mackinlay & Wake, 1965). Also the gradual disappearance of the major casein components during progressively longer cheese ripening suggested that the residual casein degradation products may have been derived from corresponding casein components. However, it may not be completely accurate to identify the appearance of new electrophoretic bands as to the type of casein from which it originated.

The action of rennet was noted immediately on the electrophoretic pattern obtained from samples of fresh curd by the appearance of para-K casein. Para-K casein is the only protein of any significance in the milk system that has a net positive charge at pH 9.0; therefore, its

migration toward the cathode is conspicious. The primary effect of rennet on cheese manufacture is well documented.

The first apparent change in PAGE that was visible occurred during overnight ripening. A band of slightly greater mobility than α_S-casein appeared and became more intense during the initial ripening period. This appeared to be a slight degradation product of α_S-casein that was likely due to rennet and/or starter culture enzymes. Ledford, O'Sullivan, and Nath (1966) observed a similar band and attributed the new band to the degradation of α_S-casein by rennet since cheese made without starter cultures had an identical band. β-casein appeared to be unaffected by rennet and starter culture enzymes under the conditions employed in quick ripening. Likewise, the β- and para-K casein did not appear to change. Young and Ashworth (1960) found that some nitrogenous material released by rennin migrated at the same rate as the intact casein fractions in paper electrophoresis, thereby producing no change in the electrophoretic patterns. Ledford et al. (1966) revealed that most microbial enzymes appeared alike, in that α-casein was degraded in almost all cheeses and β-casein was resistant to proteolysis in some types of cheese and not in others. Creamer (1971) suggested that the major factor in determining the relative rates of α- and β-casein degradation in Cheddar cheese was the activity of the water in the cheese. Whether this was because of

the effect of water activity on the rennet enzymes or the caseins was not determined.

The next changes that were noticeable in PAGE occurred simultaneously with increases in pH and NPN values. The most apparent change that occurred was the disappearance of para-K-casein. This coincided with a general weakening of intensities in the α_S- and β-casein regions. The γ, K-casein region was of particular interest since the protein zones became slightly more intense at this time. If α-casein was due to β-casein degradation, as it now appears to be (Gordon et al., 1972) this might explain the increase in intensity of the γ-casein region as β-casein zones weakened. It also might explain the absence of any new zones in the β-casein area during ripening. The fact that para-K was the first component to vanish could be explained several ways. In comparison to the other types of casein, para-K was a smaller molecular weight component and was present in lesser quantities, or perhaps it possessed bonds which were more susceptible to cleavage by the proteases involved.

As ripening continued the remaining constituents were degraded. Generally, β-casein disappeared before the α_S- or γ, K-casein. The last traces of these latter components usually disappeared simultaneously. The last days of ripening in QR Blue cheese were distinguished by the complete absence of any electrophoretically noticeable bands. Blue cheese ripened by conventional methods was

differentiated by conspicious bands in all the casein regions. This was significant since it implied that the casein in such cheese was not degraded to the same extent as in QR Blue cheese.

Lindqvist and Storgards (1959) used moving boundary electrophoresis to classify cheese ripening as α-, β-, or nonspecific, depending on the relative rates of α- and β-casein breakdown. Using these criteria, they classified Cheddar cheese as α-ripening and Roquefort cheese as non-specific. QR Blue cheese could be defined as a non-specific ripening since α_S- and β-casein were hydrolyzed at much the same rate when analyzed by PAGE.

Results and Discussion for the Modifications Attempted

In an effort to clarify the discussion, the cheese will be discussed under subtitles. All modifications of QR Blue cheese were made in duplicate.

Control Blue Cheese

QR Blue cheese was manufactured by the procedure of Kondrup and Hedrick (1963). The only change made was that a frozen concentrated starter culture was used. This QR cheese served as a control by which the effect of processing variables could be assessed. Two commercial Blue cheeses were also used as controls.

The data presented in Table 1 revealed the pH and NPN measurements for a QR Blue cheese manufactured by the

Table 1.--pH and NPN Values of Blue Cheese During Quick Ripening at 62 F (17 C) and Salted on Days 4, 5, and 6.

Day of Ripening	pH	NPN
0	5.17	0.8
1	4.78	1.8
2	4.66	2.8
3	5.28	10.4
4	5.69	28.2
5	5.93	40.8
6	6.09	44.8
7	5.92	45.2

method of Kondrup and Hedrick (1963). The hydrogen ion concentration reached a maximum on the second day and NPN values increased only slightly through this day. This was due to production of lactic acid and the weak proteolytic activity of starter culture enzymes and rennet. Between the second and the sixth day there was a rapid increase in both pH and NPN. This effect is the result of protease activity of P. roqueforti. The final days of ripening were characterized by a slight decrease in pH and a small increase in NPN. This demonstrated that the cheese proteins were still being degraded but the formation of free fatty acids overshadowed proteolysis and caused a slight decline in the pH.

The average pH and NPN values for the commercial Blue cheese were 5.79 and 40.6 percent respectively. These values were only slightly lower than the data obtained for QR Blue cheese.

Figure 1 shows the changes that occurred in the cheese proteins during ripening as observed in PAGE patterns. The most notable change that occurred in the patterns was the abrupt weakening of the protein zones on day four of ripening and the complete disappearance on day five. These observations compare favorably with data from pH and NPN values since the rapid increase in these latter values occurred during the third to fifth days of ripening.

Other points of interests to be noted from the data in Figure 1 are the appearance of new bands in the α_S-casein region, the deficiency of new bands appearing in the β-casein region and the enhancement of bands in the γ, K-casein area. Since α_S-casein was a more highly charged protein it could be presumed that the development of degradation products would also be highly charged and still migrate closely to the original protein. The lack of new zones in the β-casein region may be correlated with the increasing intensities of zones in the γ, K-casein area since γ-casein has been reported as arising from β-casein.

Figure 2 exhibits the PAGE patterns obtained from commercial Blue cheese ripened by conventional methods. These gels showed weak bands in all the casein regions except the para-K-casein area which was devoid of any

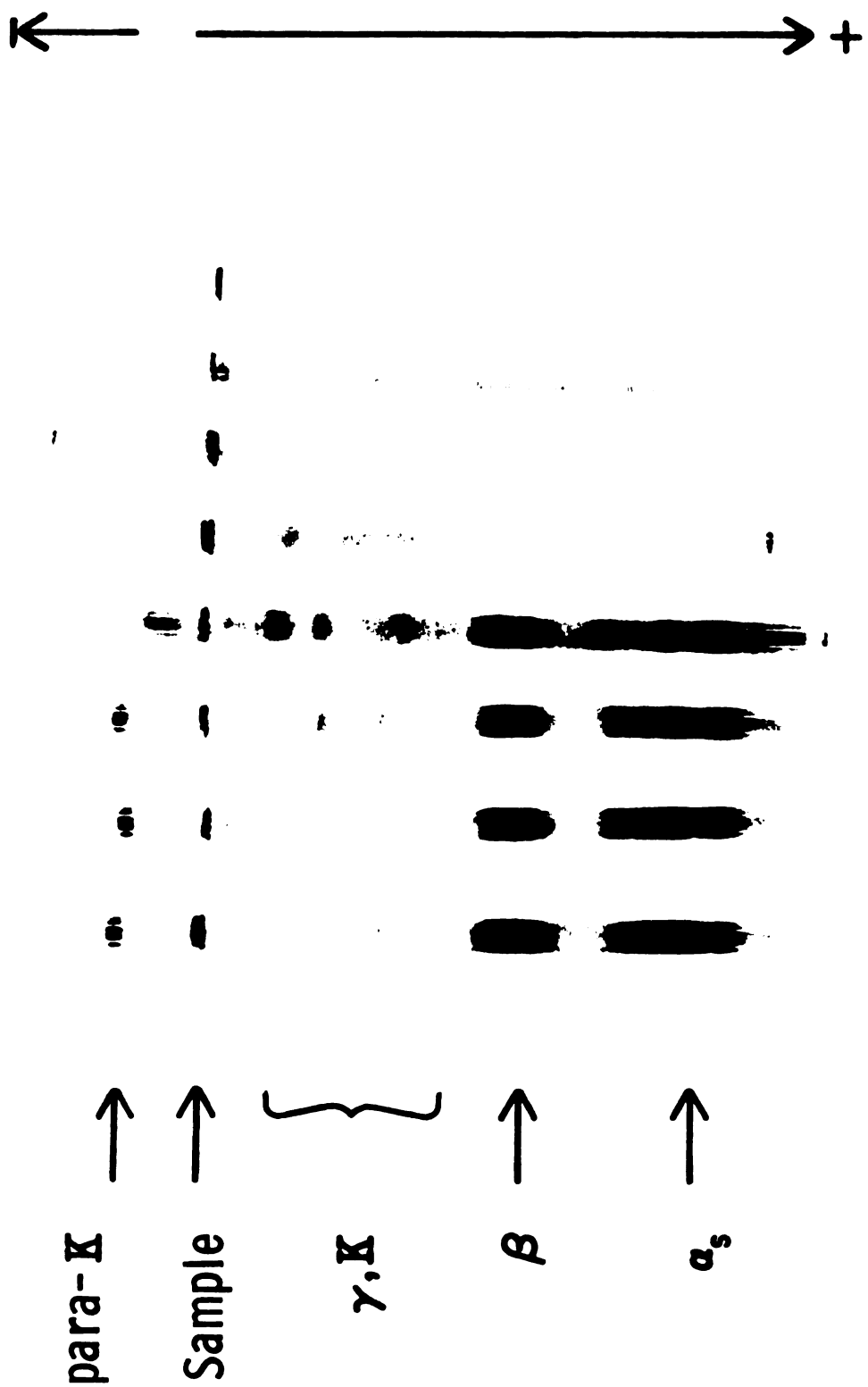

Fig. 1. PAGE patterns of Blue cheese ripened at 62 F (17 C) and salted on days 4, 5, and 6.

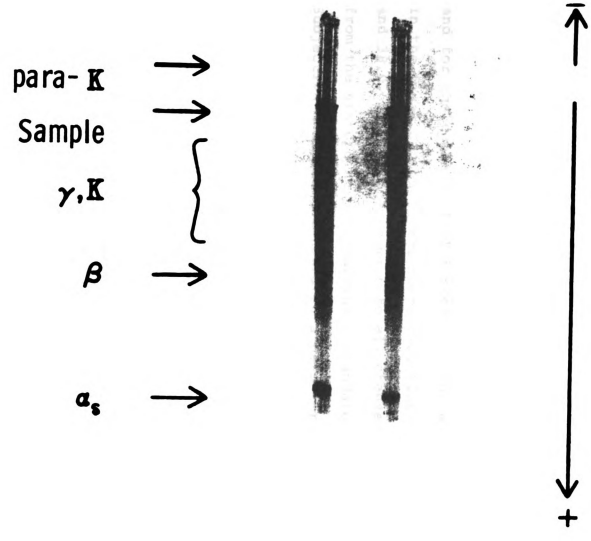

Fig. 2. PAGE patterns of two different Blue cheeses ripened by conventional methods.

protein zones. This may explain the texture differences exhibited between QR Blue cheese and commercial Blue cheese. The weak texture associated with QR Blue cheese was probably due to the lack of appreciable protein integrity.

Table 2 presents subjective analyses for two batches of QR Blue cheese on the seventh day of ripening and for the commercial Blue cheeses. The wide variation in the final scores of the commercial Blue cheeses, 32.7 and 38.0, is a problem common to all cheese manufactured from the standpoint of producing a standardized product. Subjective analysis demonstrated a need for improvement in QR Blue cheese. The QR cheese was criticized for mild flavor, excessive mold color, and a weak texture. The same samples were analyzed after two weeks of storage at 40 F (4 C) and a definite improvement in flavor was noted. This could be due to an increase in ketone formation. The cheese after storage was still criticized as containing excessive mold growth and a weak texture.

Effect of Ripening Temperature on QR Blue Cheese

In an attempt to discover the optimum temperature for ripening QR Blue cheese, four different temperatures were used. Each of the cheese batches was salted when sporulation occurred which was evident by the development of blue pigmentation. The salting involved 3 applications on successive days in the ratio of 1/4, 1/4, and 1/2 of

Table 2.--Subjective Analyses of Control Blue Cheeses.

Cheese	Flavor (20)	Texture (10)	Color (15)	Total (45)
Commercial	13.7	8.0	11.0	32.7
	Moldy	Sl.* mealy	Lacking	
Commercial	18.0	7.0	13.0	38.0
	Excellent	Weak	Sl. lacking	
QR Blue Cheese	13.4	6.3	12.0	29.2
	Mild	Weak	Excessive	
QR Blue Cheese	12.0	6.3	12.0	34.6
	Mild	Weak	Excessive	

*Sl.=slightly.

the total salt respectively. The temperatures involved with the days of salting, which are in parenthesis, were 48 F (11, 12, 13), 52 F (7, 8, 9), 62 F (4, 5, 6), and 72 F (3, 4, 5).

The most obvious effect of temperature on cheese ripening was that as temperatures were increased there was a comparable decrease in the time required for ripening. Figure 3 reveals the effect of temperature on changes in pH during ripening. All the curves shown exhibited an initial increase in hydrogen ion concentration, presumably due to lactic acid production by the starter cultures. The minimum pH attained by all the temperature variables was quite similar, however, the rapidity of minimum pH attainment was directly related to the temperature employed in ripening. The 72 F (22 C) cheese reached a minimum pH on

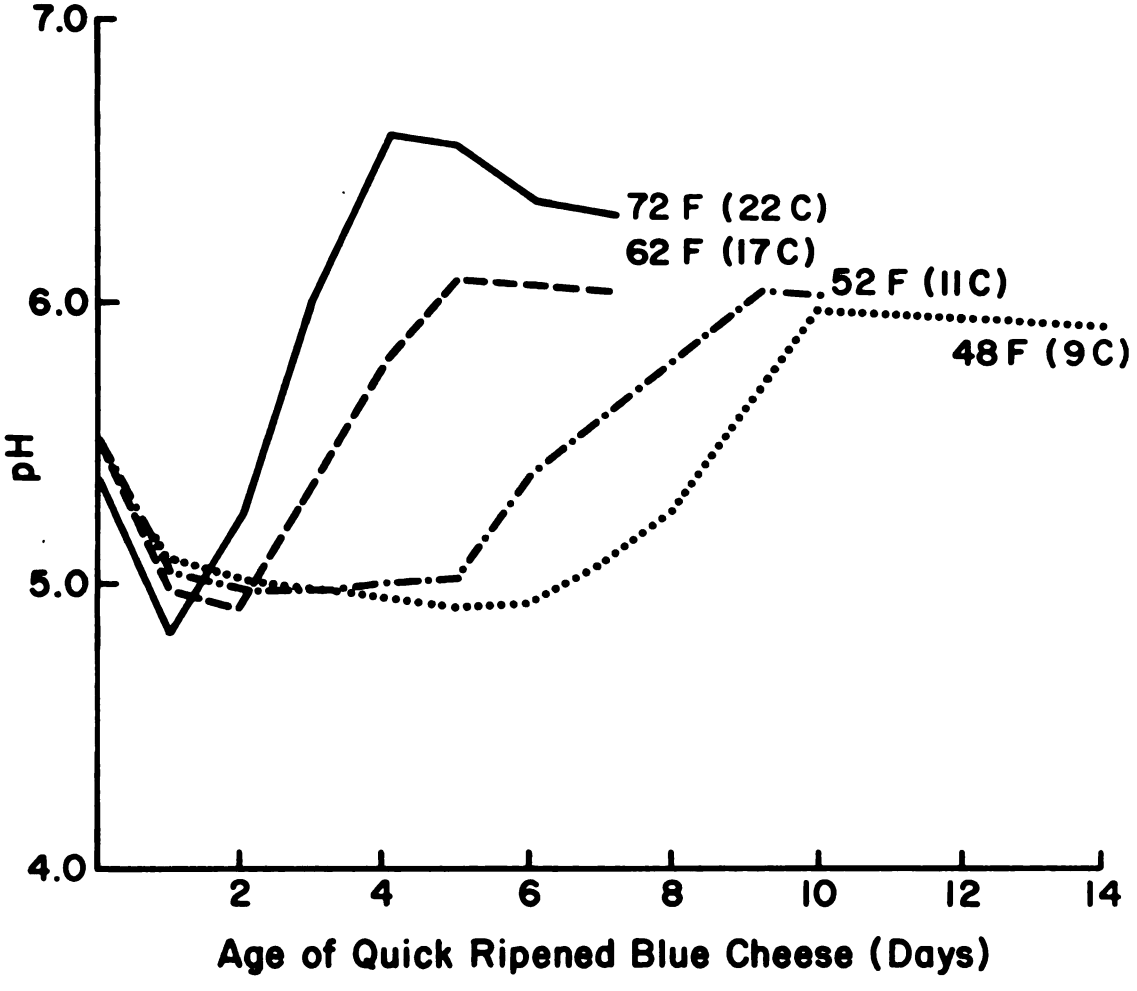

Fig. 3. The effect of curing temperature on changes in pH of Blue cheese curd.

the first day and 6 days were required for the 48 F (9 C) cheese to obtain a comparable hydrogen ion concentration. This was likely due to increased activity of the starter cultures at elevated temperatures.

The rapid increase in pH, which was attributed to proteolysis, was delayed and more gradual as the temperature was lowered. This observation can be explained by the effect of temperature on protease activity. Modler et al. (1974) demonstrated the optimum temperature for casein hydrolysis to be 46 C using an extracellular protease of P. roqueforti.

The final pH obtained by the different cheeses was similar except for the 72 F (22 C) cheese which exhibited a higher pH followed by a substantial decrease during the final days of ripening. This could be due to a more rapid and extensive proteolysis followed by a slightly slower development of free fatty acids and other factors which contribute to the overall acidity of QR Blue cheese.

Figure 4 demonstrates the effect of temperature on changes in NPN during ripening of Blue cheese at various temperatures. There was a direct relationship between temperature and casein degradation as is shown in this figure. The final level of NPN exhibited only small variations between different temperatures of ripening.

Figures 5 through 8 pictorially illustrate the protein degradation in QR Blue cheese manufactured at different temperatures. The patterns were similar from the

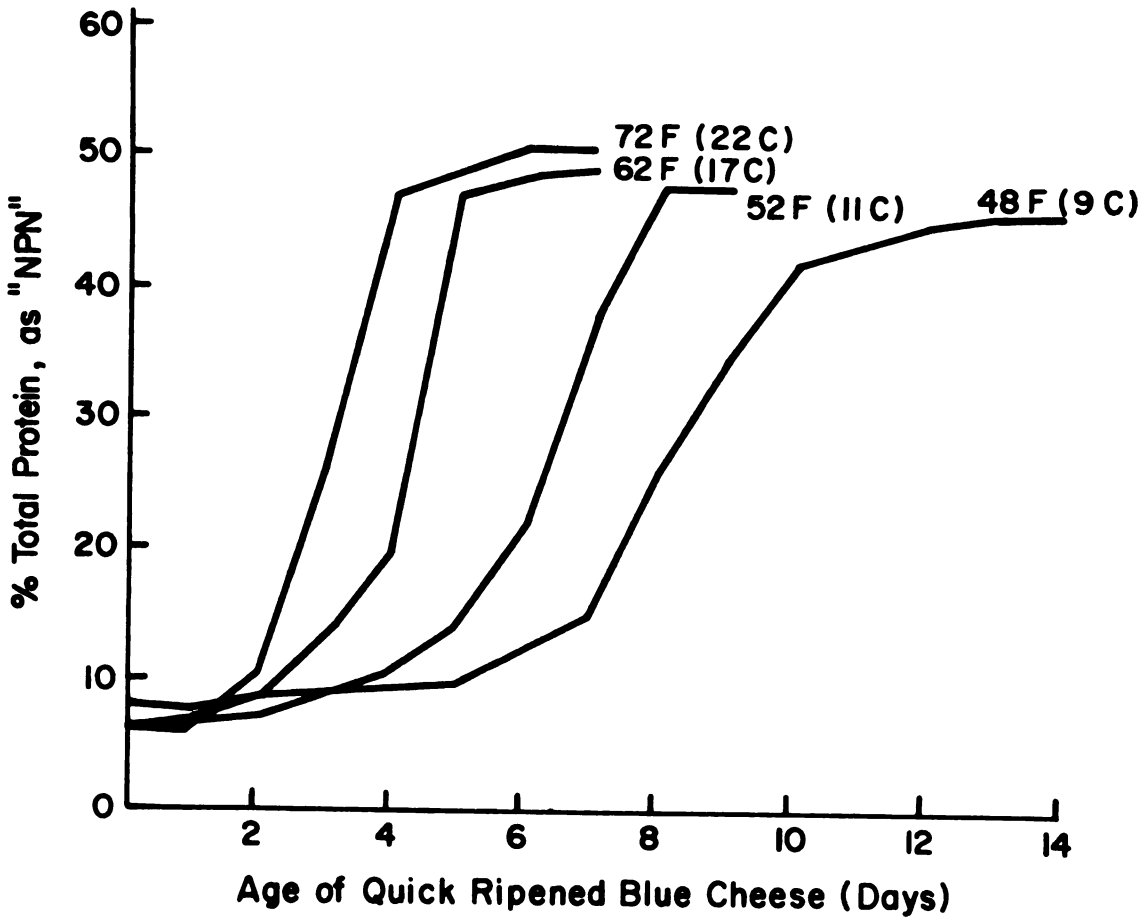

Fig. 4. Effect of curing temperature on changes in NPN of Blue cheese curd.

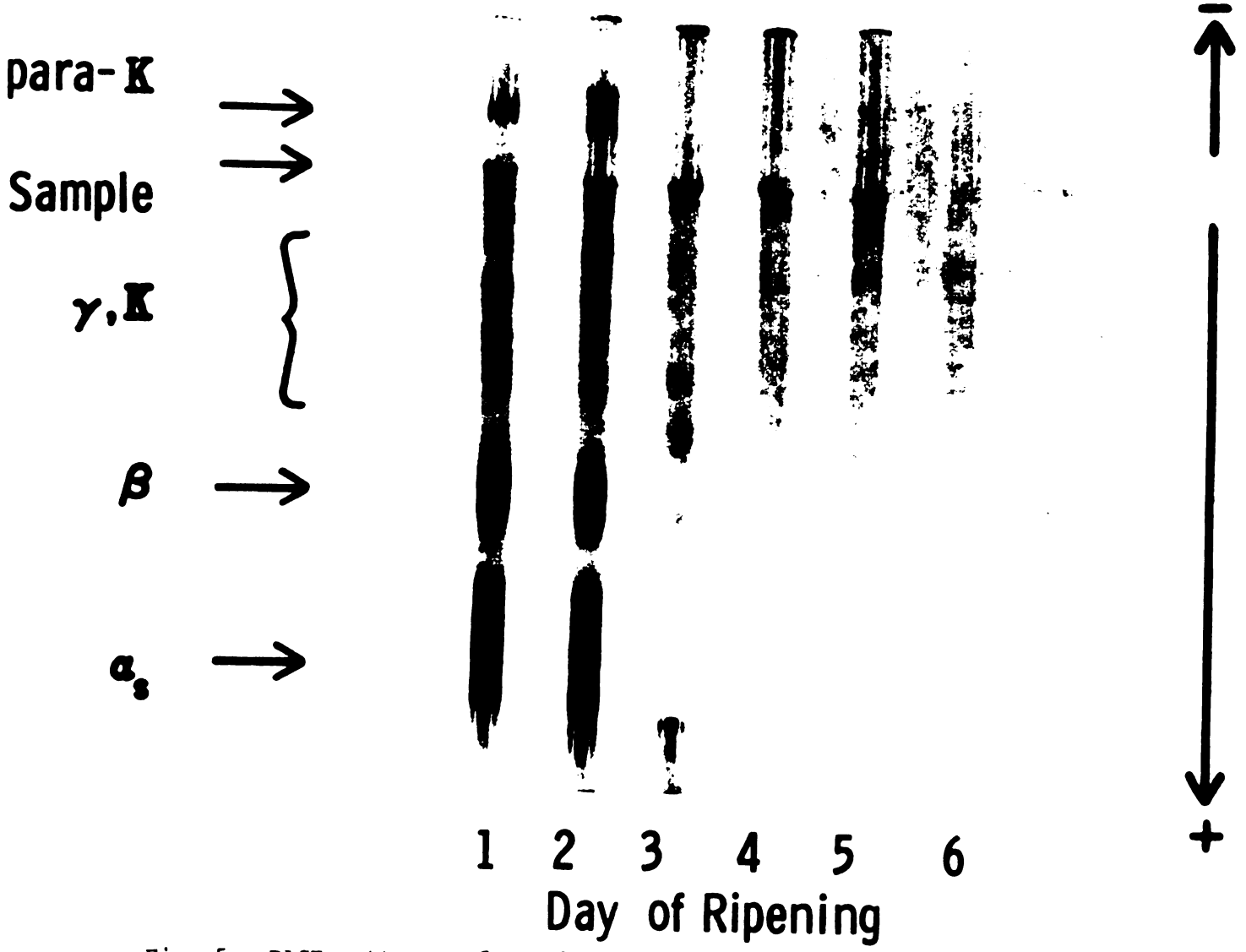

Fig. 5. PAGE patterns of QR Blue cheese ripened at 72 F (22 C) and salted on days 3, 4, and 5.

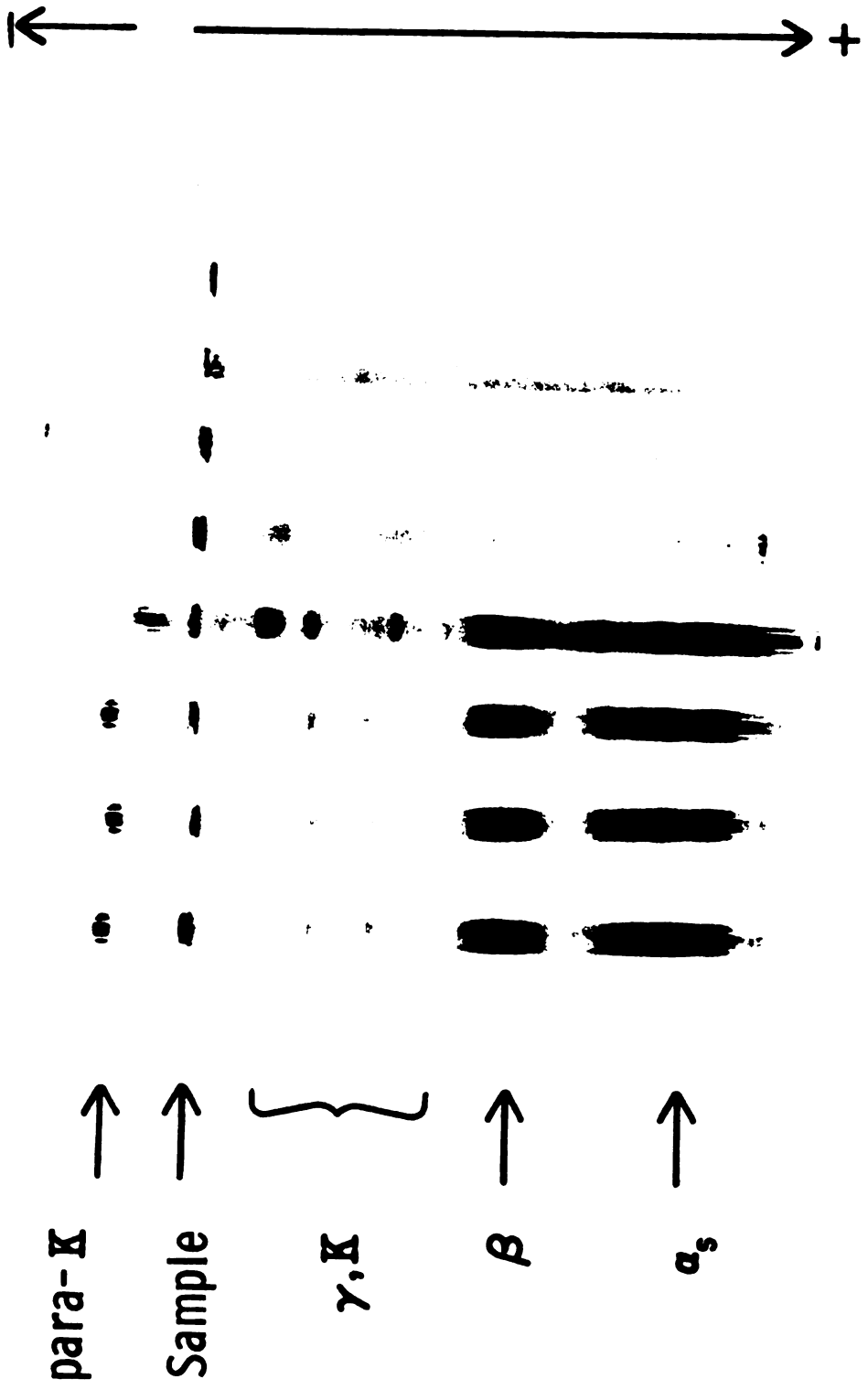

Fig. 6. PAGE patterns of QR Blue cheese ripened at 62 F (17 C) and salted on days 4, 5, and 6.

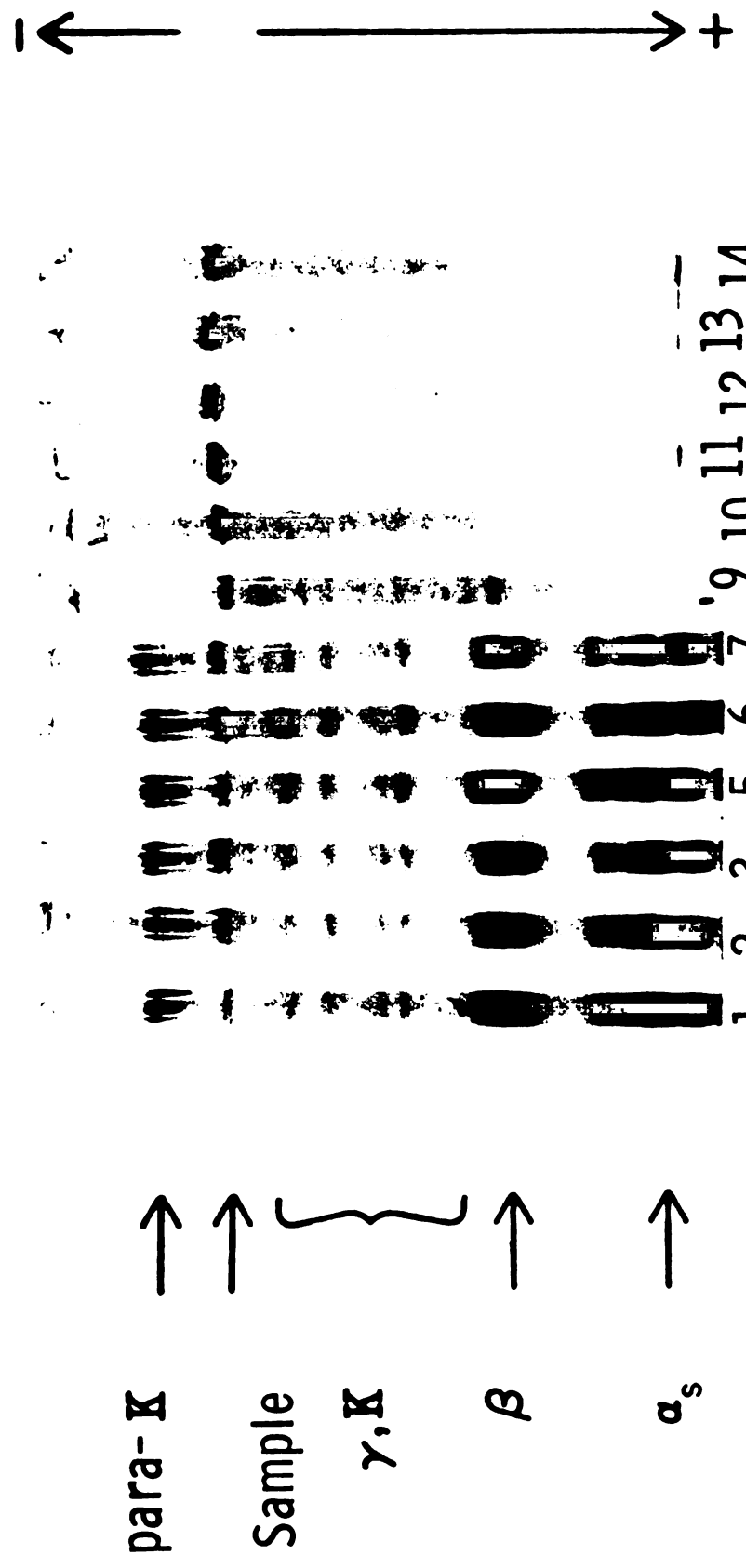

Fig. 7. PAGE patterns of QR Blue cheese ripened at 48 F (9 C) and salted on days 11, 12, and 13.

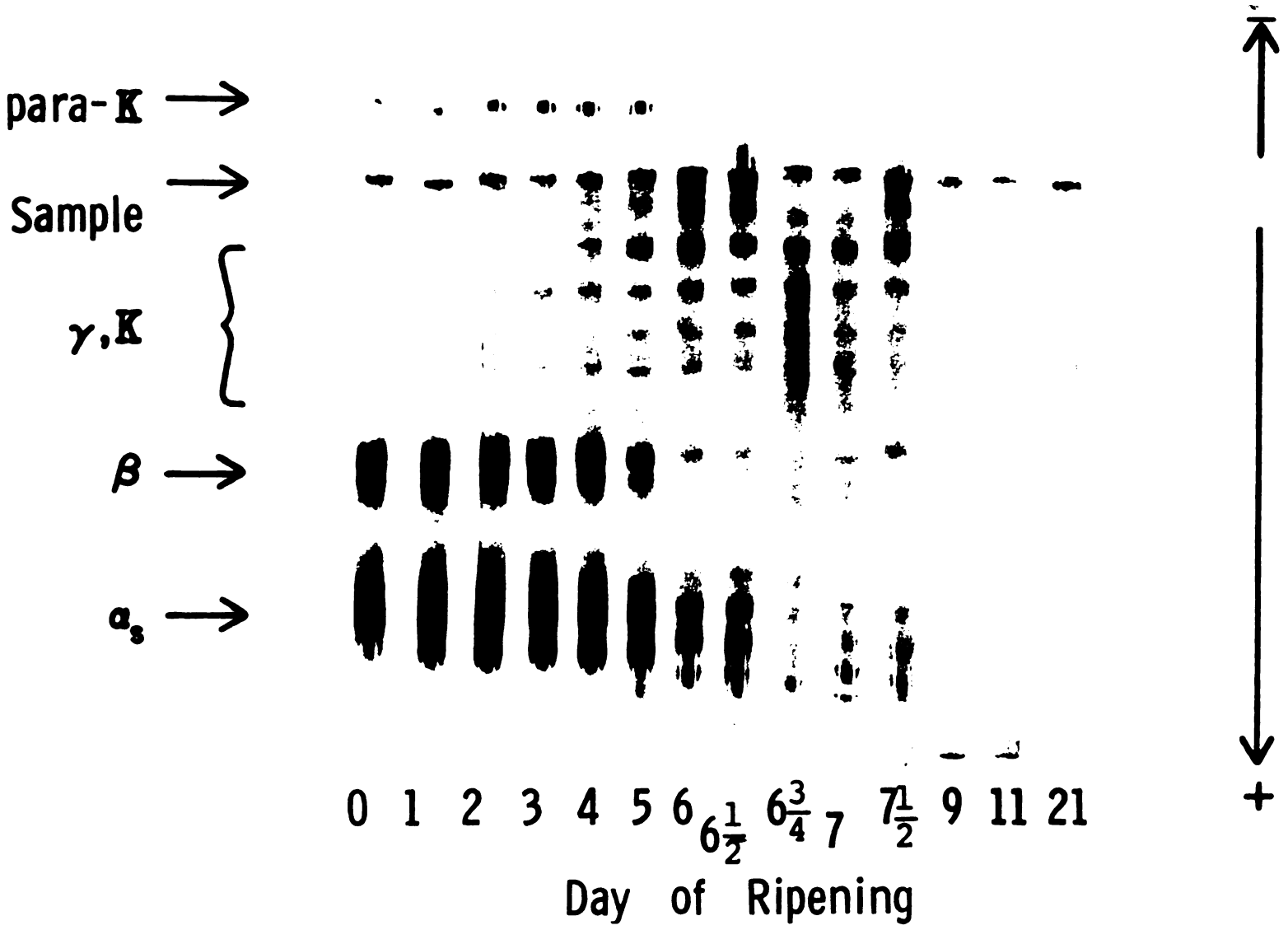

Fig. 8. PAGE patterns of QR Blue cheese ripened at 52 F (11 C) and salted on days 7, 8, and 9.

standpoint of common bands and differ only in the time of occurrence. It was notable that the last traces of protein zones disappeared from the gels simultaneously with the leveling effects noted previously in pH (Figure 3) and NPN (Figure 4).

Subjective analyses on QR Blue cheese ripened at the different temperatures is presented in Table 3. All the samples were criticized for a weak texture, presumably due to the extensive protein degradation. This was especially true for the cheese ripened at 72 F (22 C). This particular cheese was heavily criticized in all quality departments. At this ripening temperature, fermentation was so intense that, even with early salting, very little control over the reactions could be exerted. The 48 F (9 C) cheese graded superior to all the other cheeses manufactured at different temperatures. It was a well balanced, moderately flavored cheese with no traces of any off flavors. Blue cheese ripened at 62 F (17 C) and 52 F (11 C) proved to be good cheese with the major fault being a mild flavor.

Some conclusions can be extrapolated from the data obtained from cheese ripened at different temperatures. The cheese improved in flavor as the temperature was lowered. The lower ripening temperature probably retarded contaminating organisms more than it retarded the desired fermentation caused by P. roqueforti. It was questionable whether the slight increase in quality as the temperature

Table 3.--Subjective Analyses of QR Blue Cheese Manufactured at Different Temperatures.

Temperature of Ripening	Flavor (20)	Texture (10)	Color (15)	Total (45)
72 F (22 C)	8.0 Bitter	3.7 Gummy	6.5 Slight	18.2
62 F (17 C)	13.4 Mild	6.3 Weak	12.0 Excessive	31.7
52 F (11 C)	13.0 Mild	7.7 Sl. weak	13.0 Lacking	33.7
48 F (9 C)	15.0 Sl.* mild	7.0 Weak	12.5 Lacking	35.0

*Sl.=slightly.

decreased from 62 (17) to 48 F (9 C) was worthwhile since the ripening period was extended by 7 days.

Effect of Salt on QR Blue Cheese

The levels and times of salting played significant roles in the manufacture of QR Blue cheese. Lipolysis, proteolysis and growth of contaminating organisms are all affected by salt concentration.

Four QR Blue cheeses were manufactured with varying salting times. The days of salting, with the relative amounts of total salt added each day in parenthesis, were: cheese A--1,2,3 (1/4,1/4,1/2); cheese B--1,4,5 (1/4,1/4, 1/2); cheese C--0,4,6 (1/2,1/4,1/4); and cheese D--2,4,5,6 (1/4,1/4,1,/4,1/4). These were compared directly to the control cheese which was salted on days 4,5, and 6

(1/4,1/4,1/2). Data collected on cheese B will not be pictorially illustrated nor discussed fully due to the similarity of results to cheese D.

The effect of the various salting modifications on changes in pH during ripening is presented in Figure 9. All modifications showed an initial decline in pH as lactic acid was produced. Cheese A attained the highest hydrogen ion concentration on day 3 while the other cheeses had similar concentrations on day 2. The cheese which received 50 percent of the salt on day 0 showed a very slight increase in pH from day 2 to day 3. It appeared that an early salt application retarded the ability of P. roqueforti to sporulate and, therefore, produce the enzymes required in Blue cheese ripening.

It was of considerable interest to compare the pH data obtained for cheese A to the NPN data for the same cheese as shown in Figure 10. The cheese showed a rapid increase in pH from day 4 through day 6, however, there was only a slight increase in NPN during the same time. If proteolysis was the causative agent in elevating pH as shown heretofore, other factors must also be involved in the change of pH noted. A possible explanation was that few acid producing substances were being formed due to the salt concentration and hence the slight proteolysis was magnified in large increases in pH.

It appeared that the early application of salt retarded proteolysis only when at least 50 percent of the

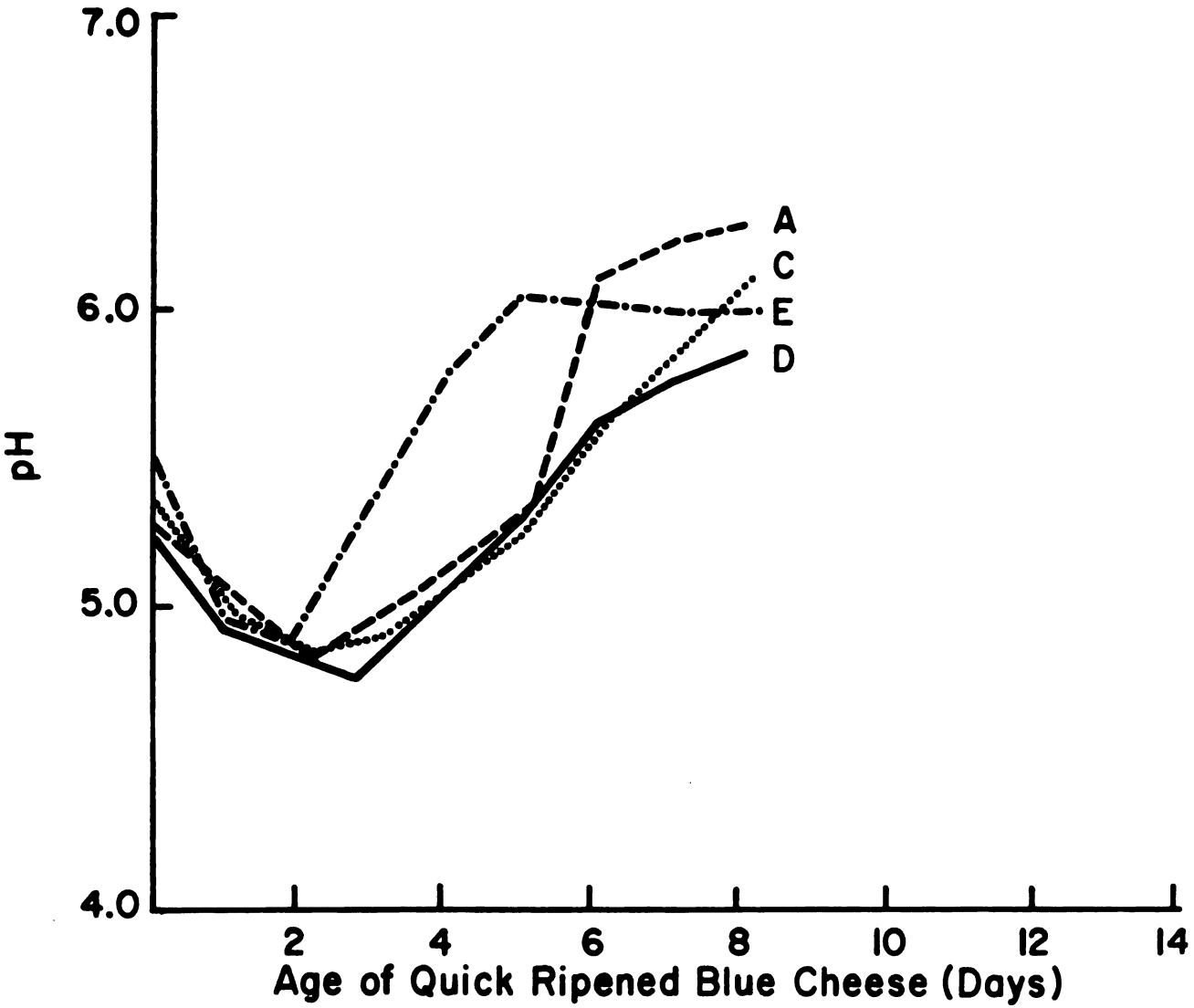

Fig. 9. The effect of salting times on changes in pH of Blue cheese curd. Cheese A: salted on days 1,2, and 3 with (1/4),(1/4), and (1/2) of the total respectively; C: 0,4,6 (1/2,1/4,1/4); D: 2,4,5,6 (1/4,1/4,1/4,1/4); E: 4,5,6 (1/4,1/4,1/2).

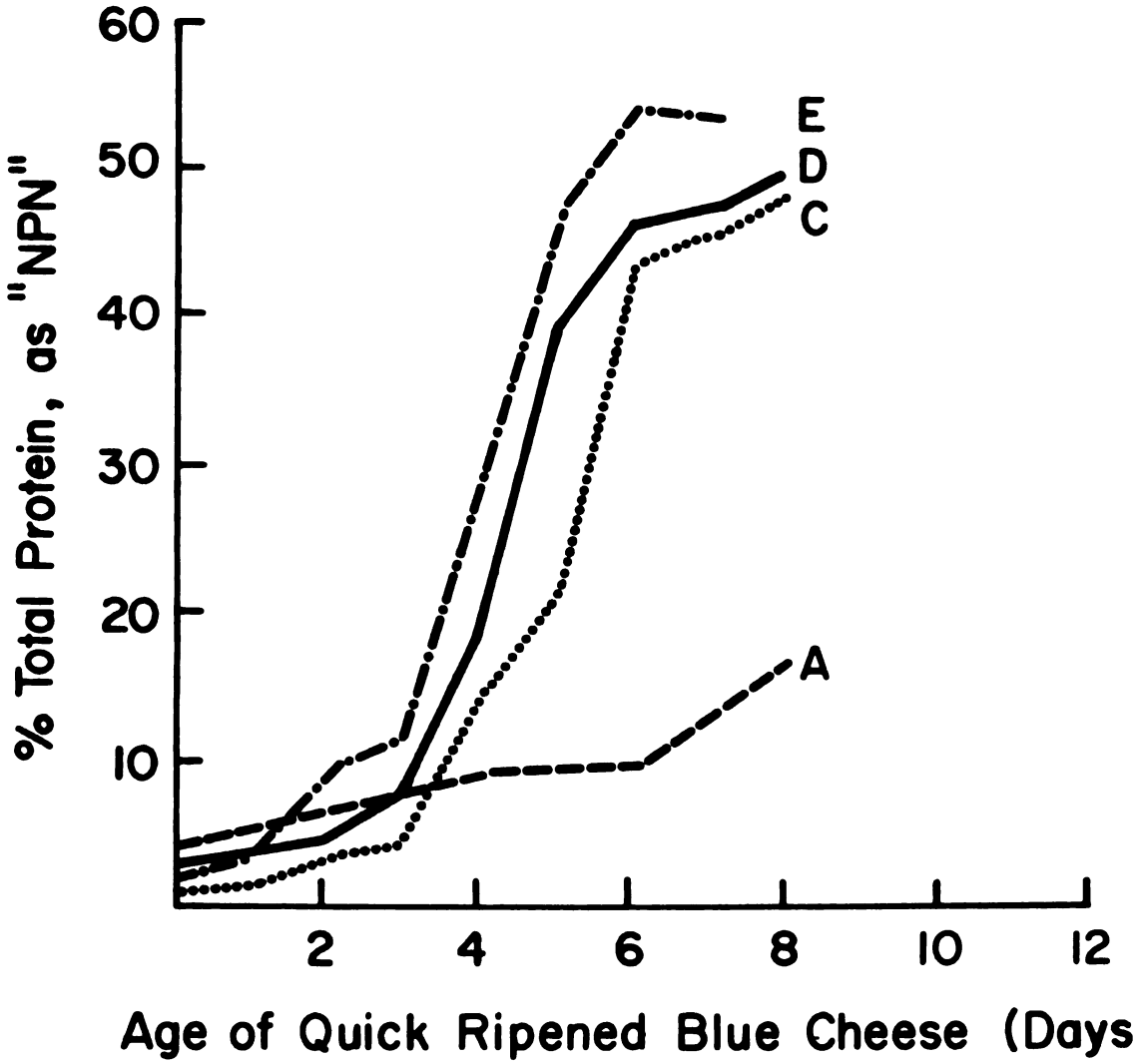

Fig. 10. The effect of salting times on changes in NPN of Blue cheese curd. Cheese A: salted on days 1,2, and 3 with (1/4),(1/4), and (1/2) of the total respectively; C: 0,4,6 (1/2,1/4,1/4); D: 2,4,5,6 (1/4,1/4,1/4,1/4); E: 4,5,6 (1/4,1/4,1/2).

NaCl had been added prior to the fourth day of ripening at 62 F (17 C). This was evident when comparing the NPN values obtained for cheese A and cheese C (Fig. 10). Even by adding 50 percent of the total salt to the fresh curd before ripening, proteolysis was not retarded after 7 days. A better representation of these data can be observed in Figures 11 and 12. These figures show the PAGE patterns on cheese A and cheese C respectively. When all the salt was added by the third day, as in cheese A, the PAGE patterns after 7 days were about equal to the patters on the fourth day in cheese C.

Figure 13 reveals the PAGE patterns obtained from cheese D, which were very similar to those of cheese C. These cheeses also exhibited common pH and NPN values throughout ripening. Figure 14 shows the PAGE patterns of QR Blue cheese which served as the control.

Subjective analyses on the cheese ripened at 62 F (17 C) with various salting applications are shown in Table 4. The cheeses were criticized for being mild or slightly bitter in flavor. This was probably due to a low methyl ketone content. The cheese manufactured when all of the salt was added by day 3 was critized for being mealy. This was probably due to the lack of complete protein degradation. The other cheeses were criticized as having a weak texture. A correlation may be made between color development and proteolytic activity. Cheese which

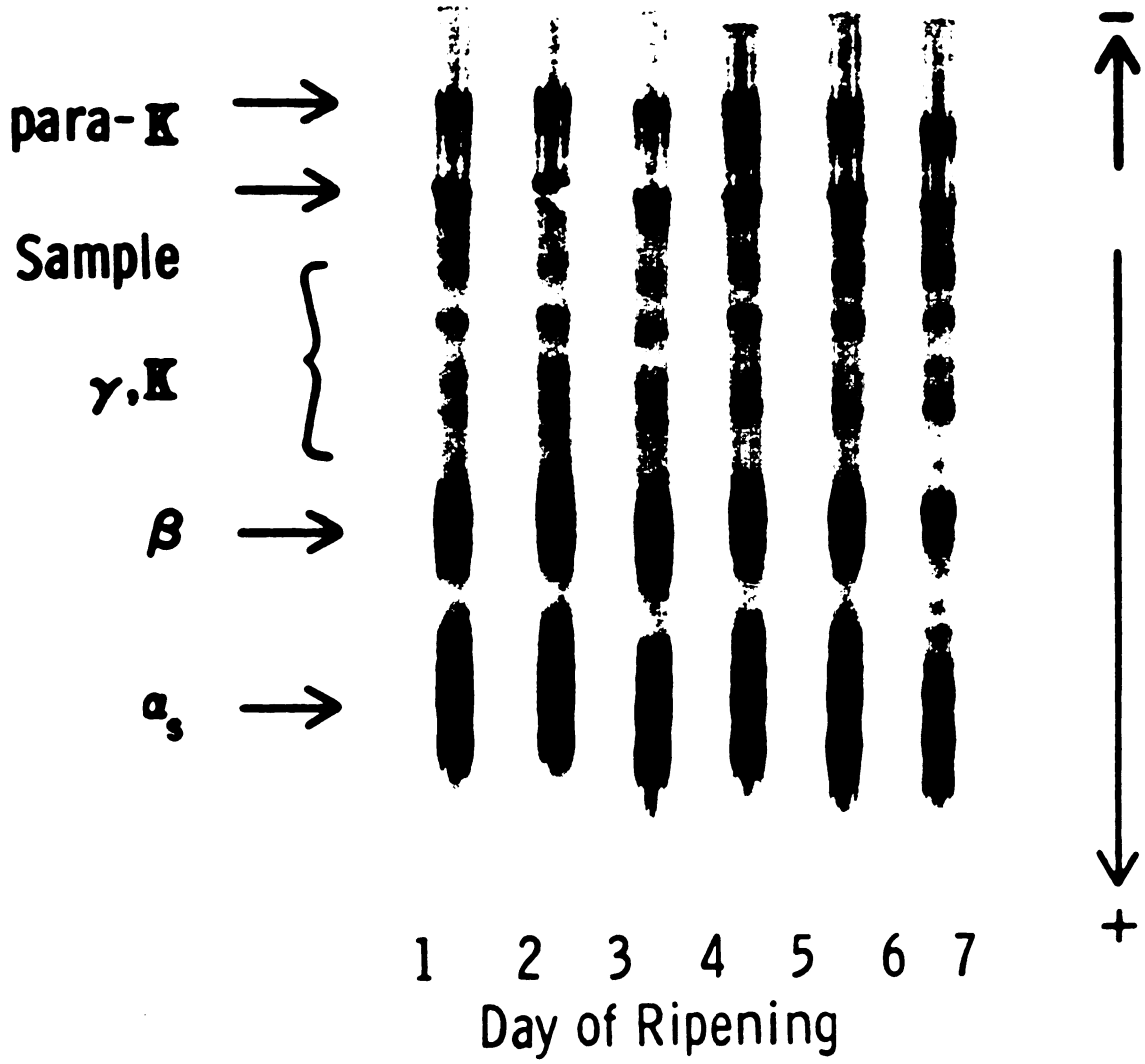

Fig. 11. PAGE patterns of QR Blue cheese ripened at 62 F (17 C) with 1/4 of the total salt added on day 1, 1/4 on day 2, and 1/2 on day 3.

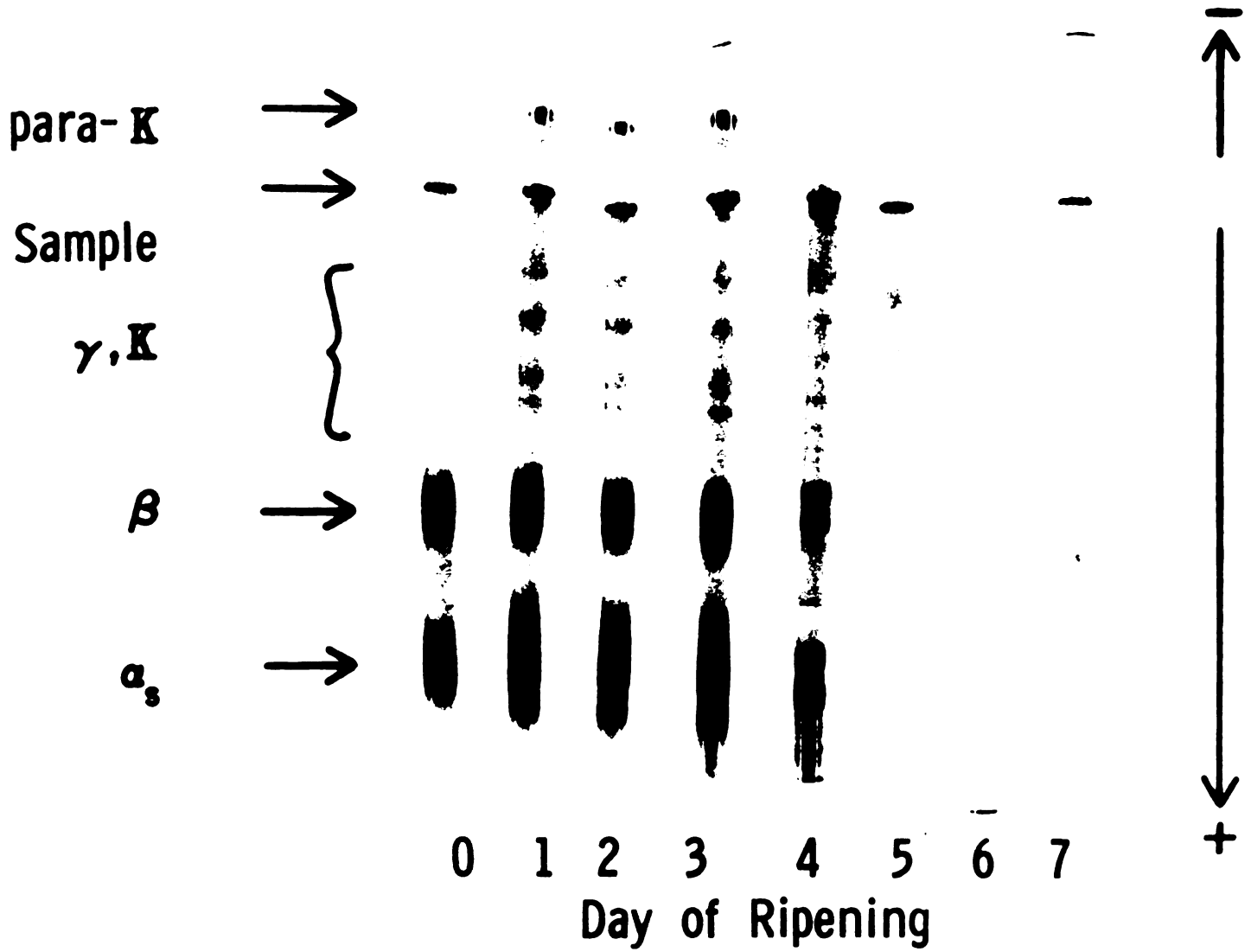

Fig. 12. PAGE patterns of QR Blue cheese ripened at 62 F (17 C) with 1/2 of the total salt added on day 0, 1/4 on day 4, and 1/4 on day 6.

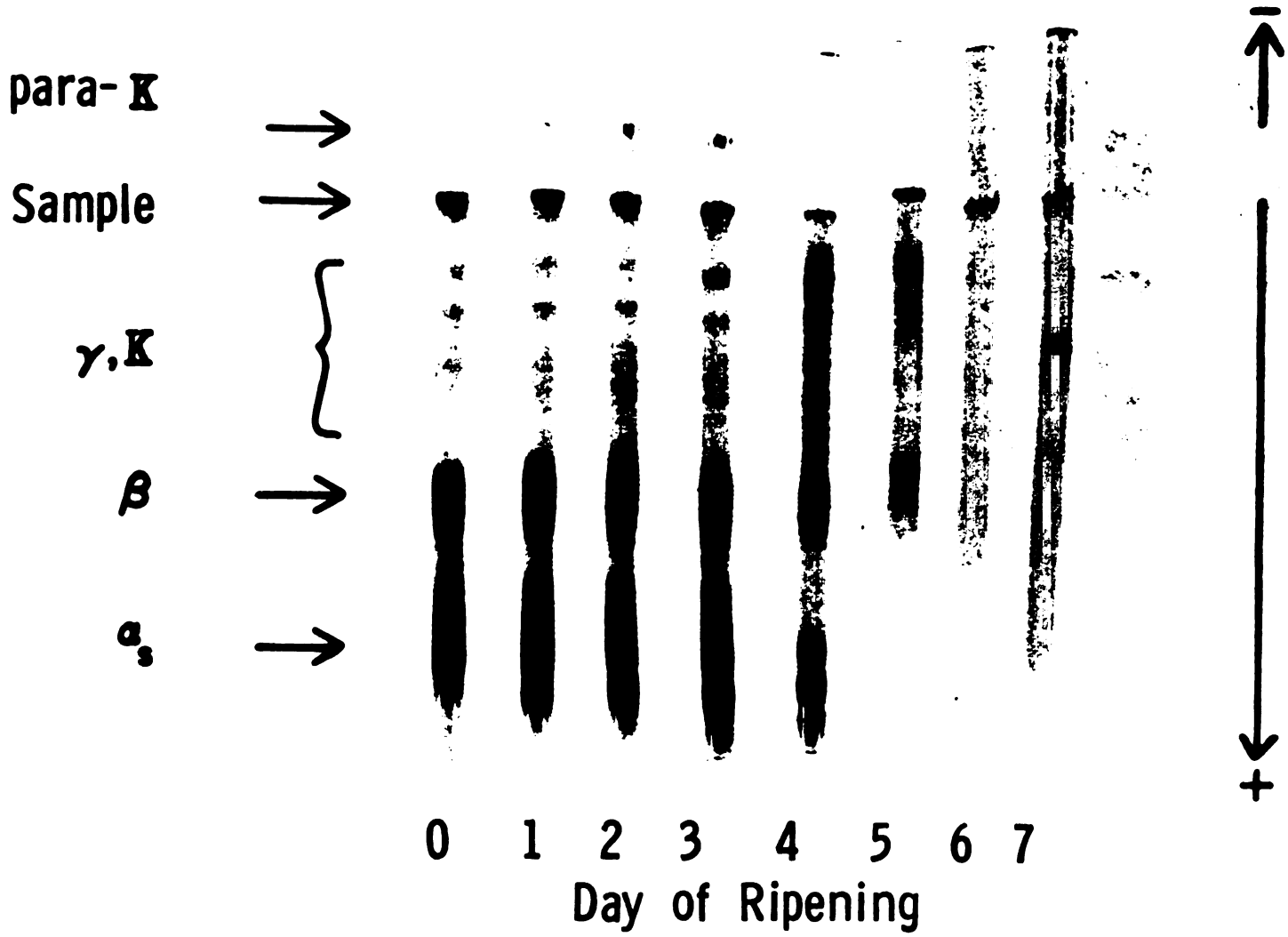

Fig. 13. PAGE patterns of QR Blue cheese ripened at 62 F (17 C) with 1/4 of the total salt added on each of days 2, 4, 5, and 6.

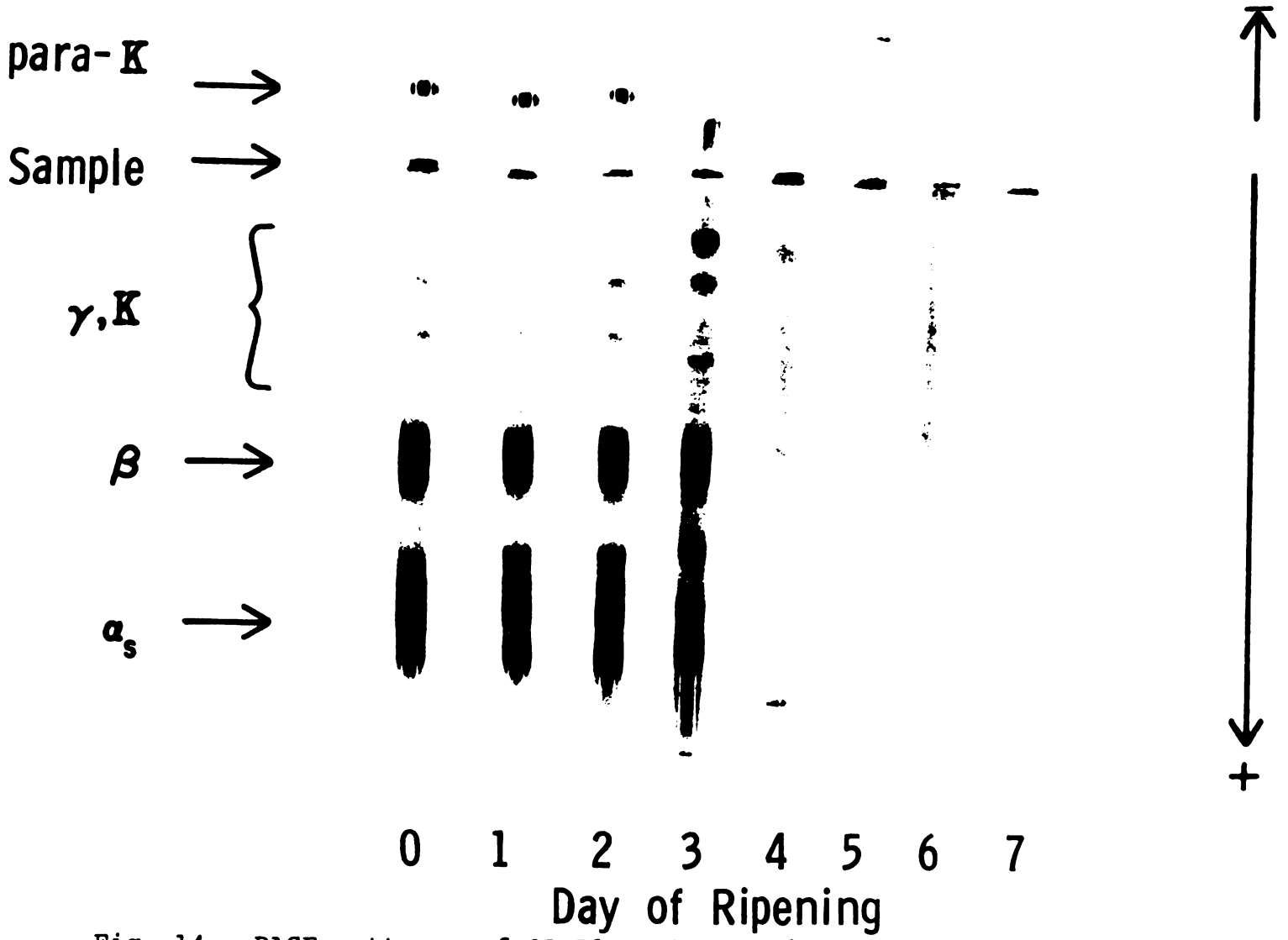

Fig. 14. PAGE patterns of QR Blue cheese ripened at 62 F (17 C) with 1/4 of the total salt added on day 4, 1/4 on day 5, and 1/2 on day 6.

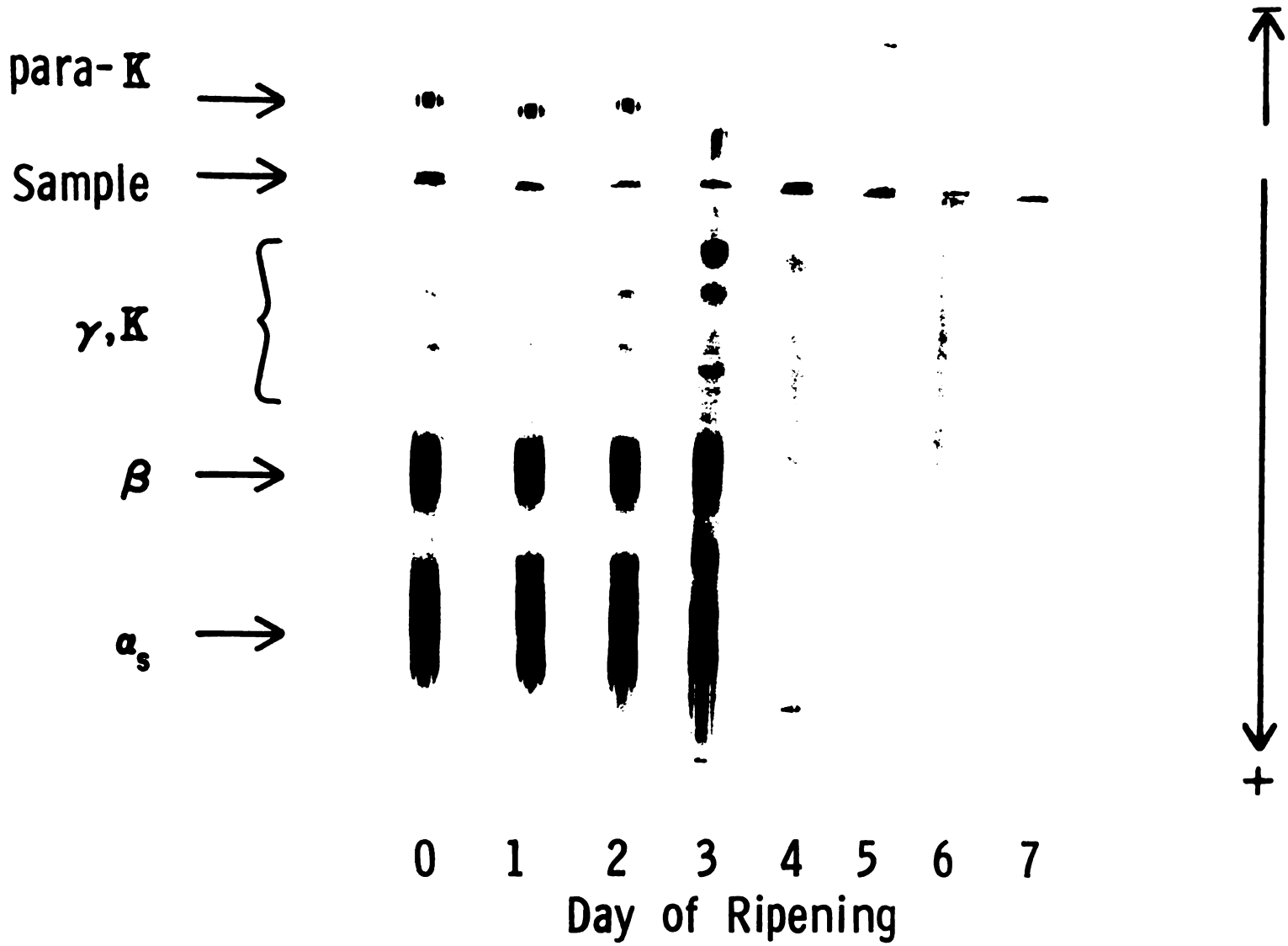

Fig. 14. PAGE patterns of QR Blue cheese ripened at 62 F (17 C) with 1/4 of the total salt added on day 4, 1/4 on day 5, and 1/2 on day 6.

Table 4.--Subjective Analyses of QR Blue Cheese Ripened at 62 F (17 C) With Different Salting Times.

Salt		Flavor (20)	Texture (10)	Color (15)	Total (45)
Days	Proportion				
1,2,3	1/4,1/4,1/2	10.7	5.6	12.7	29.2
		Mild	Mealy	Lacking	
2,4,5,6	1/4,1/4,1/4,1/4	15.3	7.3	12.0	34.6
		Sl.* bitter	Sl. weak	Excessive	
0,4,6	1/2,1/4,1/4	13.3	6.0	12.5	31.8
		Sl. bitter	Weak	Sl. lacking	
4,5,6	1/4,1/4,1/2	13.4	6.3	12.0	31.7
		Mild	Weak	Excessive	
1,4,5	1/4,1/4,1/2	12.7	6.0	10.5	29.2
		Mild	Weak	Excessive	

*Sl.=slightly.

exhibited little pigmentation was also characterized by little protein breakdown.

Effect of Salt on QR Blue Cheese Ripened at Temperatures Other Than 62 F (17 C)

Since the temperature of ripening and salting applications had profound effects on QR Blue cheese, several cheeses were made utilizing temperatures lower than 62 F (17 C) and various salting times. The first temperature selected was 48 F (9 C) with salt added on days 11, 12, and 13 in one batch and days 4, 5, and 6 in another. The other ripening temperature utilized was 52 F (11 C) with salt being added on days 7, 8, and 9 and days 1, 9, and 10. One-fourth of the total salt was always added on the first day indicated, followed by 1/4 and 1/2 respectively.

Figure 15 shows the pH patterns obtained at the lower ripening temperatures of 48 F (9 C) and 52 F (11 C). The 48 F (9 C) cheeses were distinguished by a prolonged ripening period. The pH values obtained from the cheeses ripened at 52 F (11 C) were similar indicating that the early salt application had little effect on the hydrogen ion concentration. This is verified by the data in Figure 16. Even though the early salt application slightly hindered the release of NPN the values obtained the final day of ripening in each batch were much alike. The cheeses ripened at 48 F (9 C) exhibited the same characteristics

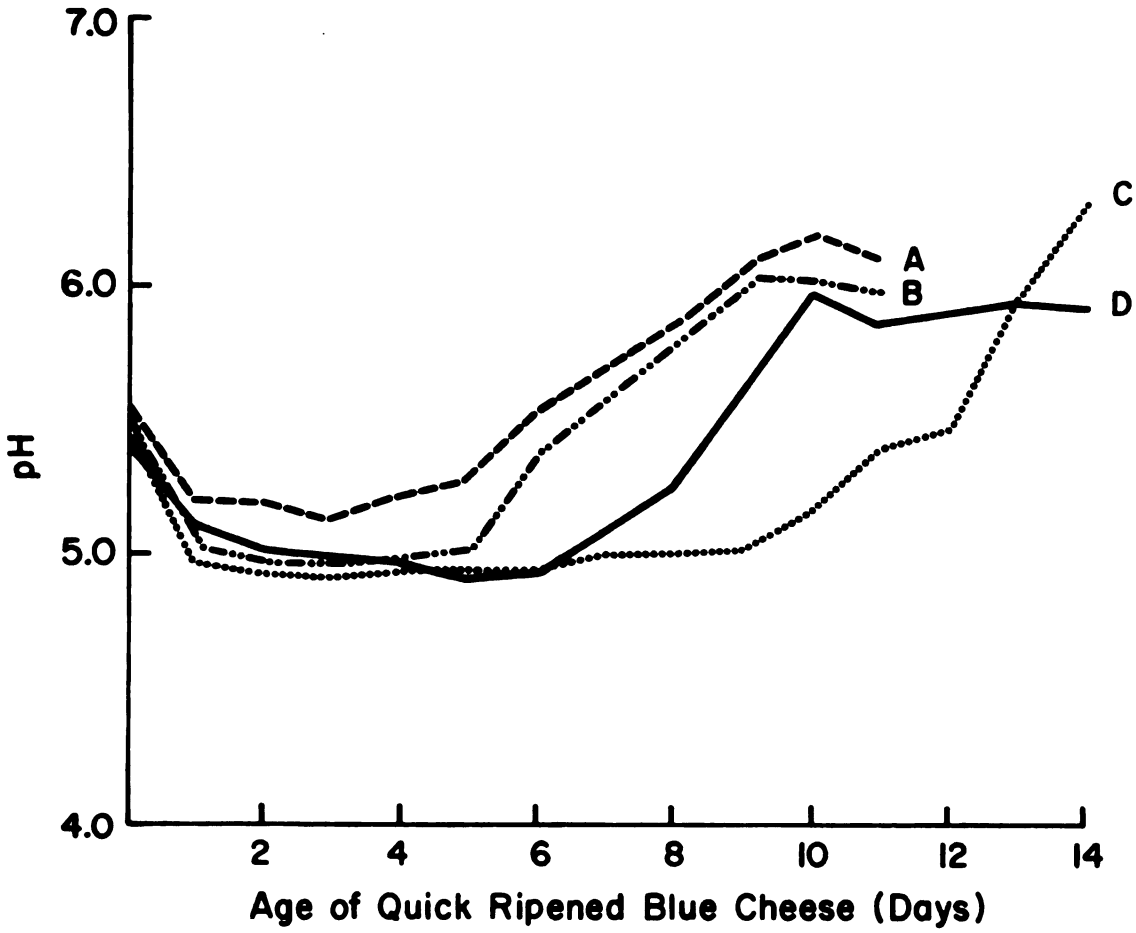

Fig. 15. The effect of lowering curing temperatures and various salting times on changes in pH during curing of Blue cheese curd.

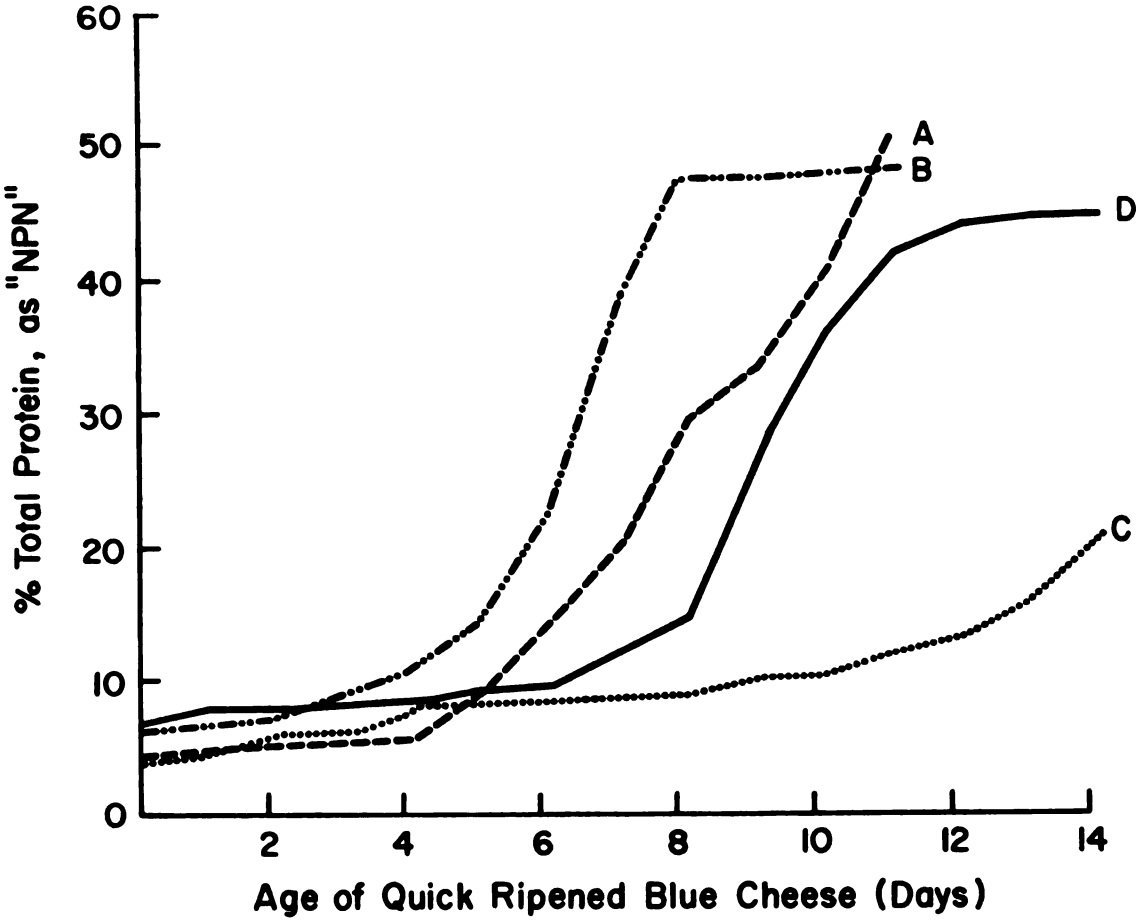

Fig. 16. The effect of lowering curing temperatures and various salting times on changes in NPN during curing of Blue cheese curd.

as the cheeses ripened at 62 F (17 C) and salted on days 1, 2, and 3. That is, the pH values at the end of ripening were high but NPN values were quite low. A 4 percent final salt concentration in the cheese may have inhibited lipolysis and, therefore, the formation of free fatty acids that perhaps aid in controlling the pH. Gould (1941) and Stadhoudes (1956) demonstrated the retarding effect of sodium chloride on lipase activity. Willart and Sjostrom (1959) found that when the salt content was increased from 1.5 to 4.0 percent, the amount of liberated free fatty acids decreased with increasing salt concentrations. An increase in the salt content from 3.0 to 3.9 percent was found by Poznanski, Jaworsky, and D'Obyrn (1967) to reduce the fat acidity in Roquefort cheese.

Another observation on the cheese ripened at 48 F (9 C) and salted on days 11, 12, and 13 was the fact that NPN values reached a peak on day 10 and then leveled off at this point. This indicated that the leveling effect usually observed in NPN data at the end of ripening was not due to the proteases of P. roqueforti being inhibited by salt. Early salt addition to the cheese appeared to retard the growth of the mold and, therefore, the liberation of proteolytic enzymes capable of hydrolyzing the cheese proteins.

Figures 17 and 18 demonstrate the differences in PAGE in Blue cheese ripened at 48 F (9 C) and salted at different times. The last traces of protein zones in the

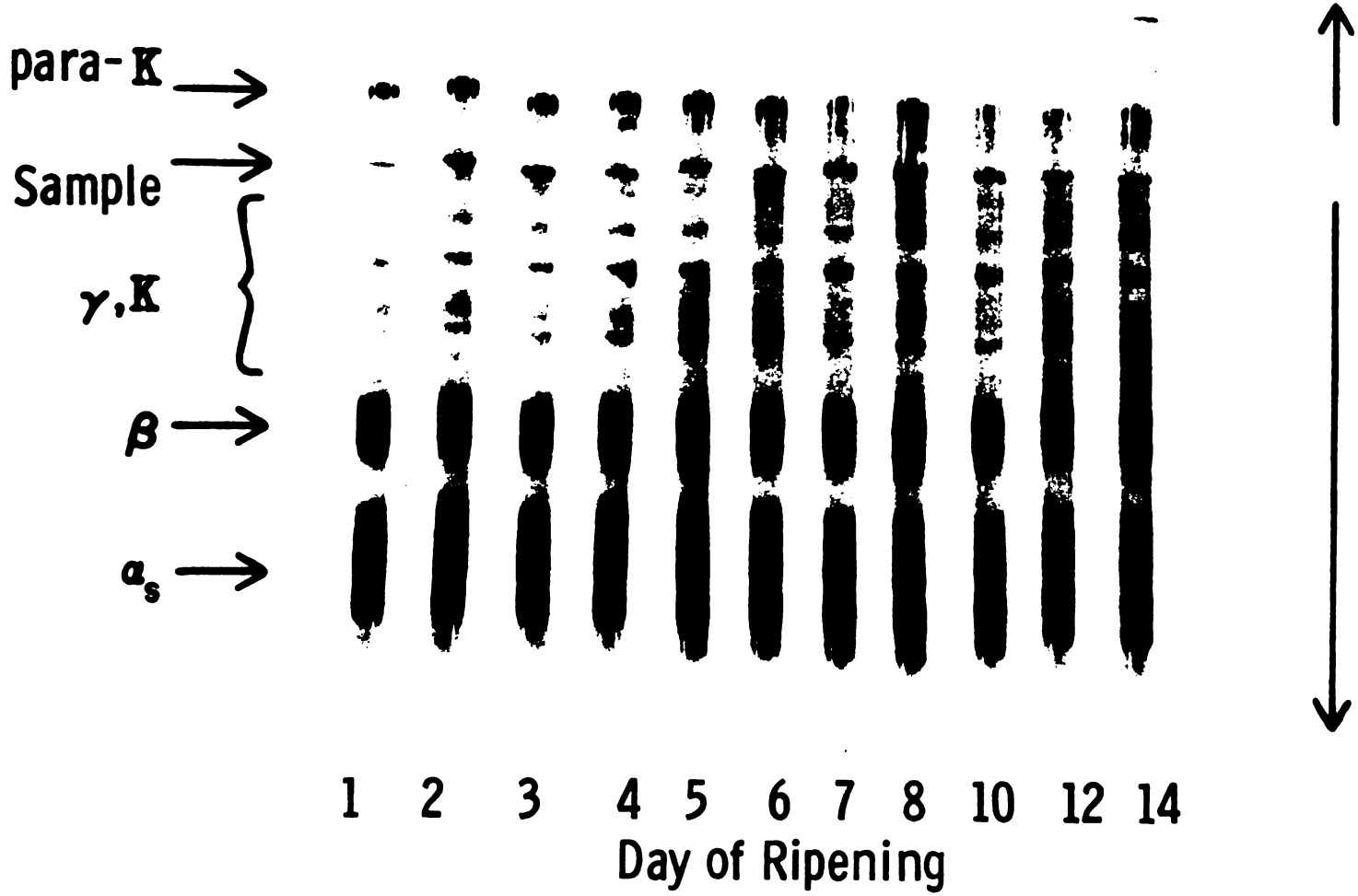

Fig. 17. PAGE patterns of Blue cheese ripened at 48 F (9 C) and salted on days 4, 5, and 6.

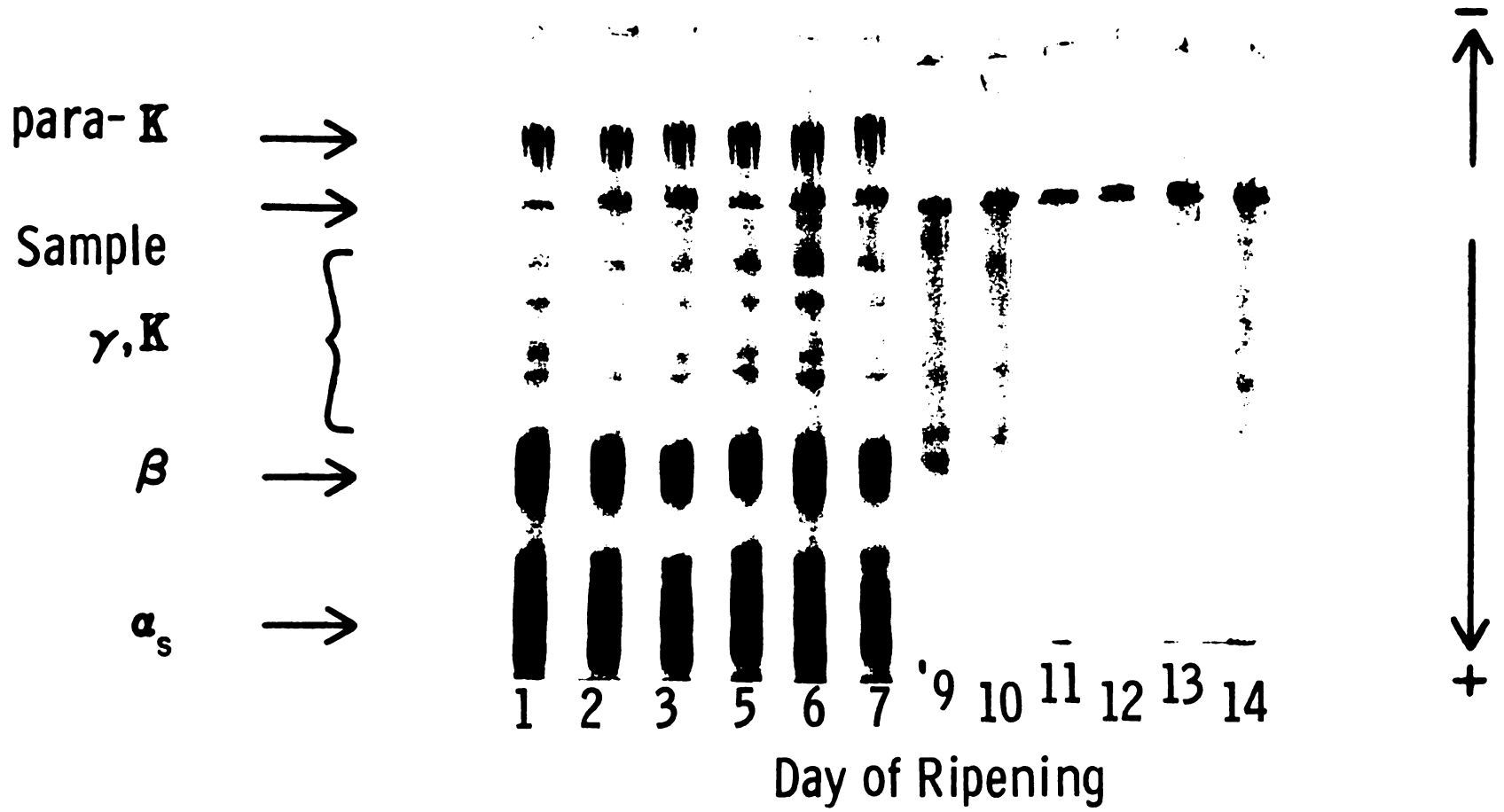

Fig. 18. PAGE patterns of Blue cheese ripened at 48 F (9 C) and salted on days 11, 12, and 13.

cheese salted on days 11, 12, and 13 occurred on day 9 while cheese salted on days 4, 5, and 6 revealed intense bands after two weeks of ripening. The application of 25 percent of the total salt content during early stages of ripening at 52 F (11 C) is illustrated in the PAGE patterns presented in Figure 19. Comparing these patterns to the cheese in which sodium chloride was added later during the ripening period (Fig. 20), it became evident that protein degradation was retarded by approximately one day. The last protein zones disappeared on day 8 in the 52 F (11 C) cheese salted on days 7, 8, and 9. This compared to day 9 in the cheese ripened at an identical temperature and salted on days 1, 9, and 10.

All the cheeses graded quite high when subjectively analyzed, except the 48 F (9 C) cheese that was salted early in ripening. These data are presented in Table 5.

Effect of Homogenization and Pasteurization on QR Blue Cheese

Homogenized raw milk, pasteurized, unhomogenized milk and milk held one hour after homogenization prior to pasteurization were investigated as variables in the preparation of QR Blue cheese. These variables were primarily attempted to increase lipolysis and thereby increase the sharpness of flavor in the QR Blue cheese. Lane and Hammer (1938) found that homogenized milk produced cheese that was lighter in color, softer in texture and

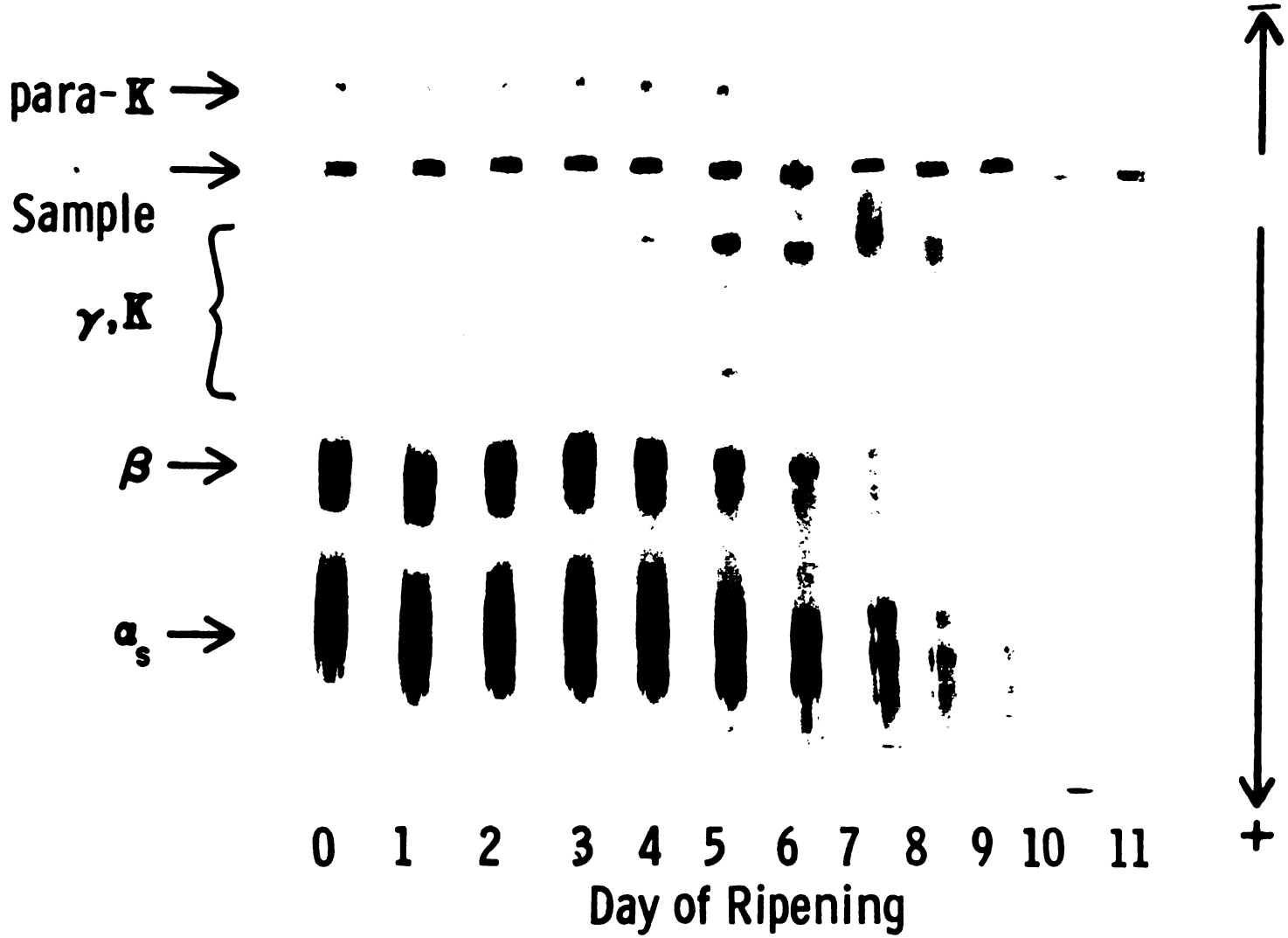

Fig. 19. PAGE patterns of Blue cheese ripened at 52 F (11 C) and salted on days 1, 9, and 10.

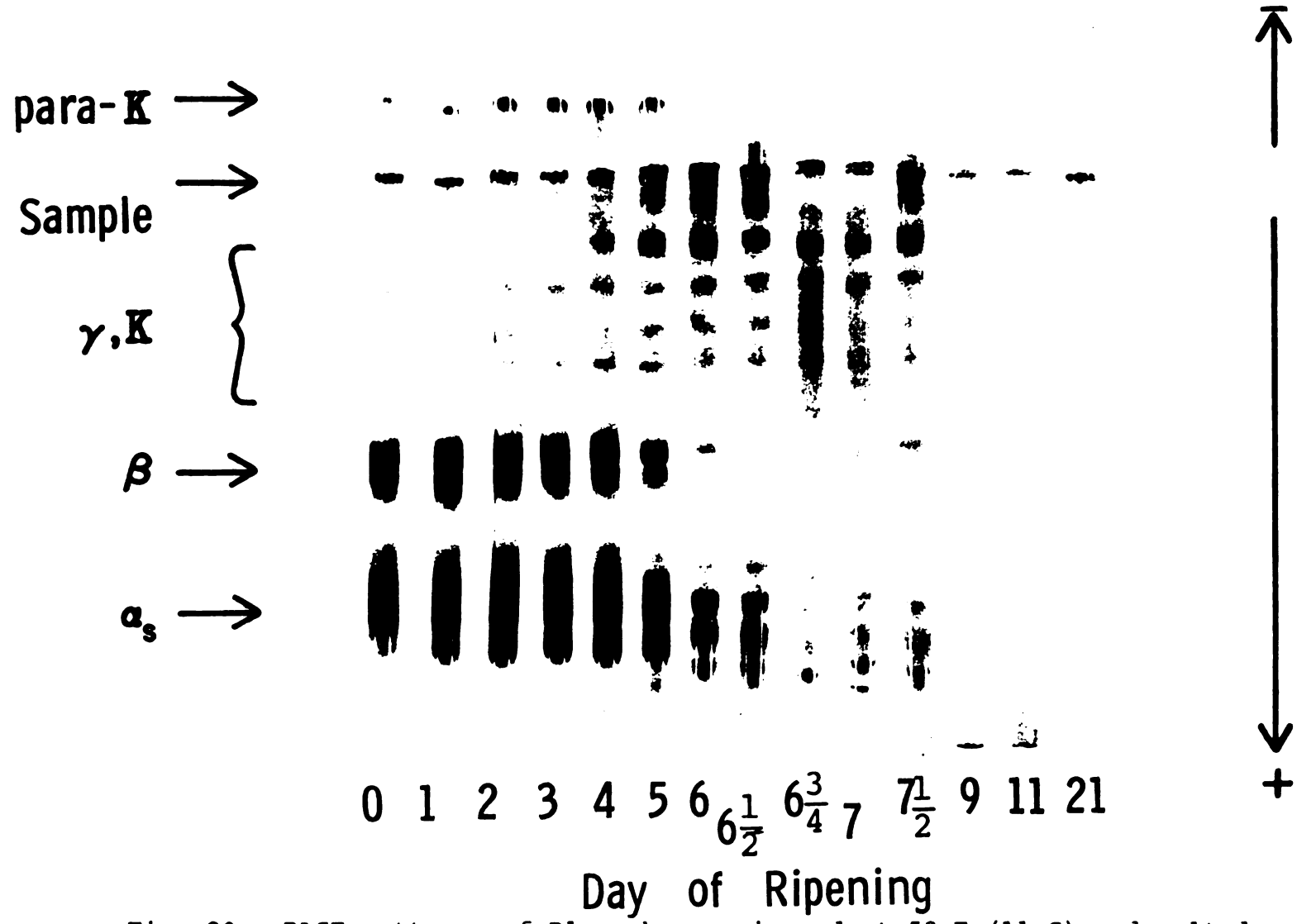

Fig. 20. PAGE patterns of Blue cheese ripened at 52 F (11 C) and salted on days 7, 8, and 9.

Table 5.--Subjective Analyses of QR Blue Cheese Ripened at Different Temperatures and Salting Times.

Temperature	Day of Salting	Flavor (20)	Texture (10)	Color (15)	Total (45)
48 F (9 C)	11,12,13	15.6	7.0	12.5	35.0
		Sl.* mild	Weak	Sl. lacking	
48 F (9 C)	4,5,6	8.0	3.3	4.8	16.1
		Extremely mild	Mealy	Lacking	
52 F (11 C)	7,8,9	13.0	7.7	13.0	33.7
		Mild	Sl. weak	Lacking	
52 F (11 C)	1,9,10	14.0	8.0	12.0	34.0
		Mild	Sl. weak	Lacking	

*Sl.=slightly.

**The salt was added on the days indicated with amount of the total salt always in the order of 1/4, 1/4, and 1/2.

that ripened faster. It was also discovered by these authors (1938) that pasteurization of milk prior to homogenization resulted in longer ripening times for cheese made from such milk.

The pH, NPN, and PAGE patterns obtained for the cheeses made with homogenized raw milk and unhomogenized milk demonstrated trends similar to data already presented. This indicated that there was not any major differences in the protein degradation during ripening due to these variables.

Lipolysis by the natural milk lipase system and the P. roqueforti mold should be increased by homogenization since homogenization increases the surface area of the fat. Morris et al. (1963) demonstrated that pasteurization destroyed the natural milk lipases. Therefore, QR Blue cheese manufactured with milk that was held one hour at 120-125 F (49-52 C) prior to pasteurization should exhibit maximum lipolysis and probably increased ketone production through subsequent beta-oxidation. However, the Blue cheese manufactured from such milk exhibited an atypical fermentation. The pH values during ripening were erratic and the highest level of soluble protein amounted to only 27 percent after seven days of ripening. The PAGE patterns are shown in Figure 21. The gels demonstrated the lack of extensive protein degradation which normally was characteristic of QR Blue cheese. This cheese appeared to be highly contaminated with undesirable organisms. The one hour holding period at a temperature of about 125 F (52 C) would have provided an ideal medium for the proliferation of organisms normally present in milk. This presumable contamination could have inhibited mold growth and thereby produced an atypical cheese. Additional research is needed to clarify this matter.

Table 6 shows the subjective analyses of the cheeses produced with changes in pasteurization and homogenization. Poor, if any, mold growth was evident by the low score given the cheese that was produced when milk

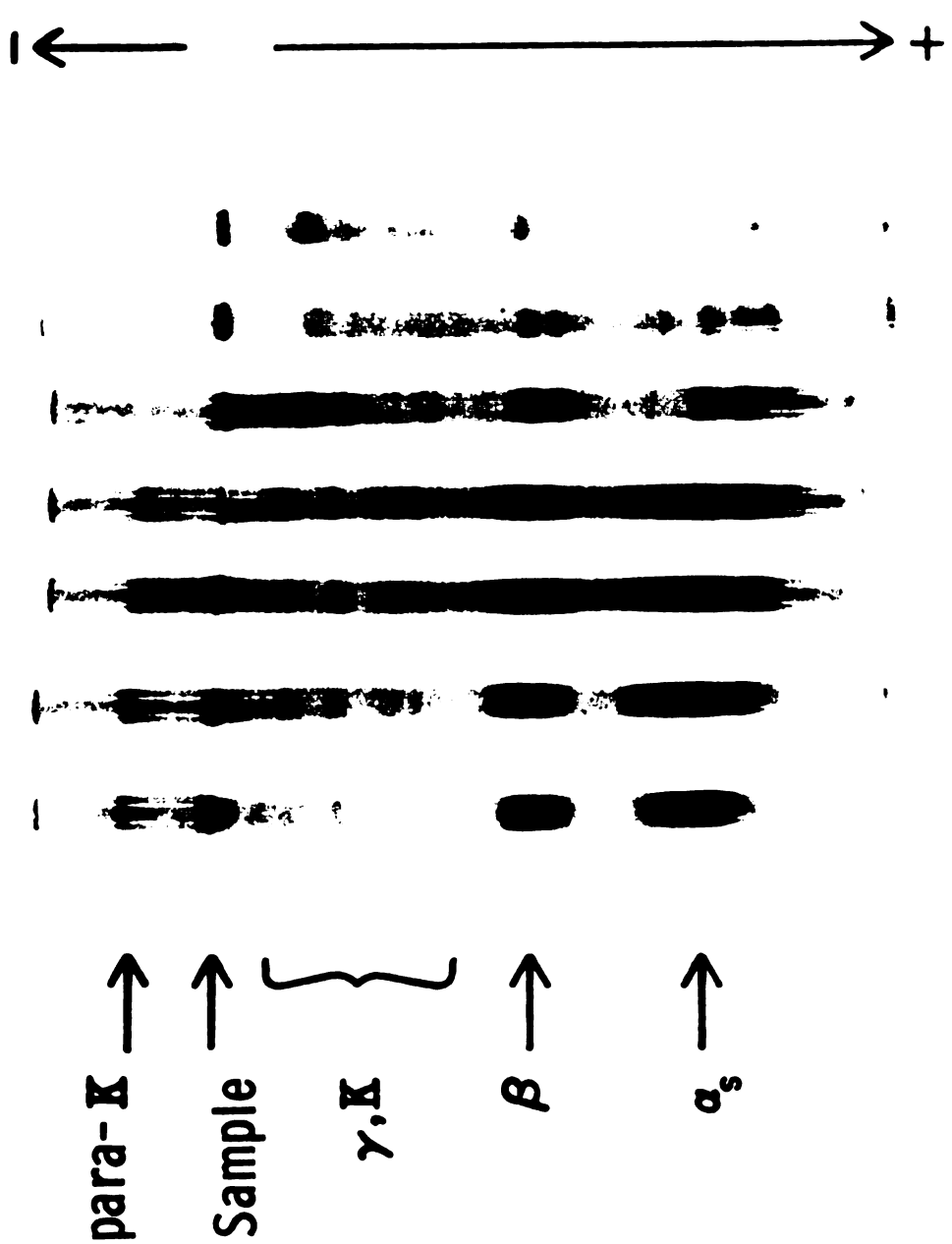

Fig. 21. PAGE patterns obtained from Blue cheese in which the milk was held one hour after homogenization prior to pasteurization, ripened at 62 F (17 C) and salted on days 4, 5, and 6.

Table 6.--Subjective Analyses of QR Blue Cheese Ripened at 62 F (17 C) With Salt Added on Days 4, 5, and 6 With Changes in Pasteurization and Homogenization.

Cheese Variable	Flavor (20)	Texture (10)	Color (45)	Total (45)
Homogenized Raw Milk	12.0	5.7	10.0	27.7
	Sl.* bitter	Sl. mealy	Excessive	
Pasteurized-Unhomogenized	9.4	5.0	5.4	19.8
	Soapy	Sl. mealy	Lacking	
Homogenized-1 hr delay	7.5	4.5	5.0	17.0
	Atypical	Gummy	No color	

*Sl.=slightly.

was held at 120-125 F (49-52 C) after homogenizing. The importance of homogenization was indicated when comparing the pasteurized, unhomogenized cheese with other cheeses manufactured under identical conditions and homogenized. The flavor of cheese was not improved by omitting the pasteurization process. The data in this table suggest that both pasteurization and homogenization are necessary if a high quality QR Blue cheese is to be produced.

Effect of Cooking the Curd and the Addition of a Commercial Lipase on QR Blue Cheese

A commercial lipase preparation was added to cheese milk after pasteurization and the cheese was ripened according to the method of Kondrup and Hedrick (1963). It was hoped the lipase would encourage the liberation of

fatty acids and thereby improve the flavor. A problem that occurred in the manufacture of QR Blue cheese was shattering of the curd prior to drainage. This resulted in poor drainage and a loss of cheese fines. In an attempt to alleviate this problem the curd was cooked to firm the curd particles. The curd was allowed to sit undisturbed for 15 min following cutting and the temperature was then raised gradually to 100 F (38 C) in 30 min, with continuous gentle stirring, and held for an additional 15 min.

The data collected pertaining to pH, NPN and PAGE did not demonstrate any major differences from the control cheese. Table 7 shows the subjective analyses for the two modified cheeses. The cheese produced with the addition of lipase produced a high total score as a result of a superior color, however, the flavor and texture were not superior. The short ripening time involved in QR Blue cheese may not permit the lipase to function to the extent that it does in conventional curing. An improvement in the quality of cheese was not expected by cooking the curd. This modification, however, did improve the drainage of whey and slightly improved the yield due to less loss of curd fines. Shehata (1966) reported that cooking the curd made it stronger which resulted in less shattering.

Effect of Direct Acidification on QR Blue Cheese

Three lots of QR Blue cheese were manufactured by direct acidification of the cheese milk with hydrochloric

Table 7.--Subjective Analyses of QR Blue Cheese Ripened at 62 F (17 C) and Salted on Days 4, 5, and 6 With Slight Modifications in the Procedure.

Cheese Variable	Flavor (20)	Texture (10)	Color (15)	Total (45)
Added Lipase	12.0	7.7	14.8	34.5
	Soapy	Sl. mealy	Excellent	
Cooking	14.3	7.0	11.0	32.3
	Sl.* bitter	Weak	Excessive	

*Sl.=slightly.

acid, lactic acid and by a combination of lactic acid and starter culture. All cheeses were ripened at 62 F (17 C) for 7 days and salted on days 4, 5, and 6. The cheeses were acidified to a pH of 5.7. This was approximately the pH used by Singh and Kristoffersen (1972) in developing cheese flavor. The starter culture was added to the one batch of cheese at a concentration of 1 percent.

The cheeses directly acidified with hydrochloric and lactic acids were very similar in pH, NPN, and PAGE patterns. The pH curves for the cheese directly acidified with lactic acid and starter culture added are shown in Figure 22. The cheese that had only lactic acid added demonstrated an unusual pH curve. Except for a slight increase on day 5 the cheese showed a gradual decrease in pH throughout ripening. The lactic acid plus starter culture cheese had a pH profile more nearly resembling the

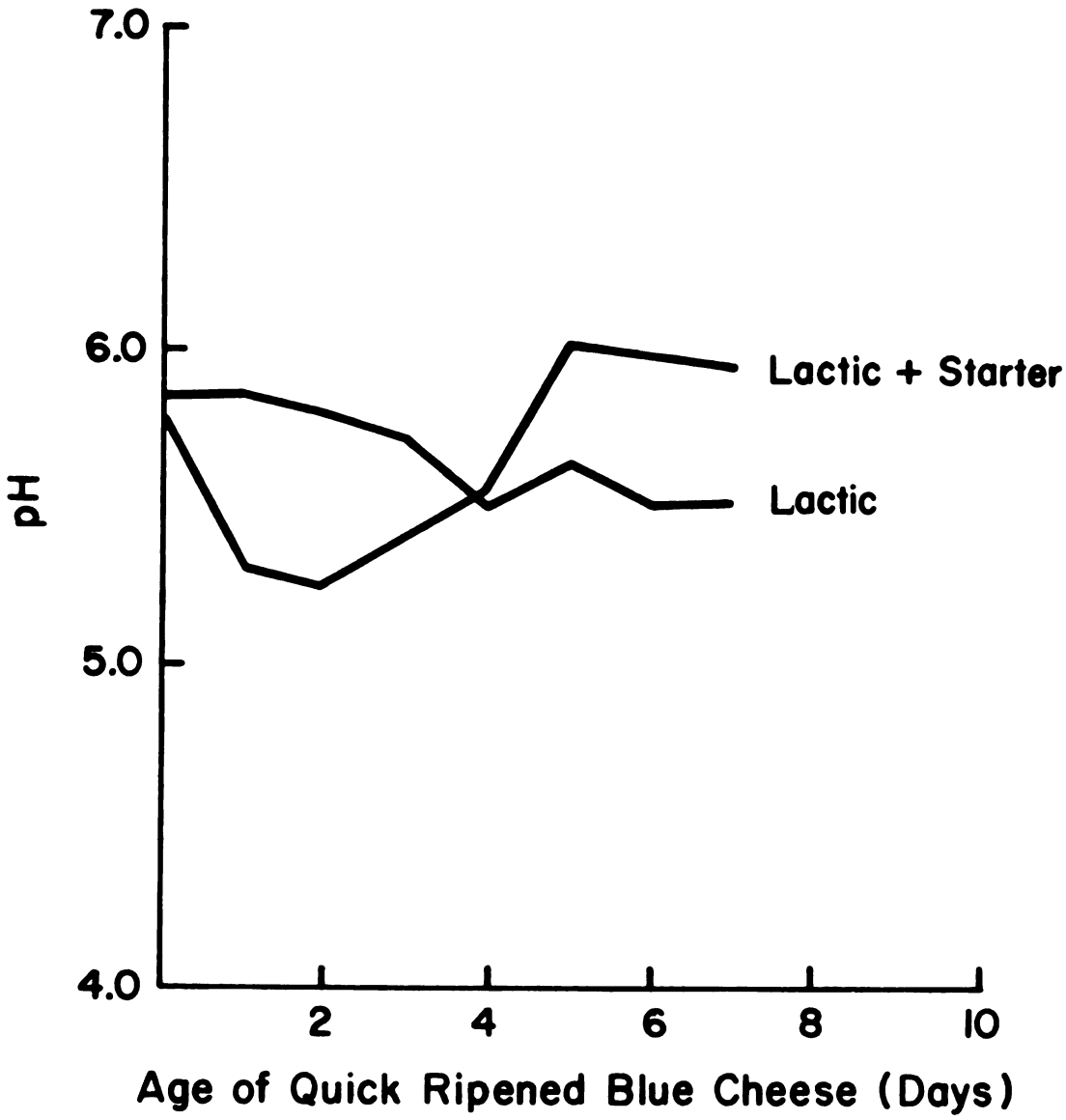

Fig. 22. The effect of direct acidification on changes in pH of Blue cheese curd.

control cheese. This was probably due to the starter culture. Figure 23 demonstrates the soluble protein changes during ripening of the two cheeses. The NPN curve of cheese made with lactic acid and starter culture was similar to the control cheese, however, protein degradation was slightly less extensive. The lactic acid cheese demonstrated only about 30 percent soluble protein after 7 days of ripening.

Protein degradation is more clearly represented with PAGE shown in Figures 24 and 25. The QR Blue cheese directly acidified with lactic acid showed an unusual PAGE pattern. The presence of three bands in the α-casein area and the absence of any zones in the γ, K-casein area indicated that the ripening of this cheese was not typical of QR Blue cheese. The data collected for the lactic acid cheese suggested that mold growth was lacking and contamination may have been responsible for the unusual ripening characteristics. The importance of starter cultures was evident for producing a cheese which was more typical insofar as protein degradation was concerned. The last traces of protein zones disappeared on the fifth day which was the same as the control cheeses.

Table 8 shows the subjective analyses of the directly acidified cheeses. None of these cheeses prepared by these methods developed any blue color. The body and texture were criticized as being gummy. The flavor of all cheeses was described as being atypical.

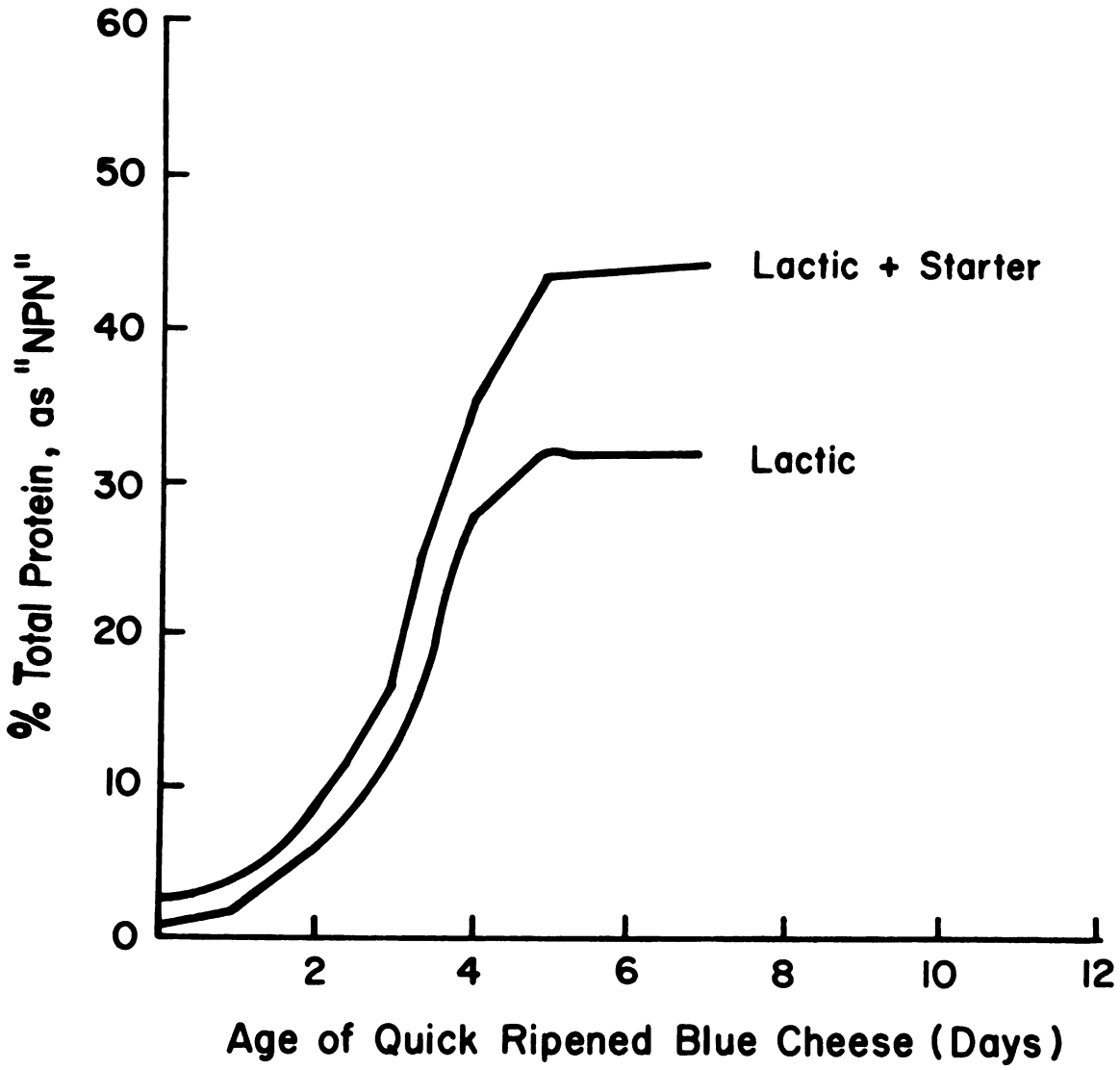

Fig. 23. The effect of direct acidification on changes in NPN of Blue cheese curd.

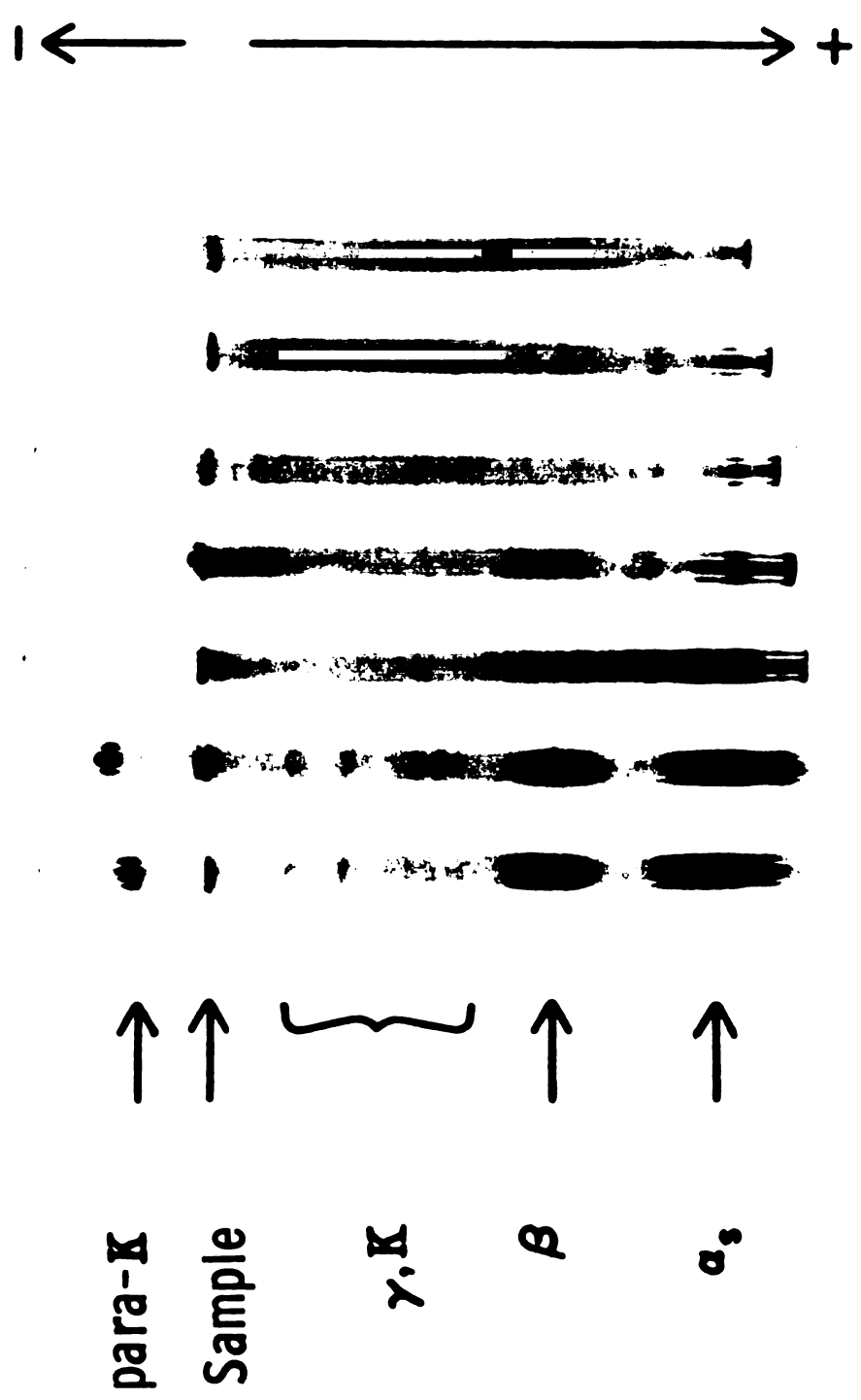

Fig. 24. PAGE patterns of Blue cheese directly acidified with lactic acid and ripened at 62 F (17 C) with salt added on day 4, 5, and 6.

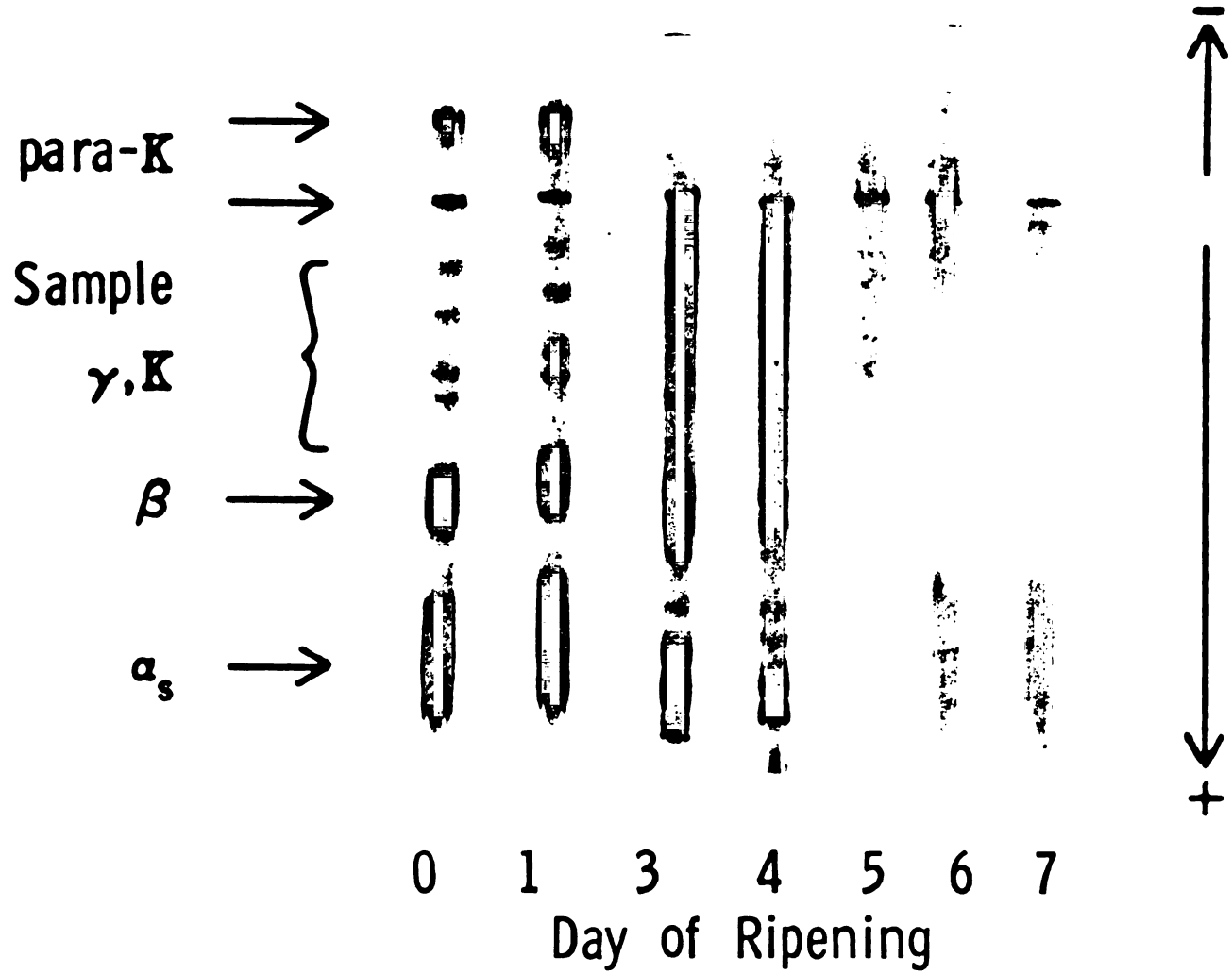

Fig. 25. PAGE patterns of Blue cheese directly acidified with lactic acid and 1 percent starter culture and ripened at 62 F (17 C) with salt added on days 4, 5, and 6.

Table 8.--Subjective Analyses of QR Blue Cheese Manufactured Using Direct Acidification.

Acid	Flavor (20)	Texture (10)	Color (15)	Total (45)
Hydrochloric	6.7	4.7	5.0	16.4
	Atypical	Gummy	Lacking	
Lactic	7.3	5.0	4.5	16.8
	Atypical	Gummy	Lacking	
Lactic + Starter	8.7	5.0	4.5	18.2
	Atypical	Gummy	Lacking	

Direct acidification of Blue cheese was shown by Shehata (1966), to produce a curd of good body and texture. The flavor was not evaluated since the cheese was not ripened. The manufacture of QR Blue cheese by direct acidification failed to produce a cheese of typical flavor and color. It appeared that an active starter culture was a prerequisite for good mold growth in QR Blue cheese.

Effect of Benzoyl Peroxide as a Decolorizer in QR Blue Cheese

In an effort to obtain a cheese that did not possess a slight off-white color after ripening was complete, a potent decolorizer, benzoyl peroxide, was used to replace the decolorizer oridinarily used in the manufacturing process. The concentration allowed and applied in the manufacture was 0.02 percent based on the weight of

the cheese milk. At this level, there was no noticeable improvement in the color of QR Blue cheese. The cause of the off-white color development in QR Blue cheese was not determined.

Recommendations for the Future Manufacture of QR Blue Cheese

Based upon the information obtained from the preceeding trials the procedure of Kondrup and Hedrick (1963) was modified for the production of QR Blue cheese. The following modifications are suggested. Lowering the ripening temperature to 52 F (11 C) was chosen because it was low enough to retard some undesirable contamination. A 48 F (9 C) ripening temperature was not selected because the cheese requires a minimum of 14 days to acquire the desired characteristics. The lower ripening temperature of 52 F (11 C) also appeared to produce a cheese which was more reproducible from batch to batch. Two different salting applications proved to be acceptable. Salting on days 7, 8, and 9 or days 1, 9, and 10 were compatible and aided in producing cheese of high quality. Another modification instituted during manufacture was the process of cooking the curd after cutting. This provided better whey drainage and a higher yield due to less loss of fines.

Sensory evaluation of the cheese manufactured with the incorporated modifications demonstrated improvement in flavor when stored at 40 F (4 C) following ripening. The flavor improved through 3 weeks of storage before a slight

decrease was noted after 4 weeks. Color and texture remained quite constant throughout the storage period of 4 weeks.

Preparation of QR White Mold Cheese

Knight et al. (1950) produced a mutant strain of P. roqueforti with white pigmenting spores. The use of the mutant has been recommended for the manufacture of salad dressing and for the production of cheese acceptable to consumers who dislike the appearance of the blue mold.

Two batches of cheese were made with the white mutant. Both batches were ripened at 52 F (11 C) with one batch salted on days 5, 6, and 7 and the other batch on days 1, 6, and 7. The time of salting was a difficult aspect of manufacturing cheese with the white mutant since color development was used as a guide in QR Blue cheese manufacture.

Figure 26 presents the changes in pH during ripening in cheese manufactured using the white mutant. The pH profile during ripening of the white mutant cheeses was similar to pH values for QR Blue cheese ripened at an identical temperature. The white mutant cheese showed an abrupt increase occurring on day 4. A similar effect was noted in Blue cheese on day 5. This indicated that proteolysis is consummated earlier in ripening. Figure 27 demonstrates a rapid increase in NPN on day 4 in both batches of white cheese. The white mutant cheese that was

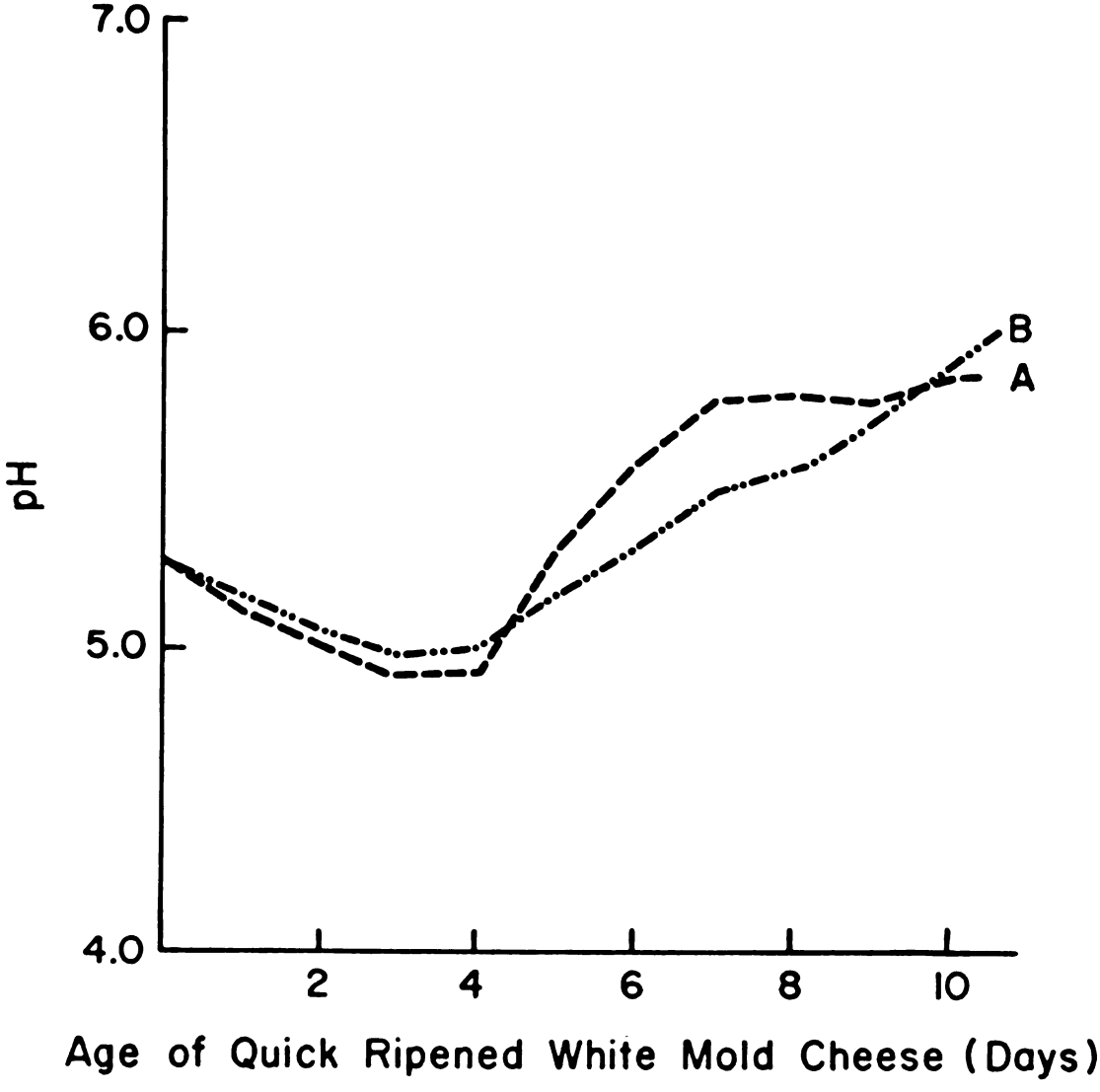

Fig. 26. The effect of the white mutant of P. roqueforti on changes in pH of cheese cured at a temperature of 52 F (11 C) and salted on days 5, 6, 7(A) and 1, 6, 7(B).

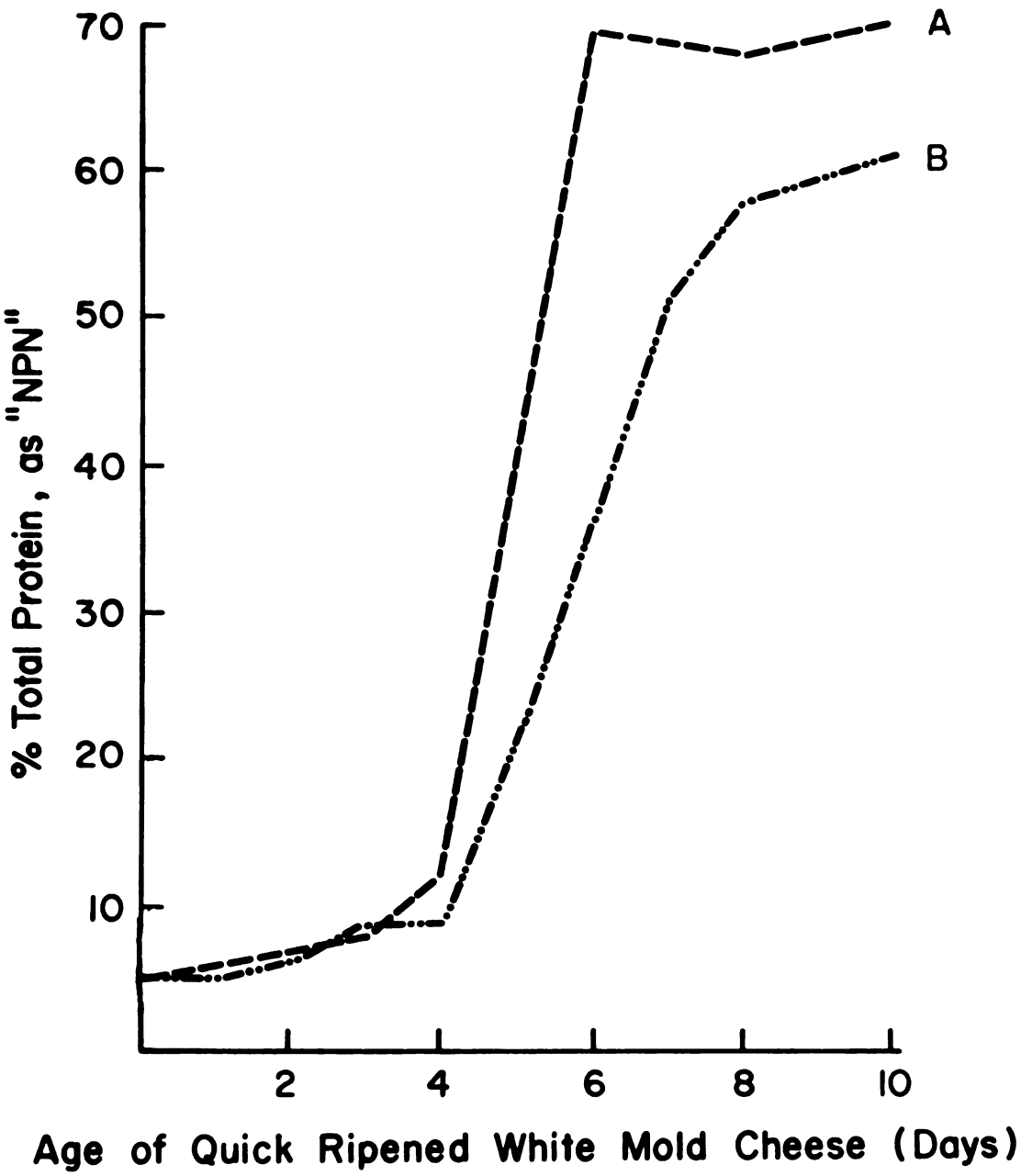

Fig. 27. The effect of the white mutant of P. roqueforti on changes in NPN of cheese cured at a temperature of 52 F (11 C) and salted on days 5, 6, 7(A) and 1, 6, 7(B).

salted on days 1, 6, and 7 demonstrated a slightly more gradual increase in soluble protein when compared to the cheese salted on days 5, 6, and 7. This was likely due to a slight retardation of mold growth. However, both cheeses proved to release more protein in less time than a comparable cheese salted at a later time manufactured from the blue mold. This suggested that the white mutant was more proteolytic than the conventional mold. Morris, Jezeski, and Combs (1954) also noted that the white mutant was more proteolytic than the parent strain. Other than a more rapid disappearance of protein zones, the PAGE patterns shown in Figures 28 and 29, are identical with PAGE patterns observed in the conventional QR Blue cheese.

Table 9 shows the results of subjective analyses of white mold cheeses. Both variations were criticized for being mild in flavor and weak in texture. The QR white mold cheese had a flavor typical of a mild Blue cheese. It was concluded that the white mutant of P. roqueforti could be used to produce a cheese with Blue cheese flavor devoid of any blue pigmentation.

Peptide Mapping and Molecular Weight Estimations of Protein Degradation Products in Blue Cheese

It was observed during PAGE that differences sometimes existed in protein degradation during ripening. Usually the PAGE patterns were devoid of any protein zones in the final days of ripening indicating extensive

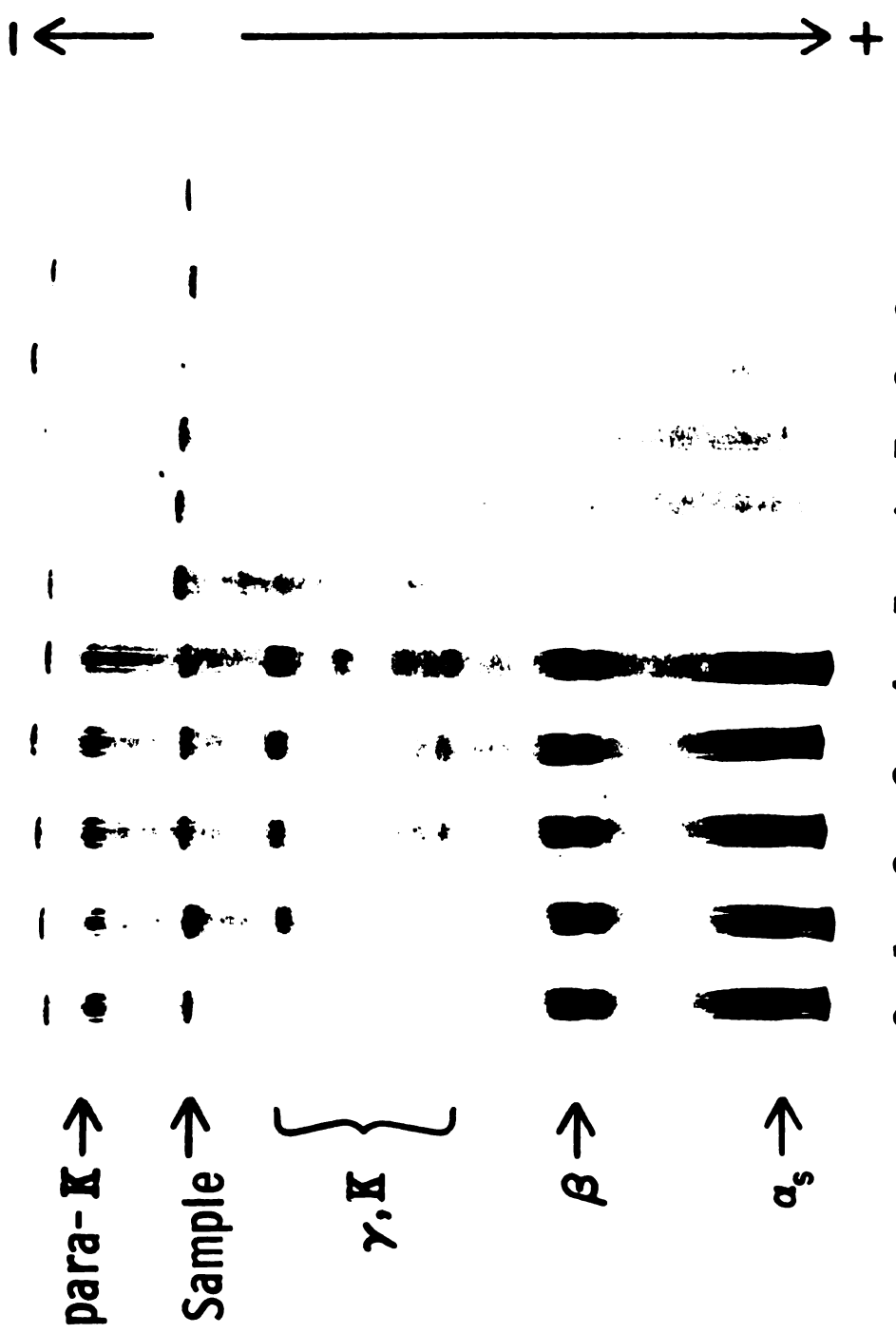

Fig. 28. PAGE patterns of cheese manufactured using the white mutant of P. roqueforti and ripened at 52 F (11 C) with salt added on days 5, 6, and 7.

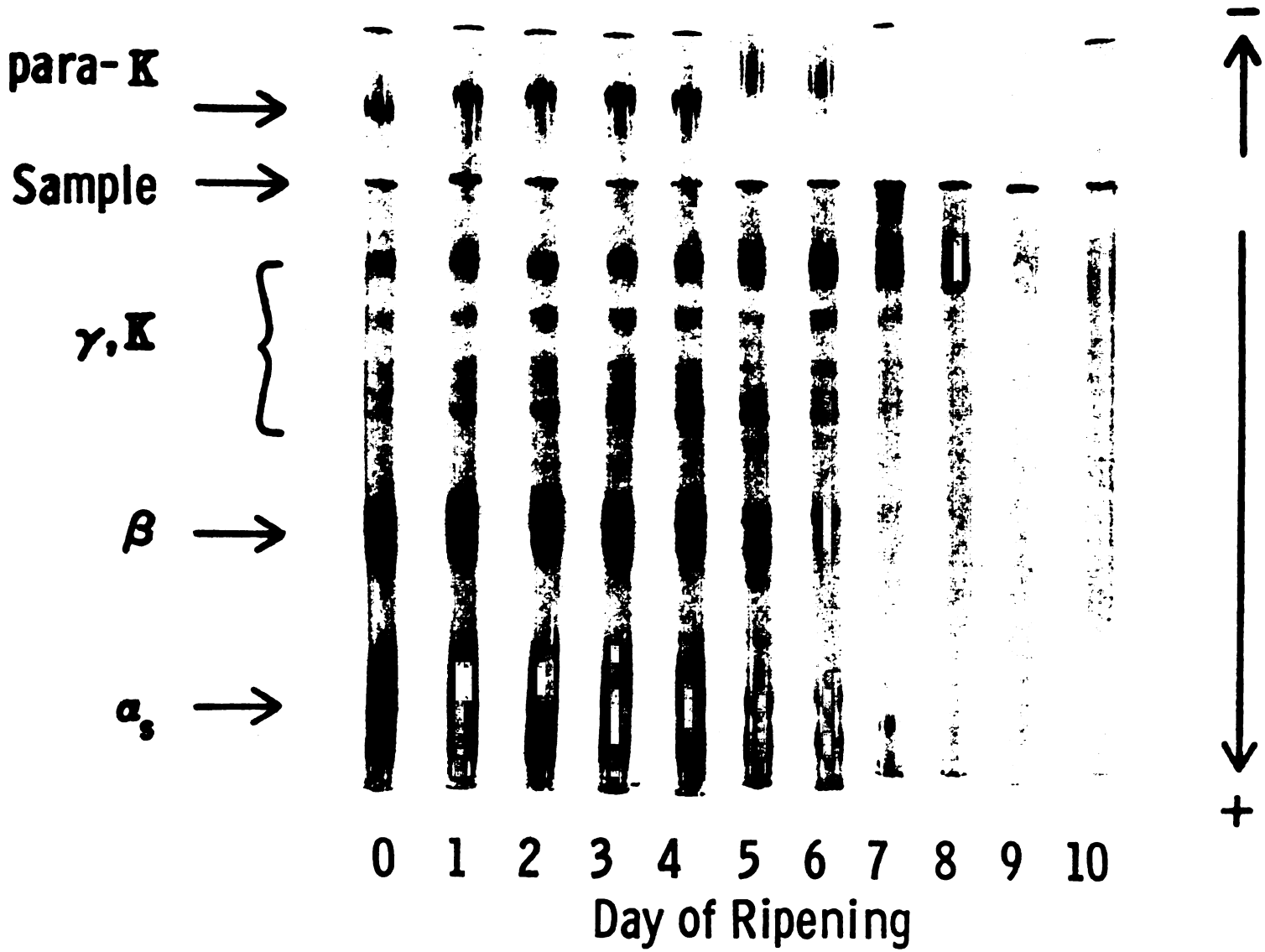

Fig. 29. PAGE patterns of cheese manufactured using the white mutant of P. roqueforti and ripened at 52 F (11 C) with salt added on days 1, 6, and 7.

Table 9.--Subjective Analyses of QR Cheese Manufactured With the White Mutant of P. roqueforti and Ripened at 52 F (11 C).

Salt	Flavor (20)	Texture (10)	Color (15)	Total (45)
1,6,7	10	7.3	12.3	29.6
	Mild	Gummy	Sl.* off color	
5,6,7	9.7	7.3	11.0	28.0
	Mild	Gummy	Sl. off color	

Sl.*=slightly.

fragmentation of the proteins to smaller molecular weight compounds. Peptide mapping and molecular weight estimations were used, therefore, to ascertain if any major differences existed in peptides that were formed during ripening. Storgards and Lindqvist (1953) and others have referred to the importance of peptides in providing important flavor sensations in cheese.

Peptide mapping on cheese has usually been accomplished by precipitating the majority of proteins with strong acids like phosphotungstic or TCA. The precipitating agents usually allowed amino acids to remain in solution and the resulting peptide maps were congested with many spots when subjected to ascending chromotography. To alleviate this problem, gel filtration chromotography was used to isolate the peptides that had an approximate molecular weight range of 700 to 5000. By the inclusion

of this isolation procedure it became a simple matter to analyze the complete molecular weight profile of Blue cheese.

Table 10 presents the molecular weight profile of selected cheeses. The two commercial cheeses showed somewhat of a variation in their degradation, especially in the range of products of molecular weight less than 700. Both of these cheeses, however, are characterized by a large portion of protein with molecular weights greater than 5000. This explained the evidence of protein zones in PAGE of these cheeses. There were not any substantial differences in the QR Blue cheese or the QR cheese produced with the white mutant. These cheeses were represented by a small percent of the total protein with molecular weights greater than 5000. Both QR cheese samples that were salted on day 1 demonstrated a slightly higher percent of proteins of less than 700 molecular weight. This slight difference was probably not of any significance.

The peptide maps obtained from all the cheeses analyzed were very similar. Figure 30 schematically demonstrates this point. The majority of spots were in identical positions in the cheeses compared. The cheese manufactured with the mutant of P. roqueforti showed the presence of a few different spots. The similarity in peptide maps may be indicative of Blue cheese in general, and may represent peptides that were the most resistant to degradation.

Table 10.--Molecular Weight Profiles Obtained by Gel Filtration Chromotography on Blue Cheese.

Cheese Description	Molecular Weights		
	%<than 700	%700-5000	%>5000
Commercial (crumbled)	22.1	35.2	42.5
Commercial (pressed)	15.2	59.7	27.0
62 F (17 C); salted 4,5,6; day 7 sample	24.2	62.8	12.2
52 F (11 C); salted 7,8,9; day 11 sample	26.0	59.4	14.5
52F (11 C); salted 1,9,10; day 11 sample	29.0	51.9	18.9
White mutant-52 F (11 C); salted 5,6,7; day 10 sample	23.2	62.0	14.8
White mutant-52 F (11 C); salted 1,6,7; day 10 sample	26.5	61.1	13.3

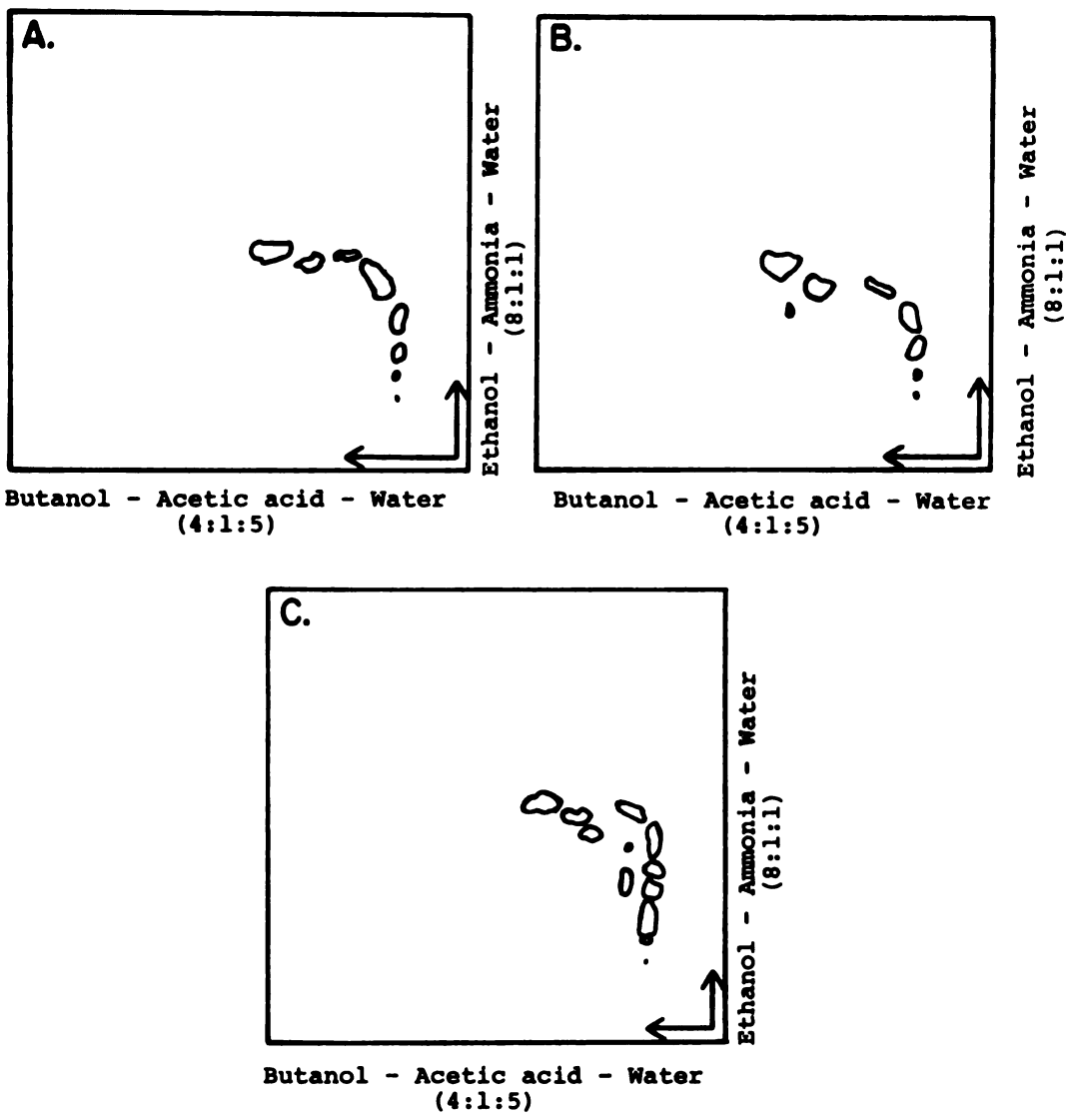

Fig. 30. Schematic representations of peptide maps obtained from commercial Blue cheese, A; QR Blue cheese, B; and QR white cheese, C.

Accumulation of Free Amino Acids in Blue Cheese

The quantity of free amino acids accumulated during the ripening period was the dual result of a liberation of amino acids from the casein and a transformation of the amino acids already liberated into further decomposition products. Free amino acids occur in all ripened cheese varieties, although in very different amounts and in highly variable relationships.

To further elucidate the degradation of proteins in QR Blue cheese, amino acids analysis were performed on cheese during ripening and on cheese after ripening was complete.

Table 11 presents the accumulation of free amino acids during the ripening of QR Blue cheese. This cheese was manufactured with the suggested modifications incorporated, that is, a ripening temperature of 52 F (11 C) and salted on days 7, 8, and 9. At the end of 11 days this cheese was stored in a polyethlene bag at 40 F (4 C). Ammonia is included in this table since ammonia is often regarded as an important component of cheese aroma (Schromüller, 1968). The presence of methionine sulphoxide was indicative of oxidation taking place. It was difficult to determine if methionine sulphoxide arose due to oxidation in the cheese or if it arose during the isolation procedure. All the amino acids continued to increase in concentration throughout the ripening period with the exception of

Table 11.--Liberation of Free Amino Acids of QR Blue Cheese Cured at 52 F (11 C) and Salted on Days 7, 8, 9 and Stored at 40 F (4 C) After 11 Days.

Amino Acids	Age in Days		
	7	11	39
	mg free amino residue/10 gm protein		
Lysine	83.43	125.22	248.21
Histidine	1.77	88.22	138.94
Ammonia	62.80	222.72	261.52
Arginine	35.82	26.73	97.04
Aspartic	13.56	50.43	102.02
Threonine	14.40	76.53	131.58
Serine	28.25	84.50	130.59
Glutamic acid	63.34	168.12	400.79
Proline	62.34	70.23	185.09
Glycine	5.67	24.76	47.43
Alanine	25.17	32.75	71.43
Cysteine	4.68	58.68	103.82
Valine	48.00	86.87	164.59
Methionine	18.24	37.31	73.64
Isoleucine	23.36	82.99	140.09
Leucine	69.39	128.36	218.94
Tyrosine	51.34	51.57	43.62
Phenylalanine	40.94	105.55	160.68
Tryptophan	7.04	8.28	23.45
Methionine sulfoxide	5.69	13.51	59.98
Total	665.14	1543.33	2803.45

tyrosine. Day 7 of ripening corresponded with the day that NPN values showed a rapid increase. The liberation of free amino acids from day 7 to day 11 proceeded at a rapid rate. The fact that free amino acids continued to accumulate during storage at 40 F (4 C) from day 7 through day 39 suggested that proteolysis continued but at a somewhat reduced rate.

There was an insufficient amount of data to predict any specificity of the liberation of amino acids by the proteases involved, however, Table 12 compared the relative percentages of each amino acid occurring during ripening to the relative rates of amino acids found in whole casein.

On the seventh day of ripening, lysine, arginine, proline, alanine, valine, methionine, leucine, tyrosine, and phenylalanine were present in a higher percentage than they were in whole casein. By the thirty-ninth day of ripening lysine, threonine, glycine, alanine, methionine, leucine, and phenylalaine were higher in relative percentages. Generally the amino acids that were present in high concentrations in casein were also present in high levels in the cheese. This would suggest non-specific action by the proteolytic enzymes. Tyrosine was present in high relative values after 7 days but had decreased significantly after 39 days. It was also the only amino acid to show a decrease in the total quantity (Table 11) during ripening. This indicated that this amino acid was further decomposing.

Table 12.--Relative Percentages of Each Amino Acid Found in Ripening Cheese Compared to Whole Casein (Cheese Ripened at 52 F, Salted on Days 7, 8, 9 and Stored at 40 F After Day 11).

Amino Acid	Age in Days			
	7	11	39	Casein*
	%	%	%	%
Lysine	13.82	9.49	9.76	7.53
Histidine	0.29	6.68	5.47	2.87
Arginine	5.93	2.02	3.82	3.84
Aspartic acid	2.25	3.82	4.01	6.43
Threonine	2.38	5.80	5.18	4.36
Serine	4.68	6.40	5.14	5.47
Glutamic acid	10.49	12.74	15.77	20.57
Proline	10.33	5.32	7.28	9.36
Glycine	0.94	1.88	1.87	1.59
Alanine	4.17	2.48	2.81	2.67
Cystine & Cysteine	0.77	4.45	4.08	0.30
Valine	7.95	6.58	6.48	6.38
Methionine	3.02	2.83	2.90	2.58
Isoleucine	3.87	6.29	5.51	5.51
Leucine	11.50	9.73	8.61	8.32
Tyrosine	8.50	3.91	1.72	5.94
Phenylalaine	6.78	8.00	6.32	4.67
Tryptophan	1.37	0.92	0.53	1.62
Methionine sulphoxide	0.94	1.02	2.36	. .

*Gordon et al., Journal of American Chemistry Society 72, 4282 (1950) and 75, 1678 (1953).

It was presumable that if tyrosine was undergoing further reactions the other amino acids were also behaving similarly.

Table 13 shows the accumulation of free amino acids in various cheeses. About 10-16 percent of the total protein was present as free amino acids in these cheeses. Undoubtedly this increases upon further storage as evidenced by approximately 28 percent of the protein present as free amino acids in the sample stored 39 days. The QR cheeses varied significantly from the commercial cheeses in the concentration of histidine, ammonia, glycine, alanine, and tyrosine. No definite conclusions could be made as a result of the different salting times. The cheese made with the white mutant had significantly lower concentrations of aspartic acid, threonine, glutamic acid, and phenylalanine. Methionine sulphoxide was present in higher concentrations in the QR Blue cheese than in the white mutant cheese or commercial cheese.

The data accumulated on free amino acids suggested that there may be as much difference in individual concentrations in identical samples as between samples manufactured by different techniques. This suggested a non-specific protease system involved in ripening.

Analysis of Tyramine in QR Blue Cheese

There have been many reports that attacks of forceful heart beat, severe headache and hypertension are related to treatment with monamine oxidase inhibitory

Table 13.--Accumulation of Free Amino Acids in Various Cheese Samples at the End of Ripening.

Amino Acids	Cheese*				
	A	B	C	D	E
	mg free amino acid/10 gm protein				
Lysine	121.48	190.00	180.25	264.06	125.22
Histidine	55.73	3.77	72.72	61.38	88.22
Ammonia	164.33	72.67	144.17	168.19	222.72
Arginine	104.99	66.28	28.99	55.45	26.73
Aspartic	136.98	47.92	20.28	21.24	50.43
Threonine	44.95	46.06	41.36	24.25	76.53
Serine	38.47	33.62	65.47	33.19	8.450
Glutamic	193.72	255.52	83.91	83.72	168.12
Proline	54.45	39.47	38.93	23.68	70.23
Glycine	16.13	5.79	16.81	15.45	24.76
Alamine	28.09	7.52	22.15	26.76	32.75
Cysteine	56.92	52.70	28.89	59.16	58.68
Valine	108.99	94.32	61.78	59.02	86.87
Methionine	59.63	48.91	25.66	20.19	37.31
Isoleucine	77.41	77.15	45.88	36.81	82.99
Leucine	214.97	169.93	59.91	51.08	128.36
Tyrosine	22.33	81.23	72.78	73.19	51.57
Phenylalanine	97.67	92.52	60.21	52.72	105.55
Tryptophan	28.98	9.54	5.43	10.30	8.28
Methionine sulphoxide	16.10	8.69	7.37	7.72	13.51
Total	1642.32	1405.60	1082.24	1147.57	1543.33

*A. 52 F (11 C); salted 1,9,10; day 11 sample.

B. Commercial Blue cheese (average of two samples).

C. White mutant; 52 F (11 C); salted 5,6,7; day 10 sample.

D. White mutant; 52 F (11 C); salted 1,6,7; day 10 sample.

D. 52 F (11 C); salted 5,6,7; day 11 sample.

drugs. In most cases, the onset has been noted after a meal of cheese or yeast products (Blackwell, Marley, & Ryle, 1964). The toxicity of various foodstuffs to patients receiving monoamine oxidase inhibitors has been attributed to the presence of amines, such as tyramine. Under normal conditions, tyramine is oxidized by monamine oxidase to a harmless phenolic acid; however, in patients receiving monamine oxidase inhibitors this oxidation step is markedly inhibited and may produce severe hypertension effects.

Dahlberg and Kosikowski (1948) showed a relationship between the tyramine content and the intensity of Cheddar cheese flavor. Dacre (1953) stated that many good cheeses were devoid of any tyramine. Sen (1969) reported values varying from 31 to 256 ug of tyramine per gram of Blue cheese. The amount of tyramine formed during cheese ripening depends on the ripening temperature (Dahlberg & Kosikowski, 1949), the amount of free tyramine liberated during the maturation process and on the concentration of tyrosine decarboxylase present (Dacre, 1953).

It was observed in the amino acid analysis that tyrosine decreased in concentration during ripening. Because of the possible danger of tyramine absorption in susceptible patients an analysis for this amino acid degradation product was made. It is also reasonable to assume that if tyrosine is undergoing degradation, some of the other amino acids are behaving likewise.

Tyramine was qualitatively identified by ascending thin layer chromotography (TLC) and gas liquid chromotography (GLC). The presence of tyramine in Blue cheese was confirmed by mass spectrometry. In a solvent system of butanol-acetic acid and water (4:1:5) tyramine had an R_f value of 0.65. It also produced a purple color reaction with ninhydrin. Tyramine had a retention time of 2.75 min when subjected to analysis by GLC. Figure 31 shows the mass spectra of a tyramine standard and tyramine obtained from Blue cheese. This spectra confirmed the presence of tyramine.

Tyramine was quantitatively analyzed by GLC. Figure 32 demonstrates the increase in tyramine during ripening of QR Blue cheese ripened for 11 days at 52 F (11 C), followed by storage at 40 F (4 C) and salted on days 7, 8, and 9. The highest value obtained was 825 ug tyramine per gram of cheese on day 39. This compared to 40 ug of tyramine per gram of Blue cheese ripened by conventional methods. Even though the QR Blue cheese demonstrated high values when compared to commercial Blue cheese, it has a relatively low value when compared to data obtained by Sen (1969) for aged Cheddar and Stilton Blue cheese. This author (1969) reported values of 1530 and 2170 ug tyramine per gram of cheese for these varieties respectively.

Dahlberg and Kosikowski (1948) suggested that the occurrence of tyramine in Cheddar cheese had its source in

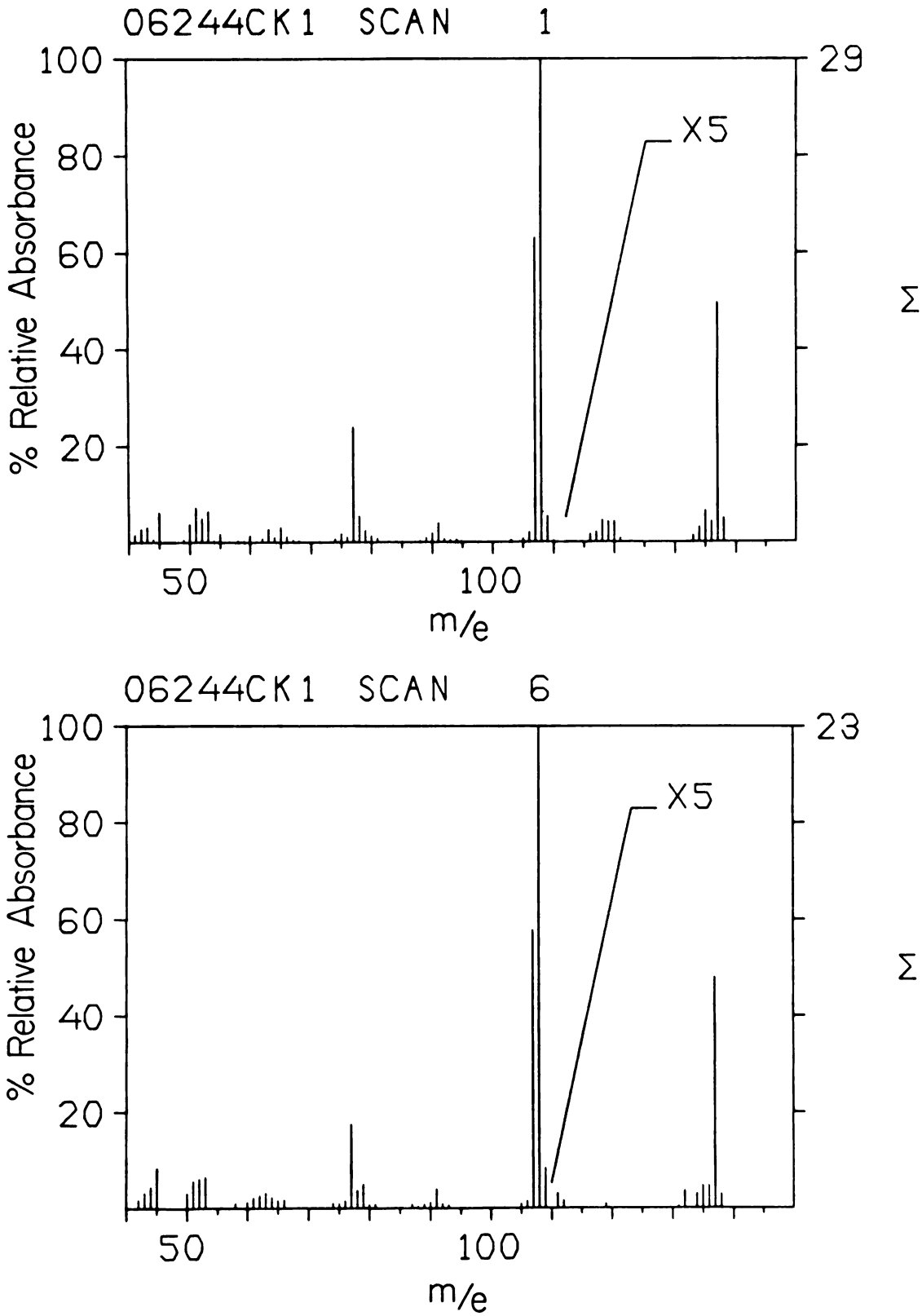

Fig. 31. Mass spectrometry patterns obtained from QR Blue cheese (A) and a tyramine standard (B).

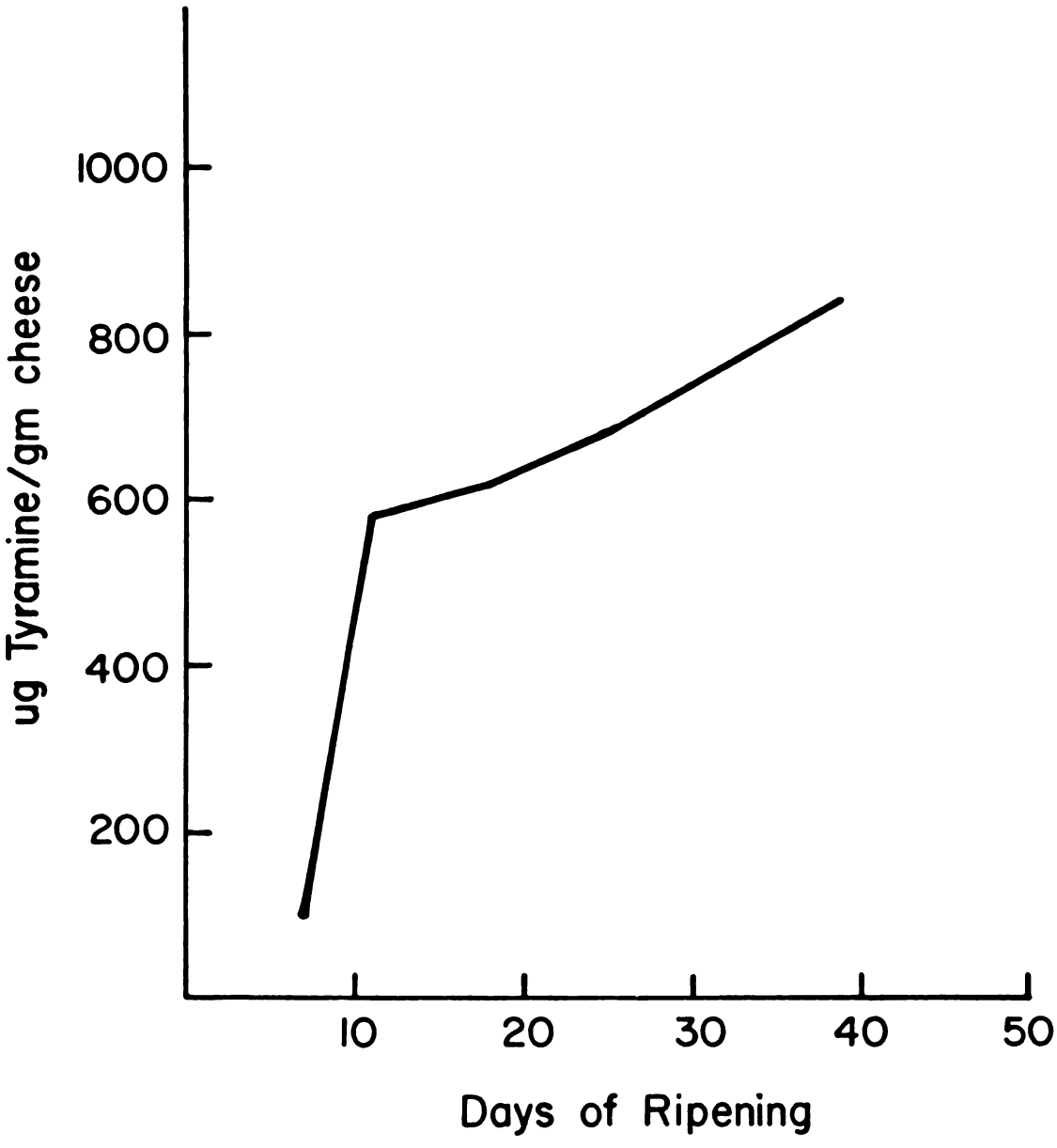

Fig. 32. The relationship between ripening and increases in tyramine in QR Blue cheese cured at 52 F (11 C) for the first 11 days followed by storage at 40 F (4 C) and salted on days 5, 6, and 7.

a corresponding tyrosine decarboxylase produced by streptococci, especially Streptococcus faecalis. However, in the case of Blue cheese it was suspected that the fungi has active decarboxylases. This appears to be correct since the rapid increased levels of tyramine on day 7 corresponds with the first signs of visible growth of the mold in the QR Blue cheese.

SUMMARY AND CONCLUSIONS

Numerous attempts were made to improve the procedure of Kondrup and Hedrick (1963) for the manufacture of QR Blue cheese so that a higher quality of cheese could be produced. The following modifications were suggested:

a. Lower the ripening temperature from 62 F (17 C) to 52 F (11 C).

b. Salt the cheese on days 1, 9, and 10 or days 7, 8, and 9. The salt was added in three applications with 1/4 of the total salt added on the first day indicated, followed by 1/4 and 1/2 for the other days respectively.

c. Cook the curd after cutting to facilitate draining and reduce loss of fine curd particles. The recommended cooking procedure consisted of allowing the curd to remain undisturbed for 15 min following cutting. Heat was then applied to raise the temperature of the curd to 100 F (38 C) in 30 min. Continuous stirring was maintained throughout this period and an additional 15 min for a total cooking time of 60 min.

d. Store the cheese for an additional 1-3 weeks at 40 F (4 C) following ripening to obtain the best flavor.

A 52 F (11 C) curing temperature was selected because a cheese of consistently high quality could be produced at this temperature. By using a ripening temperature of 52 F (11 C) instead of higher temperatures, the manufacturer should have more control over mold growth and, therefore, could more closely supervise flavor and color development.

Both salting applications proved compatible; however, there may be advantages in salting on days 1, 9, and 10. These advantages include less contamination from undesirable organisms and slightly more control over mold growth.

Other modifications in the manufacture of QR Blue cheese demonstrated the importance of homogenization, pasteurization and active starter cultures. When any of these variables were deleted in the manufacture, an atypical fermentation and/or a cheese or poor quality was produced. An acceptable QR Blue cheese could not be manufactured using direct acidification. The addition of lipases to the cheese milk did not improve the flavor of QR Blue cheese. An early salt application was shown to retard mold growth. A satisfactory cheese without any blue pigmentation could be manufactured using the suggested modifications to the procedure and the white mutant of P. roqueforti. The white

mutant proved to possess more active proteases than exhibited by the blue mold.

A correlation was shown to exist between NPN and pH values. As the NPN values increased there was a corresponding increase in pH values. The highest pH value attained was approximately 5.9 and remained near this value throughout a prolonged ripening period. Approximately 45 percent of the protein was present in the soluble form after ripening. This level was also maintained when an extended ripening period was used.

A major difference between QR Blue cheese and Blue cheese ripened by conventional methods was shown by PAGE. A more extensive degradation of proteins in QR Blue cheese was evident by the complete absence of any protein zones in PAGE. Conventionally ripened Blue cheese demonstrated some intact proteins in all the casein zones except the para-K casein region. Based upon results from PAGE, ripening could be classified as of a non-specific nature since α_S- and β-casein disappeared at approximately the same rate from PAGE patterns during cheese curing. The different casein components demonstrated specific characteristics during ripening. For example, α_S-casein was degraded to several bands that apparently had about the same mobility as the intact casein; however, the β-casein region was characterized by the presence of two bands that eventually disappeared as ripening continued. When the β-casein zones began weakening there was an apparent intensification of the bands in the

γ, K-casein region. This suggested that β-casein was being hydrolyzed to form peptides which migrated in the γ, K-casein region. This aided in confirming that the γ-casein proteins may have originated from β-casein.

Peptide mapping of Blue cheese and QR Blue cheese demonstrated that peptides in the molecular weight range of 700-5000 were similar. A molecular weight profile of QR Blue cheese showed that approximately 22-29 percent of the total protein was present as peptides of a molecular weight less than 700, 50-60 percent had a weight of 700-5000, and about 12-19 percent of the proteins had molecular weights greater than 5000. Conventional Blue cheese was characterized by having a higher percentage of the proteins with molecular weights greater than 5000.

Amino acid analysis proved that proteolysis occurred throughout ripening and storage. The content of free amino acids increased from 15 to 28 percent of the total protein from day 11 to day 39 when stored at 40 F (4 C). Generally, the amino acids that were present in high concentrations in Blue cheese were also present in high concentrations in whole casein. Analysis of several Blue cheeses demonstrated that more variation may exist between identical batches of cheese than between cheeses manufactured with slight modifications. No protease specificity could be observed by amino acid analysis.

Amino acid analysis revealed that tyrosine increased only slightly in concentration from day 7 through

day 39. Since fungi have been shown to process active amino acid decarboxylases, tyramine analyses were performed. Tyramine increased throughout ripening with concentrations after 39 days of 825 ug tyramine per gram of cheese. This compared to 40 ug of tyramine in conventional Blue cheese.

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APPENDIX

APPENDIX

Alkaline Copper Sulfate--Sodium Tartrate Solution

Two ml of 1 percent copper sulfate (w/v) were mixed with 2 ml of 2.7 percent sodium tartrate (w/v). To this, 96 ml of 0.20 N sodium hydroxide in 2.0 percent sodium bicarbonate (w/v) were added.

Ninhydrin Solution

Four hundred mg of stannous chloride dihydrate were dissolved in 250 ml of 0.2 M acetate buffer at pH 5.0. This solution was mixed with 250 ml of methyl cellosolve (ethylene glycol monomethyl ether) containing 10 g of dissolved ninhydrin (Calbiochem, A Grade). The solution was flushed with nitrogen and stored in a brown glass bottle at 4 C.

Ninhydrin Spray

Preparation of this reagent involved solvation of 0.35 g of ninhydrin (Calbiochem, B Grade) in 350 ml of absolute thanol, 135 ml of glacial acetic acid and 140 ml of collodin (2,4,6 trimethyl pyridine).

Polyacrylamide Gel Electrophoresis

Stock Buffer

The stock buffer was composed of 121 g reagent grade Tris (hydroxymethylaminomethane), 15.6 g disodium ethylenediaminetetraacetate (Na_2EDTA), and 9.2 g boric acid (H_3BO_3) diluted to four liters with distilled water. One part of the stock was diluted with two parts of water to give a pH 9.1-9.3 buffer solution.

Stock Gel

A stock gel solution was made by dissolving 70 g Cyanogum in about 400 ml buffer of the buffer, adding 270 g urea, 1 ml betadimethylamino propionitrile and diluting to one liter with buffer. The resulting solution which was 7 percent Cyanogum and 4.5 M urea, could be stored at room temperature for several weeks.

Amido Black Solution

Disc gels were stained for 15 min in a solution of 0.25 percent Amido Black 10B (E. C. Apparatus). The dye was dissolved in methanol, water and glacial acetic acid (5:5:1).

Kjeldahl Reagents

Mercuric Sulfate Solution

Mercuric sulfate (13.7 g microanalytical grade) was dissolved and diluted to 100 ml with 2 M Sulfuric acid.

Sodium Hydroxide--Sodium Thiosulfate Solution

Sodium hydroxide (200 g analytical grade) and sodium thiosulfate (12.5 g analytical grade $Na_2S_2O_3:5H_2O$) were dissolved in water and made up to 400 ml.

Boric Acid--Mixed Indicator Solution

Boric acid (20 g) was dissolved in water (ca. 800 ml). Methylene blue (6.67 mg) was dissolved in water (50 ml). Methyl red (13.3 mg) was dissolved in ethyl alcohol (10 ml). These solutions were washed into 1 liter volumetric flask and made up to volume with water.

Potassium Biiodate Solution (0.01 M)

Potassium biiodate (3.8944 g) that had been dried overnight in a desiccator was dissolved and made up to 1 liter with water.