August 12, 1959

To the Graduate Council:

I am submitting herewith a thesis written by James D. Skean entitled "Changes in Milk Proteins Associated with the Growth of Psychrophilic Bacteria." I recommend that it be accepted for nine quarter hours of credit in partial fulfillment of the requirements for the degree of Master of Science, with a major in Dairying.

W W Overcast
Major Professor

We have read this thesis
and recommend its acceptance:

B. J. Holmott

C. C. Dykie

Thos. B. Harrison

Accepted for the Council:

Dale Kaufling
Dean of the Graduate School

CHANGES IN MILK PROTEINS ASSOCIATED WITH THE GROWTH OF PSYCHROPHILIC BACTERIA

A THESIS

Submitted to
The Graduate Council
of
The University of Tennessee
in
Partial Fulfillment of the Requirements
for the degree of
Master of Science

---

by

James D. Skean

August 1959

22

33

ACKNOWLEDGMENTS

The writer is grateful to the University of Tennessee's Department of Dairying for having made possible the opportunity to do this work.

Great appreciation is expressed for the indispensible guidance and cooperation of Dr. W. W. Overcast in carrying out the work and preparing this thesis.

The writer also wishes to express appreciation for the helpful suggestions made by Professor C. E. Wylie, Professor T. B. Harrison and Dr. B. J. Demott in preparing this manuscript.

452949

TABLE OF CONTENTS

	PAGE
INTRODUCTION	1
REVIEW OF LITERATURE	3
Dairy Psychrophiles	3
Scope	3
Types and Distribution	3
Sources	6
Growth and Defects	9
Paper Electrophoresis	19
Milk Protein Analysis	19
EXPERIMENTAL MATERIALS AND METHODS	24
RESULTS AND DISCUSSION	33
SUMMARY	63
REFERENCES	65

LIST OF TABLES

TABLE PAGE

1. Average Total Counts (TC) and Psychrophilic Counts (PC) on Control and Inoculated Samples of Skimmilk Stored at 3 to 5° C. 32
2. Means of the Relative Per Cent of Each Protein Fraction in Six Control Samples of Skimmilk as Determined Paper Electrophoretically Four Times at 14-Day Intervals 34
3. The Relative Per Cent of Each Protein Fraction in Total Milk Protein as Determined by Paper Electrophoresis and Moving Boundry Electrophoresis 38
4. Average Per Cent of Deviation from the Controls for Total Casein in Skimmilks Inoculated with Pure Culture Bacteria and Subsequently Stored at 3 to 5° C. 40
5. Average Per Cent of Deviation from the Controls for Total Whey Protein in Skimmilks Inoculated with Pure Culture Bacteria and Subsequently Stored at 3 to 5° C. 41
6. Average Deviation from the Controls in the Mean Relative Per Cent of Each Casein Fraction in Skimmilks Inoculated with Pseudomonas fragi and Subsequently Stored at 3 to 5° C. 44

TABLE PAGE

7. Average Deviation from the Controls in the Mean Relative Per Cent of Each Whey Protein Fraction in Skimmilks Inoculated with Pseudomonas fragi and Subsequently Stored at 3 to 5° C. 45

8. Average Deviation from the Controls in the Mean Relative Per Cent of Each Casein Fraction in Skimmilks Inoculated with Pseudomonas fluorescens and Subsequently Stored at 3 to 5° C. 49

9. Average Deviation from the Controls in the Mean Relative Per Cent of Each Whey Protein Fraction in Skimmilks Inoculated with Pseudomonas fluorescens and Subsequently Stored at 3 to 5° C. 50

10. Average Deviation from the Controls in the Mean Relative Per Cent of Each Casein Fraction in Skimmilks Inoculated with Pseudomonas putrefaciens and Subsequently Stored at 3 to 5° C. 53

11. Average Deviation from the Controls in the Mean Relative Per Cent of Each Whey Protein Fraction in Skimmilks Inoculated with Pseudomonas putrefaciens and Subsequently Stored at 3 to 5° C. 54

12. Flavor Criticisms Assigned to Control Samples and Samples of Skimmilk Inoculated with Pseudomonas fragi and Subsequently Stored at 3 to 5° C. 59

TABLE PAGE

13. Flavor Criticisms Assigned to Control Samples and Samples of Skimmilk Inoculated with Pseudomonas fluorescens and Subsequently Stored at 3 to 5° C. 60

14. Flavor Criticisms Assigned to Control Samples and Samples of Skimmilk Inoculated with Pseudomonas putrefaciens and Subsequently Stored at 3 to 5° C. . . . 61

LIST OF FIGURES

FIGURE PAGE

1. Typical Paper Electrophoretic Patterns of the Proteins from High Quality Pasteurized Skimmilk 35
2. Representative Paper Electrophoretic Patterns of Casein from Samples of Skimmilk at the End of the Storage Period in Trials with P. fragi 46
3. Representative Paper Electrophoretic Patterns of Whey Proteins from Samples of Skimmilk at the End of the Storage Period in Trials with P. fragi 47
4. Representative Paper Electrophoretic Patterns of Casein from Samples of Skimmilk at the End of the Storage Period in Trials with P. fluorescens 51
5. Representative Paper Electrophoretic Patterns of Whey Proteins from Samples of Skimmilk at the End of the Storage Period in Trials with P. fluorescens 52
6. Representative Paper Electrophoretic Patterns of Casein from Samples of Skimmilk at the End of the Storage Period in Trials with P. putrefaciens 55
7. Representative Paper Electrophoretic Patterns of Whey Proteins from Samples of Skimmilk at the End of the Storage Period in Trials with P. putrefaciens 56

INTRODUCTION

One of the factors responsible for the loss of initial quality in adequately refrigerated, pasteurized milk and milk products is their widespread contamination with psychrophilic microorganisms. A common characteristic of the members of this taxonomically heterogeneous group is that they grow sufficiently well, though rarely optimally, at refrigeration temperatures to damage severely the organoleptic quality of dairy products in relatively short periods of time. This creates a major problem in modern dairy practice which is magnified as it becomes economically important to lengthen the time interval between production and consumption of dairy foods.

The literature on the relation of psychrophilic microorganisms to dairying is voluminous. The greater part of it treats their distribution and growth in contaminated dairy products with resulting organoleptic quality deterioration while in refrigerated storage. However, comparatively little information deals with the changes in milk constituents in dairy products due to their growth. This may be due to the fact that such changes do not share the commercial significance of organoleptic quality deterioration. Although the organoleptic detection of quality deterioration in dairy products almost invariably precedes any visible changes in milk constituents, that such changes are a part of this deterioration may be shown by other means.

Frequently the bacterial deterioration of milk and dairy products is detected organoleptically as a bitter flavor. Since this bitterness has often been associated with proteolysis, a study was made, using

paper electrophoresis, to determine the changes in milk proteins associated with the growth of psychrophilic bacteria.

REVIEW OF LITERATURE

Dairy Psychrophiles

Scope

The term "psychrophile" as used in the dairy industry has not been strictly defined in practice, being indiscriminately applied to all microorganisms growing in refrigerated dairy products. Thomas (80) listed 35 publications dealing with dairy psychrophiles wherein the reported psychrophilic colony counts were determined using plate incubation times and temperatures ranging from 2 to 28 days and 0 to 25° C., respectively. The incubation time-temperature relationship most often reported was seven days at 5° C., which is that officially recommended in the United States for the detection of more typical psychrophiles (1). It has been suggested that the definition of the term be restricted to include only those organisms capable of growing in milk at 5° C. and which are destroyed in milk by heating at 62° C. for thirty minutes (79).

Types and Distribution

The term psychrophile does not describe a particular taxonomic group of microorganisms though it may be said that most dairy psychrophiles are Gram-negative, nonsporulating rods.

A list of psychrophilic bacteria given by Elliker (20) included the genera Pseudomonas, Alcaligenes, Escherichia, Aerobacter, Achromobacter, Chromobacter and Flavobacterium, with P. fragi, P. fluorescens,

P. viscosa, P. putrefaciens, Alc. metalcaligenes and Alc. viscosus being examples of species common in dairy products.

Schultz and Olson (72) isolated species in the genera Pseudomonas, Alcaligenes, Achromobacter and Flavobacterium as well as members of the coliform group and a few yeasts and molds from commercially packaged milk, cream, chocolate drink and cottage cheese after one and two weeks of storage at 4° C. Fluorescent pseudomonads alone accounted for approximately one-half the isolates and the relative distribution of each bacterial type was fairly uniform among the various products sampled during summer and winter seasons. Foster et al. (22) state that species of asporogenous, Gram-negative rods such as Pseudomonas have been most often involved in outbreaks of spoilage at refrigeration temperatures, and occasionally species of soil and water forms like Alcaligenes, Achromobacter and Flavobacterium are found. Boyd et al. (8) reported that the majority of spoilage organisms in samples of commercially pasteurized and homogenized milk stored at 33 and 40° F. were Gram-negative rods, most of which were killed by pasteurization at 143° F. for 30 minutes. Watrous et al. (84) recognized that the psychrophiles isolated from pasteurized milk have been identified largely as Gram-negative, nonsporeforming rods with members in the genera Pseudomonas, Flavobacterium and Achromobacter predominating. Dahlberg (13) found that coliform bacteria increased more rapidly in numbers than did total counts in pasteurized market milk held under refrigeration, indicating that some coliforms also may be psychrophilic.

Sherman et al. (73) studied both raw and pasteurized milks and reported that the bacteria responsible for the spoilage of milk held

just above freezing appeared to be mainly, if not entirely, Gram-negative, nonsporeforming rods largely of the genus Pseudomonas, and that sporeformers apparently neither grew nor contributed to spoilage at these temperatures.

Davis (14) states that in clean, raw milk held at 0 to 4° C. for a few days, the psychrophilic microbes gradually predominate with the most common genera being Pseudomonas, Achromobacter, Chromobacterium and Micrococcus along with the yeast Torulopsis and molds Penicillium, Cladosporium, Mucor and Thamnidium. Marth and Frazier (45) found that the predominant bacteria in raw milks held at farm bulk tank cooling temperatures were Gram-negative rods in the genera Achromobacter, Aerobacter, Alcaligenes, Flavobacterium and Pseudomonas. Morrison and Hammer (50) reported the isolation of P. fragi from 16.5 per cent of 176 lots of milk delivered by 14 producers to an Iowa milk plant, and while no isolations were made from 17 lots delivered to a Kentucky plant in June, 16 of 40 lots delivered to the same plant in December yielded the organism.

Robinton et al. (68) studied the microbial content of 523 retail samples including both raw and pasteurized creams. They found that 24.4 per cent of the samples had agar plate counts exceeding 500,000 per milliliter when the plates were incubated four days at 8° C.

Parker et al. (61) showed that the species Alc. metalcaligenes, P. viscosa and P. fragi may be responsible for defects commonly occurring in refrigerated cottage cheese. Collins (11) also studied the role of these three species in cottage cheese spoilage.

Hussong et al. (28) reported the isolation of P. fragi from samples of abnormal milk, cream, cottage cheese and bulk condensed milk and also from homogenizer plunger packing.

Sources

Psychrophilic microorganisms are usually present in raw milk, but their presence in pasteurized dairy products is not to be condoned in view of the fact that they do not survive pasteurization, therefore, their presence denotes post-pasteurization contamination.

Watrous et al. (84) listed water, soil, bedding and utensils as probable sources of psychrophiles found in raw milks. Elliker (20) believed soil and water on the farm to be the original sources of psychrophiles. In Iowa, Morrison and Hammer (50) reported the isolation of P. fragi from 51.8 per cent of all samples of dirt and other materials examined that were likely to come in contact with milk. However, in Kentucky, only 4.1 per cent of similar samples taken in the summer yielded the organism compared to 37.4 per cent in December. The organism was found in 71.4 per cent of 35 samples of barnyard soil obtained from various states, being present in 90.9 per cent of the samples from eastern states compared to 38.5 per cent from western states (50). Such information emphasizes the wide distribution of this frequently encountered psychrophile and its possibilities as a raw milk contaminant. In fact, Olson et al. (58) stated that all who have tried to find psychrophilic bacteria in raw milk have succeeded, particularly if their analytical procedure included incubation of poured plates for a sufficient period of time within a temperature range of 5 to 10° C.

Kennedy and Weiser (32) reported that 14 of 15 pure cultures of psychrophiles, after being inoculated into milk, survived 145° F. for 30 minutes in considerable numbers. Jezeski and Macy (30) had only six of 41 cultures of psychrophilic bacteria isolated from fresh butter to survive laboratory pasteurization at 150° F. for 30 minutes. Erdman and Thornton (21) had only four of 722 psychrophilic cultures to survive laboratory pasteurization. Several other investigators (58, 61, 69, 83, 84) have presented data which indicate quite conclusively that the presently known dairy psychrophiles do not survive proper pasteurization.

The likely sources of post-pasteurization contamination of dairy products with psychrophiles are water supplies, processing equipment and containers. Rogick and Burgwald (69) found the main sources of market milk contamination to be equipment and bottles. Parker et al. (61) believed that the principal means by which cottage cheese spoilage organisms entered the product were in the curd wash water and from improperly cleaned and sanitized manufacturing equipment. Boyd (6) also blamed these two means but added that the creaming mixtures used in cottage cheese may be a common source. Watrous et al. (84) recognized improperly cleaned equipment as a source of psychrophilic contamination and recommended rigorous cleaning methods. Morrison and Hammer (50) found dairy plant equipment to be relatively free of P. fragi but 9.7 per cent of Iowa plant water supplies examined yielded the organism. Long and Hammer (41) isolated P. putrefaciens from samples of the water supplies in 14 of 29 creameries in nine midwestern states.

Harmon (26) found samples from water supplies in 14 butter and 12 cottage cheese plants to be mostly free of coliform bacteria but to contain psychrophiles. Corley et al. (12) in a study of samples from butter plant water supplies found that 5 per cent yielded P. putrefaciens and 25 per cent contained organisms that formed fluorescent colonies on agar plates incubated at 21° C.

In a study of samples of water and butter obtained from butter plants having spoilage difficulties, Jezeski and Macy (30) isolated 41 pure cultures of microorganisms, including both caseolytic and lipolytic types, capable of vigorous growth at 8° C.

Olson and Nielsen (55) concluded that there is great variation in the bacteriological quality of home water supplies used by consumers in recombining milk powders. These waters were found to contribute significantly to the flora of freshly recombined milks but the cleanliness of kitchen utensils used in the recombination process was likely the more important factor influencing the subsequent keeping quality of the milk under refrigeration.

Sieberling and Harper (74) described a high-temperature short-time heat treatment for plant water supplies, the use of which permitted one plant having cottage cheese spoilage from psychrophiles to produce a better-keeping product than previously attained with the use of chemical sanitizers.

The coliform test for post-pasteurization contamination (1) is known to be unreliable in indicating that pasteurized dairy products are free from contamination with psychrophilic microorganisms (3, 54, 84).

Growth and Defects

The development of defects in dairy products by psychrophiles may occur within periods of time involved in present marketing methods.

Prouty (66) reported that in some samples of raw milk held 48 hours at 37 to 39° F. there were psychrophilic counts exceeding one million per milliliter and may have contributed to slight flavor defects noted at this time.

Mikolajcik and Burgwald (47) found the bacterial counts on milk samples stored seven days at 0 to 1.66° C. and 7.2 to 10° C. to be much higher than the initial counts; in some instances as much as thirty times higher. Rogick and Burgwald (69) found psychrophiles in all samples of milk examined after one week of storage at 4 to 7° C. even though none were found in 4.1-ml. quantities of milk taken directly from vat or high-temperature short-time pasteurization systems. In this study flavor changes developed only in raw milk samples during one week of storage, indicating that extremely high counts were necessary to noticeable flavor changes.

Marth and Frazier (44) reported that psychrophiles grew faster, especially after two days, in low count milk having less than 50,000 bacteria per milliliter. More samples of high count milk showed appreciable growth within one, two, three, or four days at temperatures of 36, 38, and 45° F. than did low count milk. In an earlier publication (43) they reported that in milk held at 36° F. little increase in bacterial numbers occurred in three days but at 38 and 45° F. significant increases occurred in some samples within one or two days.

These workers believed that a 45° F. storage temperature for raw milk was too high. They found no correlation between farm source of the milk samples and growth of bacteria but did find that psychrophiles grew better in summer milk than in winter milk. Marth and Frazier (45) also studied the growth characteristics of several pure cultures of psychrophilic bacteria inoculated into milk and held four days at 38° F. Most of the Pseudomonas species grew rapidly and steadily during the first two days and more slowly during the last two days. Cultures of Achromobacter and Alcaligenes species grew rapidly during the first three days and more slowly during the fourth. The Flavobacterium cultures tested failed to make any appreciable growth under these conditions.

Hadfield (23) stated that psychrophilic growth in milk was much more rapid at 45 to 50° F. than at temperatures below 40° F.

Kaufmann and Andrews (31) concluded that the growth of psychrophiles varies considerably; that the growth rate of the contaminating organism may be more important in determining the length of the storage period than the initial level of contamination; and that an average initial psychrophilic count of less than 10 per milliliter is readily obtainable under good practical plant operating conditions.

Watrous et al. (84) reported that the psychrophilic count on 75 samples of raw milk after 10 and 20 days of storage at 5° C. in many cases was considerably greater than the "total count," but the "total count" on an average exceeded the psychrophilic.

Sherman et al. (73) found that pasteurized milk held at 0° C. had slower bacterial growth than did raw milk of substantially the

same quality or bacterial content, and that pasteurized milk, if not recontaminated, kept from two to three times as long as raw milk of the same quality and bacterial content.

Lawton and Nelson (35) in studying the growth characteristics of psychrophilic bacteria, some of which were isolated from commercial milk sources, found that 21 to 25° C. was the optimum growth range for the majority with their logarithmic phase of increase occurring within the first 24 hours of growth. When the temperature was lowered to 5 to 10° C. the logarithmic phase was not entered until either the 24- to 48-hour or 48- to 72-hour interval. Their data showed that low temperatures only retard the growth of some organisms and that the logarithmic phase of increase lengthens as the growth temperature is lowered from the optimum.

Boyd et al. (7) found flavor deterioration to be correlated with the growth of psychrophilic bacteria in commercially pasteurized and homogenized milk stored at 33 and 40° F. In split duplicate samples stored at these different temperatures, the average keeping quality of the milk at the lower temperature was extended an additional 11 to 14 days. In a later publication they reported that the deterioration of flavor in all commercially processed samples examined was closely associated with the growth of psychrophilic bacteria (8).

Burgwald and Josephson (9) reported that the development of psychrophilic organisms in refrigerated pasteurized milk followed a pattern similar to that of mesophiles except that after six days the psychrophiles were far greater in number. They felt that the problem

of flavor development during refrigerated storage is of little importance if the milk is consumed within seven days but is apparently of great importance during storage beyond seven days. Weese and Henderson (85) found that good quality pasteurized milk under home refrigeration retained a good flavor three to four days after delivery, but after the fourth day there was decided flavor deterioration. They observed that the average daily per cent of increase in bacterial count on the fifth and sixth day of storage was decidedly greater than that on the third and fourth day.

Babel (4) studied changes in raw milk held at 4.4° C. He found that high quality raw milk showed a significant increase in total bacteria after two to four days and that this flora was made up largely of psychrophilic types. Generally, the free fat acidity and amino-nitrogen content of the milk increased slowly and any abrupt increases were accompanied by abrupt increases in numbers of bacteria. Also, the rate of rennet coagulation varied considerably with extended holding at this temperature and showed a direct relation to the type of bacteria present.

Jezeski and Macy (30) state that psychrophilic bacteria in raw milk may produce ropiness, discoloration, proteolysis and bitter flavors.

Parker and Elliker (60) presented experimental evidence that pure cultures of P. viscosa and P. fragi, in that order of activity, caused appreciable losses in the diacetyl content of cottage cheese stored at 10° C. within 48 hours although the cheese retained its normal appearance. A similar phenomenon occurred in 10 per cent reconstituted non-fat-dry-milk

solids containing 6 p.p.m. added diacetyl, except that losses in diacetyl were accompanied by corresponding increases in acetylmethylcarbinol. These losses in diacetyl were accompanied by the development of a flat flavor. Their results indicated a direct relationship between diacetyl content and the desirable aromatic flavor in cottage cheese. Pure cultures of P. viscosa and P. fragi isolated from gelatinous cottage cheese were capable of rapidly reducing almost all the diacetyl present in cottage cheese or milk whereas a culture of Alc. metalcaligenes was much less active in such destruction. With P. viscosa and P. fragi a complete loss of aroma preceded the development of any apparent physical manifestation of spoilage. It was also demonstrated that the diacetyl destroyed was converted to acetymethylcarbinol and some possibly to 2,3-butylene glycol.

Olson et al. (57) pointed out that psychrophiles have been responsible for considerable cottage cheese losses through tapioca or slimy curd although fruity or unclean flavors are usually observed to precede any apparent physical decomposition of the curd particles.

Boyd (6) states that the development of fruity and unclean flavors and odors in cottage cheese results from the growth of psychrophilic bacteria. This also may cause a condition known as tapioca curd in which each curd particle develops a shiny, translucent surface.

Collins (11) believed that fruitness in cottage cheese caused by the growth of P. fragi was the defect most commonly limiting the storage life of commercial cottage cheese. He found this species to

be the least retarded by low temperature in a study of P. viscosa and Alc. metalcaligenes.

Claydon and Hammer (10) found that butter wash water inoculated with P. mephitica produced the typical skunk odor in experimental unsalted butter in two to three days of storage at 21° C.

Atherton et al. (3) studied bacterial growth in commercially pasteurized milk held at 4.4° C. They found that the average initial psychrophilic counts were very low but increased sharply through 15 days of storage. Observed changes in the milk samples that accompanied increases in bacterial content were losses in protein stability and significant increases in vitamin B_{12} content. No changes in phosphatase content due to microbial action, as had been reported by Hammer and Olson (25), were observed in this study. These workers concluded from their spoilage observations that the goal of milk processors should be to bottle pasteurized milk which maintains an acceptable quality during 12 days of storage at 4.4° C.

Olson et al. (56), at storage temperatures of 1, 4 and 7° C., obtained approximately 5-, 3- and 2-week periods, respectively, of satisfactory keeping quality with milk concentrates prepared under ideal sanitary conditions after pasteurization. They found that bacterial growth occurred at an appreciably greater rate in recombined milk than in the concentrate from which it was made. Both bitter and putrid flavors appearing to be associated with bacterial growth were encountered in this study. Olson and Nielsen (55) later reported that home recombined milks made from non-fat-dry-milk solids generally

deteriorated rapidly during storage at 50° F. and bacterial counts of several millions per milliliter after 48 to 72 hours of storage were not uncommon.

Powell (65) stored samples of fresh raw cream at 35° F. for ten days. He found that the acid coagulating organisms formed the largest initial group. Both this group and the acid coagulating-peptonizing group increased during storage but after eight days the peptonizers predominated. The fresh cream generally had a good flavor but became slightly bitter by the second day of storage and very bitter by the eighth day. Flash-heating the cream at 155° F. greatly reduced the number of peptonizing bacteria and they did not increase during subsequent storage.

Elliker (20) included in a list of undesirable flavors caused by psychrophilic bacteria in dairy products those described as fruity, rancid, bitter, stale, musty and sour, with rotten and putrid odors marking the advanced stages of decomposition. He stated that the ability of these organisms to produce lipase and proteolytic enzymes made them particularly objectionable from the standpoint of off-flavors.

Olson et al. (58) believed that sooner or later all commercially processed milk when held within a few degrees above freezing spoils due to metabolic products resulting from bacterial proliferation. To this they attributed the flavor defects described as putrid, fruity and unclean or sour. Changes in body and appearance may occur such as thickening or ropiness and a green or yellow surface discoloration.

Hussong et al. (28) made unsalted butter from pasteurized cream inoculated with P. fragi. They found wide variation in the initial numbers of organisms with great increases evident after three or four days of storage at 7° C. Counts of over 50 million per milliliter were rather common with a few over 100 million. They reported that the flavor production by P. fragi was inconsistent but the most common changes was a "May apple" odor followed by rancidity. Sometimes with certain strains this pattern did not occur and the butter became cheesy.

Derby and Hammer (16) and Long and Hammer (41) studied quite extensively the organisms involved in the surface-taint defect of butter. The causative organism was first tentatively designated Achromobacter putrefaciens (16) but later was classified as a member of the genus Pseudomonas (41) and renamed P. putrefaciens. They found that when this organism was inoculated into pasteurized cream and subsequently churned, the resulting butter developed the surface-taint defect within two to four days at 15.6° C. and within seven to ten days at 5° C.

Stark and Scheib (77) isolated 486 pure cultures of bacteria from butters, of which 96 per cent were capable of hydrolyzing tributyrin, 77 per cent split milk fat and 63 per cent were proteolytic in milk. These workers considered these three characteristics as criteria for the isolation of organisms capable of producing changes in the odor or flavor of butter.

Moir and Russell (49) state that proteolytic bacteria may break down the curd in butter and so adversely affect its flavor.

Parker et al. (59) state that various Pseudomonadeae are responsible for rancid, fruity, fishy and a variety of other defects in butter.

Several earlier investigators (15, 19, 27, 53, 67, 71, 76) reported on the relationships between proteolytic bacteria, proteolysis of milk proteins and the resulting off-flavors in butter bearing these protein degradation products.

Van der Zant and Moore (81) studied the proteolytic activities of P. fragi, P. fluorescens and P. putrefaciens in skimmilk held at temperatures of 5, 10, 21 and 25° C. for 16 days. All cultures were proteolytic to varying degrees as determined by the production of soluble nitrogen and the amino acids tyrosine and tryptophan. All cultures showed proteolysis at the incubation temperatures used. There was no evident relationship between level of population and proteolytic activity. There did appear to be some relationship between the soluble nitrogen content and the content of tyrosine and tryptophan. Only one of these cultures failed to show a short lag phase even at the lower holding temperatures.

Hammer and Babel (24) state that proteolysis in milk and cream is often accompanied by a bitter flavor due to the presence of "bodies" such as peptones occurring among the protein decomposition products. This bitterness may be evident before the appearance of visible signs of proteolysis. Under practical conditions it occurs most frequently in products held at a relatively low temperature.

Pelczar and Reid (62) state that microbial proteolysis, depending upon the organisms involved, results in the accumulation of varying amounts of soluble nitrogenous products imparting an alkaline reaction and bitter taste to the milk. P. viscosa and P. putrefaciens may cause this condition.

Pennington (63) investigated extensively the bacterial growth and chemical changes occurring in raw milk held at a temperature of -1.67 to -0.55° C. for periods of five and six weeks. In comparisons of "clean" or certified milk to ordinary market milk, the clean milk maintained an acceptable quality throughout the storage period while the market milk developed bitterness by the end of the fourth week and bitterness with an unpleasant odor by the end of the fifth week under these conditions. Bacterial increases, as determined by three plating methods, occurred throughout the storage period. These enumerations were made by incubating one series of plates one day at 37° C. followed by three days at room temperature, another series at 20° C. for five days and another at the sample storage temperature for four to six weeks. The analyses for nitrogen distribution showed that the casein of milk in cold storage was rapidly digested until finally more than 50 per cent of it was changed to soluble compounds such as caseoses, amino-acids and probably peptones. The rapidity of the digestion varied with different samples but was pronounced at the end of two weeks of storage. It was thought likely that proteolysis at these temperatures was caused by enzymes native to the milk rather than by those of bacterial origin, and a series of preliminary experiments seemingly substantiated this belief. In a later publication Pennington et al.

(64) presented data establishing the fact that bacterial enzymes were quite important in the degradation of the proteins in raw milk at 0° C. This work prompted the conclusion that the proteolysis of casein is primarily bacterial while that of lactalbumin is due to native milk enzymes. However, it was apparent that bacterial flora and native enzymes, by their combined action, effected more rapid proteolytic changes than were produced by either agent alone. Also, the total proteolytic activity observed in these experiments proceeded in the manner of a tryptic digestion wherein the true proteins pass through caseose and peptone forms in their degradation to amino-acids.

Paper Electrophoresis

Milk Protein Analysis

The use of paper electrophoresis in the investigation of biological fluids, particularly for qualitative and quantitative fractionations of blood serum proteins, is thoroughly discussed in at least three texts (5, 37, 87). The latest of these (37) only briefly mentioned the use of the method in research on milk proteins. The available literature to date dealing with paper electrophoretic studies of milk proteins is meager.

Zweig and Block (88) appear to be among the first to report the use of paper electrophoresis in the separation of milk proteins. Their method was an adaptation of that previously developed by Durrum (18) for the separation of blood serum proteins. They worked only with casein and its components apart from the complete milk protein system,

therefore, paper electrophoretic data on whey proteins were not included. The relative mobilities of the casein components obtained in this study compared favorably with those obtained by the Tiselius electrophoretic method. These workers believed that the concentration of gamma-casein in whole casein was too low to permit its being detected unquestionably by this method.

MacRae and Baker (42) successfully used paper electrophoresis to separate quantitatively alpha-, beta- and gamma-casein from whole casein suspended at 5 per cent concentration in veronal buffer at pH 8.6 with an ionic strength of 0.05. The analysis was done on large sheets of relatively thick filter paper horizontally placed in the apparatus. They apparently obtained positive migration of the slower-migrating gamma-casein. The separated components were visualized by staining with dyes Azocarmine or Naphthalene Black. The relative percentage of each component was determined both by direct photometry and Kjeldahl nitrogen contents. These methods gave comparable results. In estimating component percentages these workers applied correction factors, which they derived empirically, to lessen the error caused both by different dye-binding capacities of the components and deviation from Beer's law. From their corrected analysis data they estimated that whole casein contained 60 per cent alpha-, 32 per cent beta- and 9 per cent gamma-component.

Libbey and Ashworth (38) carried out the paper electrophoretic separation of casein using a phosphate buffer at pH 12.3 with an ionic strength of 0.19. They claimed that the casein was clearly

resolved into three fractions in a Spinco apparatus during 30-hour runs with 20 to 30 milliamperes of current at a temperature of about 4° C. The level of sample application was found to be critical. The best results were obtained using a 0.01- to 0.02-ml. quantity of a 10 per cent concentration of whole casein suspended in the buffer. The highest degree of resolution was obtained by placing the samples on the strips as near as possible to the cathode side of the Durrum type cell. The separated proteins were made visible by staining with bromophenol blue. These workers reported the approximate relative distribution of the casein fractions, based on areas beneath scanning curves without correcting for tailing errors, as 50 per cent alpha- and kappa-, 45 per cent beta- and 5 per cent gamma-casein.

Lindquist (39), Lindquist et al. (40) and Storgards and Lindquist (78) used paper electrophoresis as a means of studying changes in casein occurring during the ripening of different cheeses. They stressed the fact that even though a linear relationship exists between the optical density and the concentration of a specific protein when dyed, different proteins have different dye-binding capacities and there is good reason to believe that this is also true of the decomposition products of casein. They cautioned, therefore, against drawing too far-reaching conclusions from the quantitative data obtained by electrophoretic analysis. However, they believed that the method did afford several new possibilities for research in cheese chemistry.

Weigt (86) described a relatively simple procedure for the paper electrophoretic analysis of milk serum (whey) proteins. In this procedure approximately 0.08 ml of clear, supernatant whey, obtained after the isoelectric precipitation of casein in milk, was separated on each paper strip. The electrolyte used was a sodium veronal-sodium acetate buffer of pH 8.6 and ionic strength of 0.1. The electrophoretic runs were 16 hours long at 18° C. A constant voltage of 150 v. was applied to the strips. The procedure yielded characteristic curves comparable to those obtained by the Tiselius method. The separation of five protein fractions from normal milk serums was reported. The fractions were tabulated with the range of the relative per cent of each as follows: euglobulin, 5.7 to 15.7 per cent; pseudoglobulin 3.1 to 11.8 per cent; alpha-lactalbumin, 15.0 to 31.8 per cent; beta-lactoglobulin, 44.7 to 67.7 per cent; and, blood serum albumin, 1.3 to 6.2 per cent. These fractions are listed in the order of their increasing electrophoretic mobility.

Lecce and Legates (36) used paper electrophoretic analysis in studying changes in the whey protein of milk from cows with acute mastitis. They found the whey protein pattern of milk from mastitic quarters to be markedly altered from the normal. The outstanding changes in the patterns were the appearance of a fraction migrating at the rate of blood-serum albumin and a relative increase in the amount of immune globulins. For milk obtained from 43 normal quarters grouped into three categories they reported means of the per cent of the various

whey proteins ranging from 13 to 20 per cent immune globulins, 27 to 39 per cent alpha-lactalbumin, 47 to 60 per cent beta-lactoglobulin and 0 to less than 1 per cent blood-serum albumin, given in the order of increasing mobility. They also observed that whey samples from some quarters contained a trace amount of a fraction migrating faster than blood-serum albumin and another fraction migrating between beta-lactoglobulin and alpha-lactalbumin.

Modi and Owen (48) reported the use of paper electrophoresis, along with paper chromatography and fluorescence under ultra-violet light, in studying the qualitative and quantitative distribution of riboflavin and allied compounds among the milks of several mammalian species.

EXPERIMENTAL MATERIALS AND METHODS

All skimmilk used in this study was obtained from the University Creamery and had been commercially separated. Duplicate samples of approximately 1500 ml. in quantity were aseptically removed from the pasteurizing vat within five minutes after the end of the 30-minute hold period, placed in closed sterile 2-l. Erlenmeyer flasks and cooled in an ice bath. The pasteurization temperatures ranged from 145 to 146° F. while the space heater temperature at times rose to 165° F. A total of six pairs of samples were used in the study.

The flask closures were laboratory designed for ease of manipulation, for tightness during sample agitation and to reduce the danger of the sample adsorbing undesirable flavors from the external atmosphere.

When the samples had cooled to near ice bath temperature one received a 0.5 to 1.0 per cent inoculation with a pure culture of the bacterium to be studied and the other sample was held untreated as the control. The samples were subsequently stored in a refrigerator at 3 to 5° C. and examined at 14-day intervals during a 42-day period. The samples were hand agitated daily except on Sundays.

The bacteria studied were three species in the genus Pseudomonas, namely, P. fragi, P. fluorescens and P. putrefaciens. The P. fragi (4973) and P. fluorescens (11251) were obtained from the American Type Culture Collection (ATCC), 2112 M Street, N. W., Washington 7, D. C. The P. putrefaciens was obtained from the culture collection maintained by the Dairy Microbiology Laboratory at the University of Tennessee. Stock cultures of these bacteria were maintained at 3 to 5° C. on

slants of milk-protein hydrolysate (M-PH) agar. These were activated monthly by transferring to tryptose phosphate broth containing 0.1 per cent agar (17) and incubated 24 hours at 21° C. The 24-hour broth cultures were used to inoculate fresh agar slants which were incubated 24 hours at 21° C. before the cultures were returned to cold storage. Working cultures were maintained in tubes of phosphate agar broth. These were stored with the stock cultures at 3 to 5° C. and were transferred weekly, followed by 24 hours incubation at 21° C. before being placed in cold storage.

All skimmilk sample inocula were prepared by transferring a loopful of a working culture to fresh phosphate agar broth and incubating it 24 hours at 21° C. A loopful of the 24-hour broth culture was inoculated into each of two screw-cap culture tubes containing 10 ml. of skimmilk which had been heated 13 minutes in an autoclave at 15 p.s.i. pressure. The inoculated tubes of skimmilk were held in a 5° C. refrigerator and on the sixth day were used to inoculate a trial sample of pasteurized skimmilk.

The periodic examinations made at 14-day intervals on the duplicate trial samples included enumeration of contained bacterial populations, organoleptic evaluation of the milk and paper electrophoretic analyses of the milk proteins.

Two general bacterial populations were enumerated. These were differentiated on the basis of growth temperature and are referred to as total count and psychrophilic count. The total count included those bacteria which produced visible colonies in M-PH agar plates within

45 to 51 hours incubation at 32° C. while the psychrophilic count included those bacteria which had produced visible colonies in M-PH agar plates on the seventh day of incubation at 5° C. The enumerations were made essentially according to officially recognized procedures (1), except that the gelatin-phosphate dilution medium of Naylor and Smith (51) was substituted for the phosphate buffered distilled water normally used for sample dilutions.

The organoleptic evaluations were made by an experienced milk judge assisted at times by one or two others. The appearance and intensity of odor and/or flavor of the samples were recorded as given by the judge with no attempt made to assign numerical scores.

The milk proteins were obtained for electrophoretic analysis by precipitating the casein at pH 4.6 with the dropwise addition of 42.5 per cent lactic acid to 200 ml. of milk under vigorous agitation. The precipitate and whey were separated by centrifugation in 250-ml. flasks followed by decantation of the supernatant whey. The centrifugations were for thirty minutes at 2500 r.p.m. using a 4-place centrifuge head 7 inches in diameter. The recovered precipitate was twice washed by resuspension in 50 ml. of distilled water at 4 to 6° C., as suggested by MacRae and Baker (42), followed by vacuum filtration through Whatmann No. 1 paper. The washed casein was resuspended in a minimum quantity of distilled water, then freeze-dried to yield a fine, white powder containing approximately 14 per cent nitrogen. The water resuspensions of casein were made using a laboratory homogenizer. All freeze-drying was done with an apparatus usually operated at a

pressure of 20 to 50 microns of mercury as measured with a McLeod type gauge.

Immediately after decantation from the casein the whey was filtered through a Seitz filter to remove bacteria and any remaining particles of undissolved precipitate. This yielded a clear, greenish-yellow fluid. A 150-ml. quantity of the whey was then placed in cellophane dialyzer tubing of 1 1/8 inches in diameter and dialyzed 44 hours at 4 to 6° C. against six liters of distilled water, which was refreshed three times at 12-hour intervals. The pH of the whey was determined both before and after dialysis. A 100-ml. quantity of the dialyzed whey was freeze-dried to yield a product containing approximately 12 per cent nitrogen.

All nitrogen contents were determined by a micro-Kjeldahl method (2). A steam-distillation apparatus required for these determinations was constructed in the laboratory. Duplicate determinations on each sample were usually within two-tenths of 1 per cent and were averaged to arrive at the nitrogen content subsequently used in protein suspension standardization calculations.

The proteins were prepared for electrophoretic analysis by resuspending the dried materials in suitable buffers at concentrations of 1 per cent protein nitrogen. A barbiturate buffer, containing 3.7 g. of barbital (barbituric acid derivative) and 20.5 g. of sodium barbital (sodium diethylbarbituate) per liter, of pH 8.6 and ionic strength of 0.1 was used for both casein and whey protein analyses until it was learned that a borate buffer, containing 4.96 g. of

boric acid and 4.0 g. of sodium hydroxide per liter, of pH 11.5 and ionic strength of 0.1 permitted improved resolution of the casein fractions. The borate buffer was employed for the casein analyses during the latter two-thirds of the study, including four of the six trials. Whey protein analyses were made in the barbiturate buffer throughout the study.

The electrophoretic analyses were made using a Spinco Model R Paper Electrophoresis System. This apparatus was equipped with one electrophoretic cell which analyzed eight strips per run. The strips were of Schleicher and Schull 2043A mgl paper. A 0.01-ml. quantity of a buffer suspension of protein from the control and inoculated sample was applied to each of four strips during an electrophoretic run. The electrophoretic separations were allowed to continue for 16 hours at 4 to 6° C., the cell being operated in a room held at this temperature. A constant voltage was applied to the strips. The caseins were separated at 140 v. with a mean initial current of 5.8 milliamperes (ma.) which increased to a mean terminal current of 7.0 ma. The whey proteins were separated at 200 v. with a mean initial current of 7.0 ma. which increased to a mean terminal current of 8.9 ma.

At the end of the electrophoretic runs the strips were prepared for scanning by a modified Spinco procedure for blood serum analysis (75). A stepwise schedule of the procedure used follows:

1. The separated protein on the strips was denatured and bound in place by heating for 30 minutes in an oven at 120 to 130° C.

2. The strips were given two 15-minute quiescent rinses in methanol.

3. They were then stained during a 1-hour immersion in a 0.1 per cent solution of bromphenol blue in methanol.

4. The stained strips were given three successive 5-minute rinses in fresh aqueous solutions of 5 per cent acetic acid.

5. The rinsed strips were gently blotted between sheets of clean blotting paper.

6. The blotted strips were heated for 15 minutes in an oven at 120 to 130° C.

7. Within 1 hour of scanning the strips were placed in an ammonia-saturated atmosphere for a 15-minute period.

The scanning of the strips in the Analytrol yielded characteristic curves from which the relative quantities of the separated protein fractions were derived. The curve peaks representing different protein fractions were delimited by sketching in their Gaussian distribution curves, then carefully drawing vertical lines perpendicular to the base line and extending through the points of intersection of the Gaussian curves (75). The vertical lines were also extended downward through the integrator trace line drawn at the bottom of the curve sheet. The quantitative data for each delimited faction were then obtained from the integrator trace line which recorded the curve area in 0.1-sq. cm. units.

No corrections were attempted for possible differences in the dye-binding capacities of the various fractions, or for the tailing effect of the more rapidly-migrating components (5).

The various protein fractions were not identified during the study and are therefore referred to by number in the text. However, the nomenclature of Jenness et al. (29) may be applied, as the electrophoretic curves obtained were quite similar to those found in the literature (33, 34, 46, 70, 86).

RESULTS AND DISCUSSION

The microbial growth which occurred in both the control and the inoculated samples throughout the 42-day storage period is represented by the data in Table 1.

The control samples showed little or no psychrophilic growth. In only one trial with P. fluorescens did a measurable increase in the psychrophilic colony count occur, and that was on the final day of the storage period. There was no good explanation for this. However, in every trial the total count tended to increase from about the midpoint to the end of the hold period. Perhaps some pasteurization-resistant organisms became acclimated to the low temperature environment with extended holding and eventually began to multiply. Davis (14) believed that this could happen. However, it did not appear likely in any trial that the mesophilic growth, as determined by the total counts, was sufficient to alter the control milk constituents to a degree measurable by the methods used in the study.

Good growth was obtained in all trials with the three pure cultures studied. This is indicated by the marked increases in the average total and psychrophilic counts on the inoculated samples. Generally, these increases occurred throughout the storage period, not being confined to any particular part of it. In the samples inoculated with P. fragi and P. putrefaciens the average psychrophilic count always exceeded the concurrent average total count. Whereas, the reverse held true for all samples inoculated with P. fluorescens. One explanation

TABLE 1

AVERAGE TOTAL COUNTS (TC) AND PSYCHROPHILIC COUNTS (PC) ON CONTROL AND INOCULATED SAMPLES OF SKIMMILK STORED AT 3 TO 5° C.

Culture	Sample	Days of Storage: 0 TC[a]	0 PC[b]	14 TC	14 PC	28 TC	28 PC	42 TC	42 PC
		(Per Milliliter[c])							
Pseudomonas fragi	C[d]	280.0	<10.0	220.0	<10.0	5.2T	<10.0	11.0T	<10.0
	I[e]	150.0T[f]	420.0T	6.3M[g]	190.0M	14.0M	350.0M	71.0M	850.0M
Pseudomonas fluorescens	C	200.0	<10.0	360.0	<10.0	1.1T	<10.0	110.0T	20.0T
	I	43.0T	29.0T	15.0T	9.4M	320.0M	79.0M	140.0M	95.0M
Pseudomonas putrefaciens	C	75.0	<10.0	70.0	<10.0	1.8T	<10.0	2.3T	<10.0
	I	4.5T	20.0T	20.0T	2.8M	2.5M	9.8M	10.0M	44.0M

[a]Plates incubated 45 to 51 hours at 32° C.

[b]Plates incubated 7 days at 5° C.

[c]Two trial average.

[d]Control sample.

[e]Inoculated sample.

[f]Thousands.

[g]Millions.

for this is that this particular culture, P. fluorescens, may have had a less psychrophilic cultural tendency.

Quantitative data obtained by the paper electrophoretic analysis of the proteins in high quality pasteurized skimmilk are given in Table 2. Typical paper electrophoretic patterns, from which these data were obtained, are illustrated in Figure 1. The data in Table 2 include the means of all determinations of the relative per cent of each protein fraction in all control samples of skimmilk used in the study. At this point, a consideration of these results in relation to those of similar work reported in the literature may add to the general understanding of the overall results obtained from this study.

Of three reports (38, 42, 88) on the paper electrophoretic analysis of whole casein from normal milk, the quantitative results obtained here (Table 1) agreed closely only with those reported by MacRae and Baker (42). However, one apparent difference was observed. These workers obtained a definite anodic migration of gamma-casein, corresponding to casein fraction one (Table 2 and Figure 1), which did not occur in this study. A possible explanation for this lies in the differences in the apparatuses used. The electrophoretic cell used by MacRae and Baker (42) held only one 8- by 16-inch sheet of filter paper in a horizontal position surrounded by a minimum of ambient atmosphere to be stabilized. The cell used in this study held 10, 3- by 30-cm. strips suspended over a ridgepole with a relatively much greater ambient atmosphere to stabilize per sq. cm. of paper. It is possible that a more accelerated evaporation-diffusion phenomenon occurred in the latter cell, which

TABLE 2

MEANS OF THE RELATIVE PER CENT OF EACH PROTEIN FRACTION IN SIX CONTROL SAMPLES OF SKIMMILK AS DETERMINED PAPER ELECTROPHORECTICALLY FOUR TIMES AT 14-DAY INTERVALS

Casein Fractions		
1	2	3
(Per Cent)		
9.34	29.53	61.13

Whey Protein Fractions					
S	1	2	3	4	5
(Per Cent)					
18.88	5.86	19.76	9.49	44.28	1.73

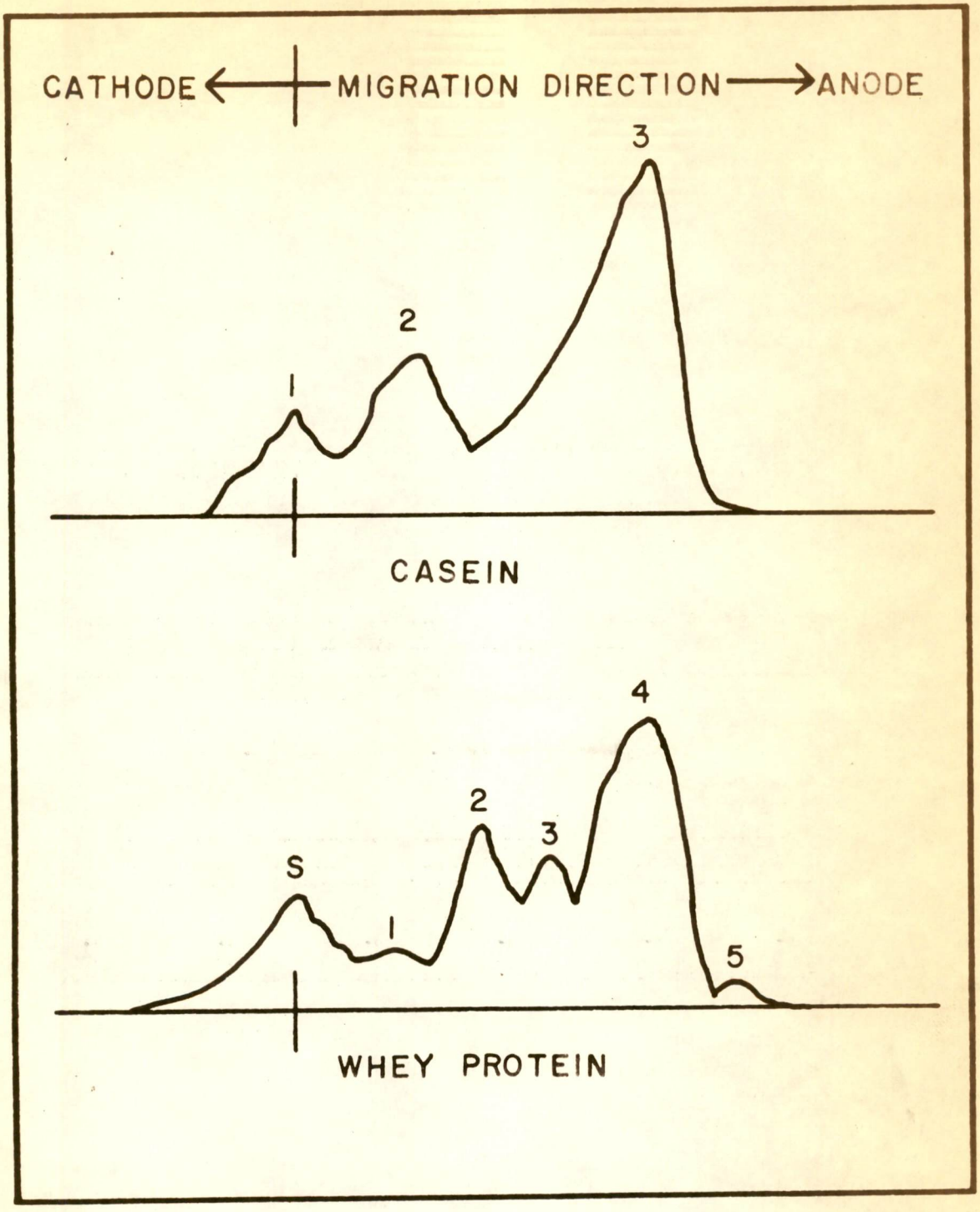


Figure 1. Typical paper electrophoretic patterns of the proteins from high quality pasteurized skimmilk.

counteracted the mobility of the slow-moving casein fraction to the extent that it was unable to undergo definite anodic migration and, therefore, remained essentially at the point of sample application. The higher pH and ionic strength of the buffer used in the present study also may have contributed to this result.

The results obtained from the analysis of whey proteins (Table 2 and Figure 1) agreed reasonably well with the corresponding results reported in the literature. The relative values given in Table 2 for whey protein fractions S, two, four, and five correspond, respectively, to the values for immune globulins, alpha-lactalbumin, beta-lactoglobulin and blood-serum albumin reported by Lecce and Legates (36). Also, they correspond to fractions 4 and 5, Immuneglobulin; 3 alpha-lactalbumin; 2, beta-lactoglobulin; and, 1, Serumalbumin as reported by Weigt (86). The whey protein paper electrophoretic patterns of the control samples in this study differed from those reported by the above workers mainly in the number of appearing fractions. Consistently, six protein fractions were clearly resolved from the control sample wheys in this study while the above workers reported the consistent appearance of only four. However, Lecce and Legates (36) occasionally observed a trace amount of a fraction migrating faster than blood-serum albumin and another fraction migrating between beta-lactoglobulin and alpha-lactalbumin but reported no pertinent quantitative data. Whey protein fraction three (Table 2 and Figure 1) corresponds to the latter occasionally appearing fraction of Lecce and Legates (36). Whey protein fraction one (Table 2 and Figure 1) apparently had no correlative

fraction in the paper electrophoretic patterns as reported by other workers. It is possible that the appearance of whey protein fractions one and three (Table 2 and Figure 1) was due to degradation and interaction products that resulted from the heat treatment of pasteurization upon the original, discrete proteins in the raw milk (34). Also, the whey proteins were analyzed after being dried and resuspended at relatively high concentration in a foreign fluid. This is likely to have affected the paper electrophoretic patterns obtained.

The paper electrophoretic patterns and relative percentages of normal milk protein fractions obtained in this study were somewhat different from others reported in the literature. However, due to differences in sample preparation techniques and electrophoretic apparatuses used, these differences do not necessarily denote fundamental disagreement. That no fundamental disagreement exists may be shown by the proper conversion of the relative per cent of each milk protein fraction, as determined paper electrophoretically by the various workers, to the per cent of the total milk protein it represents. By such conversions it is shown in Table 3 that the quantitative data on normal milk proteins obtained in this study are in line with the milk protein composition data of Jenness et al. (29), Larson and Rolleri (34) and McMeekin (46).

The paper electrophoretic patterns of casein and whey proteins obtained in this study appear susceptible to the application of the nomenclature of Jenness et al. (29) and Larson and Rolleri (34), although the protein fractions separated were not identified.

TABLE 3

THE RELATIVE PER CENT OF EACH PROTEIN FRACTION IN TOTAL MILK PROTEIN AS DETERMINED BY PAPER ELECTROPHORESIS AND MOVING BOUNDRY ELECTROPHORESIS

Milk Proteins						
			Analysis Reported by:			
Fractions						
In This Study	In the Literature: Nomenclature	Reference	This Study	Jenness et al. (29)	McMeekin (46)	Larson and Rolleri (34)
			(%[a])	(Approximate %)	(%)	(%[a])
Casein	Casein	46			80.0	
1	gamma-Casein	29, 46	7.47	3-7	2.4	
2	beta-Casein	29, 46	23.62	19-28	17.6	
3	alpha-Casein	29, 46	48.90	45-63	60.0	
Whey Proteins	Whey Proteins	46			20.0	
S	Immune Globulins	29, 34	3.78	1.4-3.1	2.0	2.6
1	"Proteose-Peptone" 3, or Component III[b]	34	1.17			0.92
		46			3.6	
2	alpha-Lactalbumin	29, 46	3.95	2-5	2.4	3.94
3	"Proteose-Peptone" 5	34	1.90			1.72
4	beta-Lactoglobulin	29, 46	8.86	7-12	11.0	8.54
5	Blood serum albumin	29, 46	0.35	0.7-1.3	1.0	0.94
	"Proteose-Peptone" 8	34				1.14

[a]Values converted from per cent of total casein or per cent of total whey protein to per cent of total protein.

[b]Considered inapplicable to the corresponding fraction in this study.

However, the electrophoretic patterns obtained in this study closely resemble those obtained by moving boundry electrophoresis (33, 34, 46). Therefore, since the nomenclature suggested for use (29, 34) is directly applicable to moving boundry electrophoretic patterns, its application to the patterns obtained in this study seems tenable. This being the case, casein fractions one, two and three (Figure 1) correspond, respectively, to alpha-, beta- and gamma-casein. And, whey protein fractions S, two, four and five correspond, respectively, to immune globulins, alpha-lactalbumin, beta-lactoglobulin and "blood" serum albumin while one and three are, as yet, unamed component members of the "proteose-peptone" fraction. These relationships are given in Table 3.

The data in Table 4 show the deviations from the controls obtained by the paper electrophoretic analyses of total casein in the control samples at the indicated storage intervals. The data in Table 5 are a similar treatment of the results of the whey protein analyses. Theoretically, it may be assumed that there was no difference between either the total casein or total whey protein of the control samples and corresponding inoculated samples when analyzed on the day of inoculation. The data show that apparent differences did exist. These differences reflect the volumetric difficulties inherent in the necessary handling of the proteins in so many different ways preceding the actual paper electrophoretic analysis. Since similar sample preparation difficulties have been reported (34, 40) they do not appear to be peculiar to this particular study. However, the mean

TABLE 4

AVERAGE PER CENT OF DEVIATION FROM THE CONTROLS FOR TOTAL CASEIN IN SKIMMILKS INOCULATED WITH PURE CULTURE BACTERIA AND SUBSEQUENTLY STORED AT 3 TO 5° C.

Days of Storage	Culture		
	Pseudomonas fragi	Pseudomonas fluorescens	Pseudomonas putrefaciens
	(Per Cent[a])		
0	8.76	0.75	2.78
14	-2.40	10.29	1.44
28	1.76	-0.20	4.82
42	-0.61	4.53	18.50

[a]Two trial average.

TABLE 5

AVERAGE PER CENT OF DEVIATION FROM THE CONTROLS FOR TOTAL WHEY PROTEIN IN SKIMMILKS INOCULATED WITH PURE CULTURE BACTERIA AND SUBSEQUENTLY STORED AT 3 TO 5° C.

Days of Storage	Culture		
	Pseudomonas fragi	Pseudomonas fluorescens	Pseudomonas putrefaciens
	(Per Cent[a])		
0	2.11	5.67	5.39
14	-3.16	0.85	-17.71
28	-1.48	0.08	-36.73
42	-5.32	-9.21	-40.66

[a]Two trial average.

of the differences in the initial casein analyses on the control and inoculated samples was 3.8 per cent, while this value for the whey protein analyses was 4.4 per cent. These values indicate that reasonably good reproducibility was obtained in the paper electrophoretic analysis of proteins from duplicate samples of skimmilk. Also, it should be stated that the minor differences obtained in the analyses of total proteins from duplicate samples are unlikely to be reflected in the relative per cent of each protein fraction.

The data in Table 4 indicate a slight decrease in total casein with the growth of P. fragi and apparent increases with the growth of P. fluorescens and P. putrefaciens at the end of the 42-day storage period. These data are misleading. Actually, average decreases in total casein occurred with the growth of all three cultures but these decreases were obscured in the analytical results due to the fact that all protein suspensions were standardized at a concentration of 1 per cent protein nitrogen before electrophoretic analysis. The apparent increases in total casein may have been caused by a greater dye-binding capacity on the part of the remaining protein.

In contrast to the results for the casein analyses, the whey protein data in Table 5 indicate that definite decreases in total whey protein had occurred by the end of the 42-day storage period, despite the fact that the whey protein suspensions were also standardized at a 1 per cent protein nitrogen content before electrophoretic analysis. This strongly suggests that not all of the nitrogen recovered in the freeze-dried whey from the inoculated samples represented protein with

the dye-binding capacity necessary to paper electrophoretic analysis. It is noted that P. fragi, P. fluorescens and P. putrefaciens effected decreases in total whey protein in that order of increasing amounts.

Quantitative data are given in Table 6 on the changes in casein and in Table 7 on the changes in whey proteins with the growth of P. fragi. These changes are illustrated in the characteristic paper electrophoretic curves in Figures 2 and 3. For casein, a marked decrease in fraction two is indicated along with a less marked decrease in fraction one. The total decrease in these fractions is balanced by a near corresponding increase in fraction three. This compensating effect, a result of sample standardization, was apparent in all casein analyses in this study.

A similar compensating effect also occurred in the whey protein analyses. The data in Table 7 indicate that sizeable decreases were effected in fractions two and three with the most marked decrease being in fraction three. The decrease in fraction four, though of nearly the same magnitude as that in fraction three, is not nearly as marked as that in fraction two and three. Fraction four was reduced only by about one-fifteenth its original content while fraction two was reduced by one-fifth and fraction three by more than one-half. Fraction "A" was an atypical fraction of cathodic migration which appeared only on the final day of analysis. This fraction may have been composed of peptides resulting from protein degradation. Peptides containing a predominance of basic amino-acids would be expected to migrate towards the cathode. Lindquist et al. (40) found that similar fractions appeared in cheese during ripening.

TABLE 6

AVERAGE DEVIATION FROM THE CONTROLS IN THE MEAN RELATIVE PER CENT OF EACH CASEIN FRACTION IN SKIMMILKS INOCULATED WITH PSEUDOMONAS FRAGI AND SUBSEQUENTLY STORED AT 3 TO 5° C.

Days of Storage	Casein Fractions		
	1	2	3
	(Per Cent[a])		
0	0.09	-0.57	0.48
14	0.40	-0.24	-0.16
28	1.05	-9.61	8.56
42	-1.79	-7.84	9.62

[a]Two trial average.

TABLE 7

AVERAGE DEVIATION FROM THE CONTROLS IN THE MEAN RELATIVE PER CENT OF EACH WHEY PROTEIN FRACTION IN SKIMMILKS INOCULATED WITH PSEUDOMONAS FRAGI AND SUBSEQUENTLY STORED AT 3 TO 5° C.

Days of Storage	Whey Protein Fractions						
	"A"	S	1	2	3	4	5
	(Per Cent[a])						
0		-0.65	0.16	-0.55	0.48	0.25	0.33
14		0.83	0.17	0.62	-0.19	-0.88	-0.55
28		3.74	0.43	-1.98	-2.19	-0.23	0.24
42	11.15	0.71	-0.44	-3.48	-5.60	-3.07	0.73

[a]Two trial average.

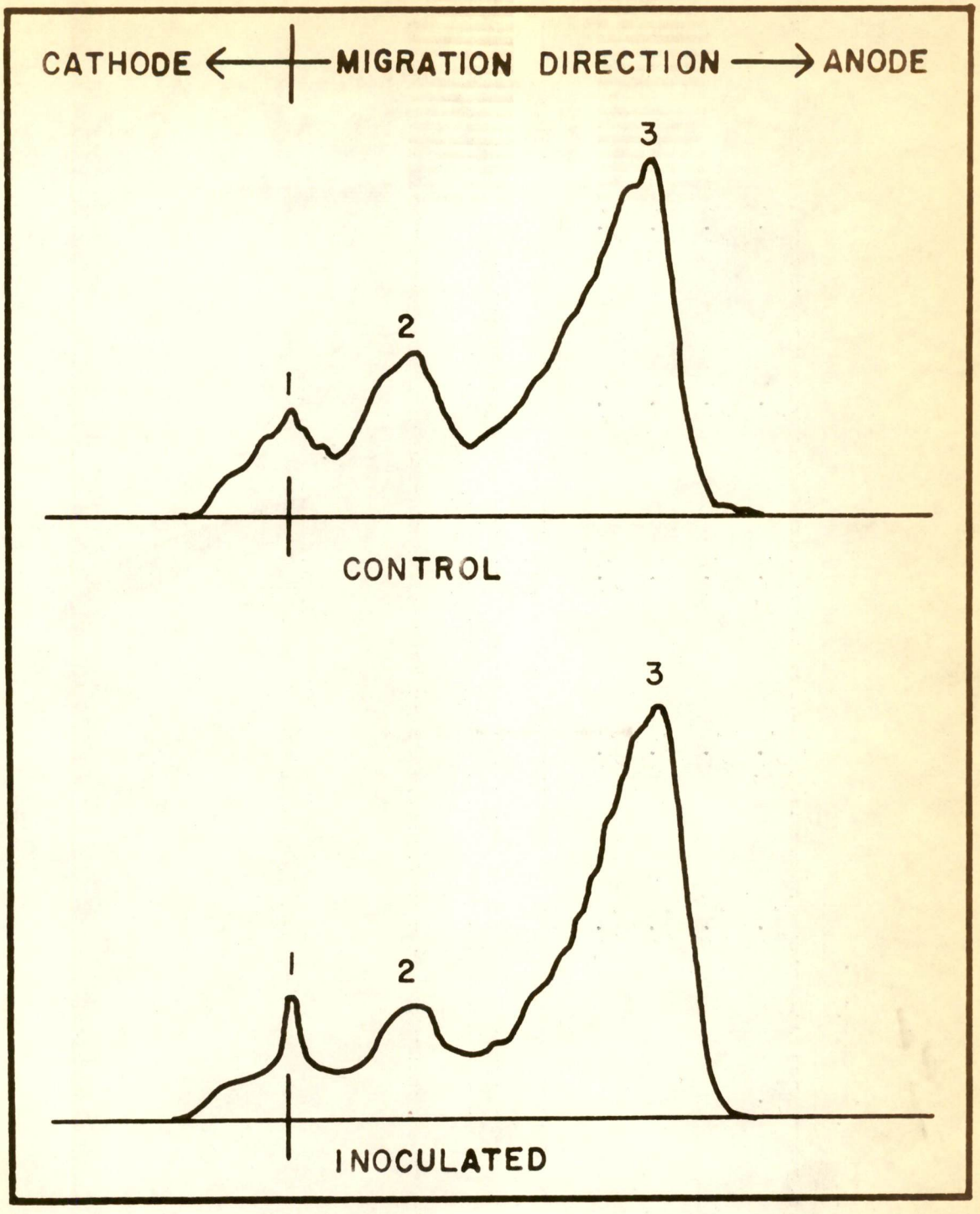


Figure 2. Representative paper electrophoretic patterns of casein from samples of skimmilk at the end of the storage period in trials with P. fragi.

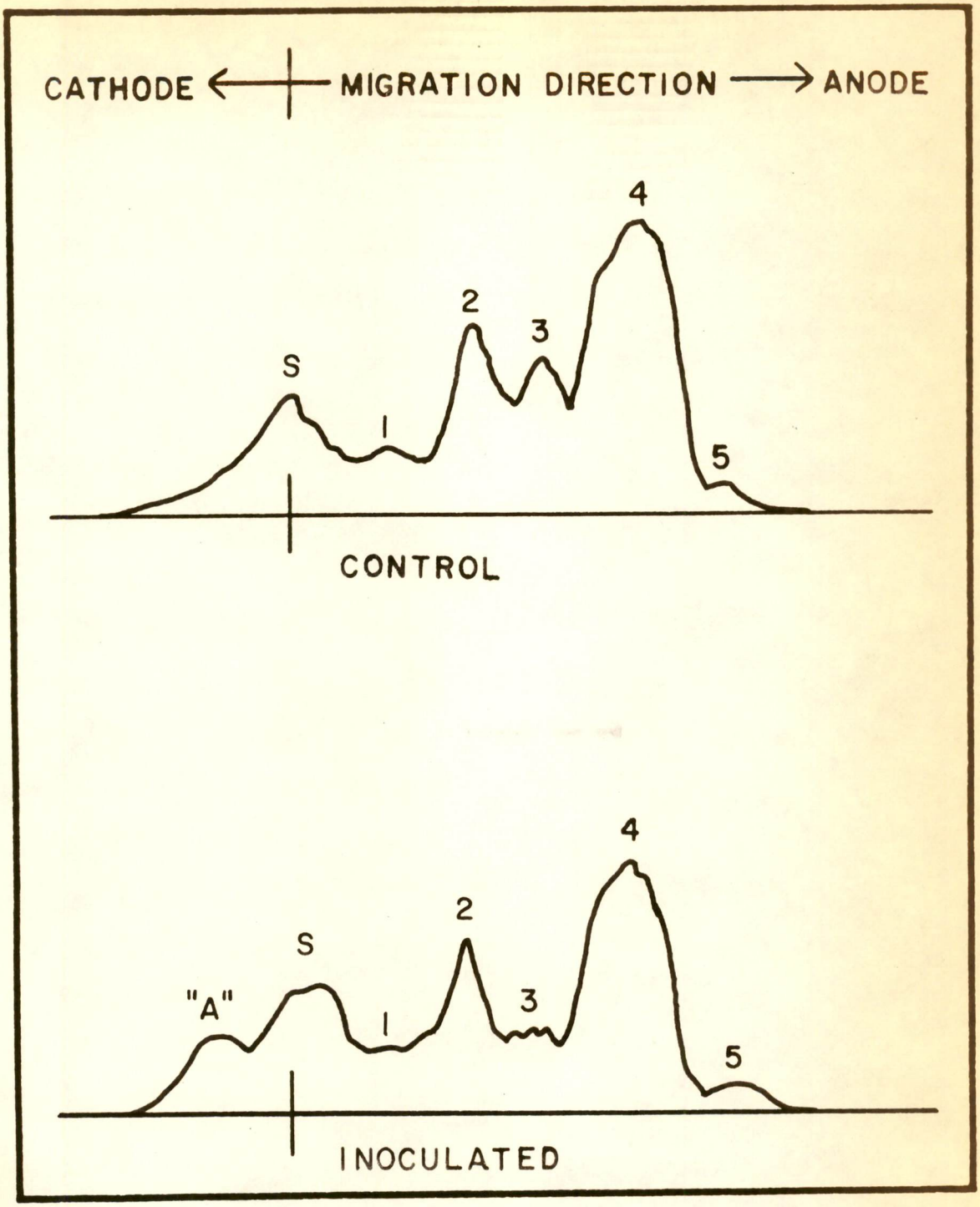


Figure 3. Representative paper electrophoretic patterns of whey proteins from samples of skimmilk at the end of the storage period in trials with P. fragi.

The quantitative data on the changes in milk proteins with the growth of P. fluorescens are given for casein in Table 8 and for whey proteins in Table 9. Figures 4 and 5 illustrate these changes. The changes accompanying the growth of this organism were very similar to those obtained with P. fragi, except that no atypical fraction appeared in the whey protein. The same casein and whey protein fractions were decreased but to a lesser degree.

The quantitative data on the changes in milk proteins with the growth of P. putrefaciens are given for casein in Table 10 and for whey proteins in Table 11. Figures 6 and 7 illustrate these changes. These data show that more marked changes in the milk proteins were effected in a shorter period of time with this organism than with the other two studied. A greater reduction in casein fractions one and two than with the other organisms is indicated. A much greater loss in total whey protein is shown by the data in Table 5. This loss is illustrated by the difference in the total area enclosed by the characteristic curves of the control and the inoculated samples (Figure 7). The atypical fraction "B" of anodic migration (Table 11 and Figure 7) appeared on the fourteenth-day analysis and was present at the end of the storage period. Fractions in this general location have been observed by other workers (33, 34, 36). Since this atypical fraction appeared to be of much greater magnitude than those reported in the literature, it may be that it is composed, at least in part, of protein degradation products. In this case the anodic migration tendency of this fraction could have been caused by a predominance of acidic amino-acids.

TABLE 8

AVERAGE DEVIATION FROM THE CONTROLS IN THE MEAN RELATIVE PER CENT OF EACH CASEIN FRACTION IN SKIMMILKS INOCULATED WITH PSEUDOMONAS FLUORESCENS AND SUBSEQUENTLY STORED AT 3 TO 5° C.

Days of Storage	Casein Fractions		
	1	2	3
	(Per Cent[a])		
0	0.40	-1.11	0.71
14	-0.15	-1.73	1.89
28	2.09	-1.79	-0.35
42	-0.88	-3.77	4.65

[a]Two trial average.

TABLE 9

AVERAGE DEVIATION FROM THE CONTROLS IN THE MEAN RELATIVE PER CENT OF EACH WHEY PROTEIN FRACTION IN SKIMMILKS INOCULATED WITH PSEUDOMONAS FLUORESCENS AND SUBSEQUENTLY STORED AT 3 TO 5° C.

Days of Storage	Whey Protein Fractions					
	S	1	2	3	4	5
	(Per Cent[a])					
0	3.75	-0.49	-2.01	-1.64	-0.17	0.56
14	-0.54	-0.37	-0.40	-0.10	2.21	0.10
28	-0.18	-0.59	-2.34	-3.30	5.88	0.53
42	4.33	-0.36	-3.27	-3.86	1.70	0.75

[a]Two trial average.

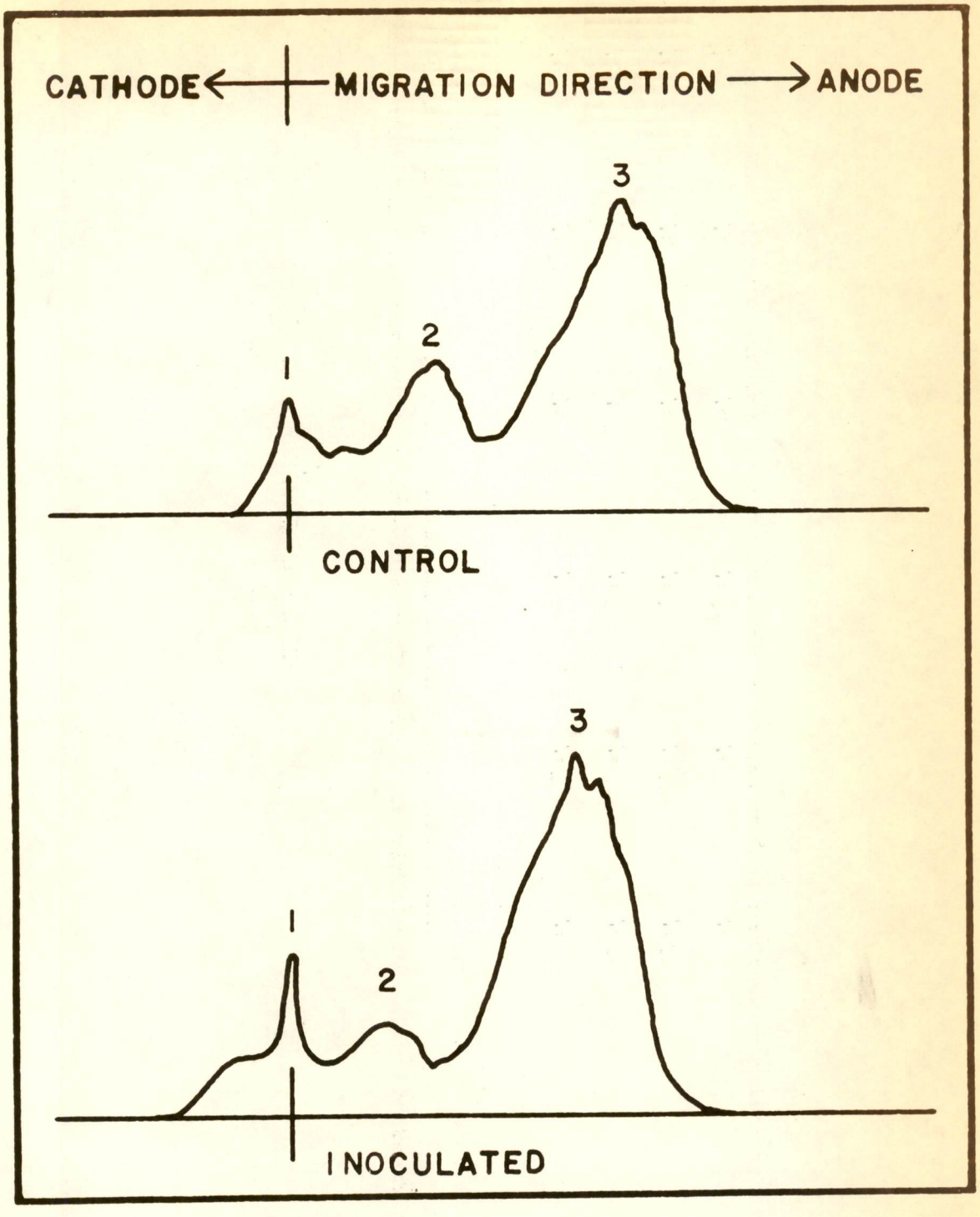


Figure 4. Representative paper electrophoretic patterns of casein from samples of skimmilk at the end of the storage period in trials with P. fluorescens.

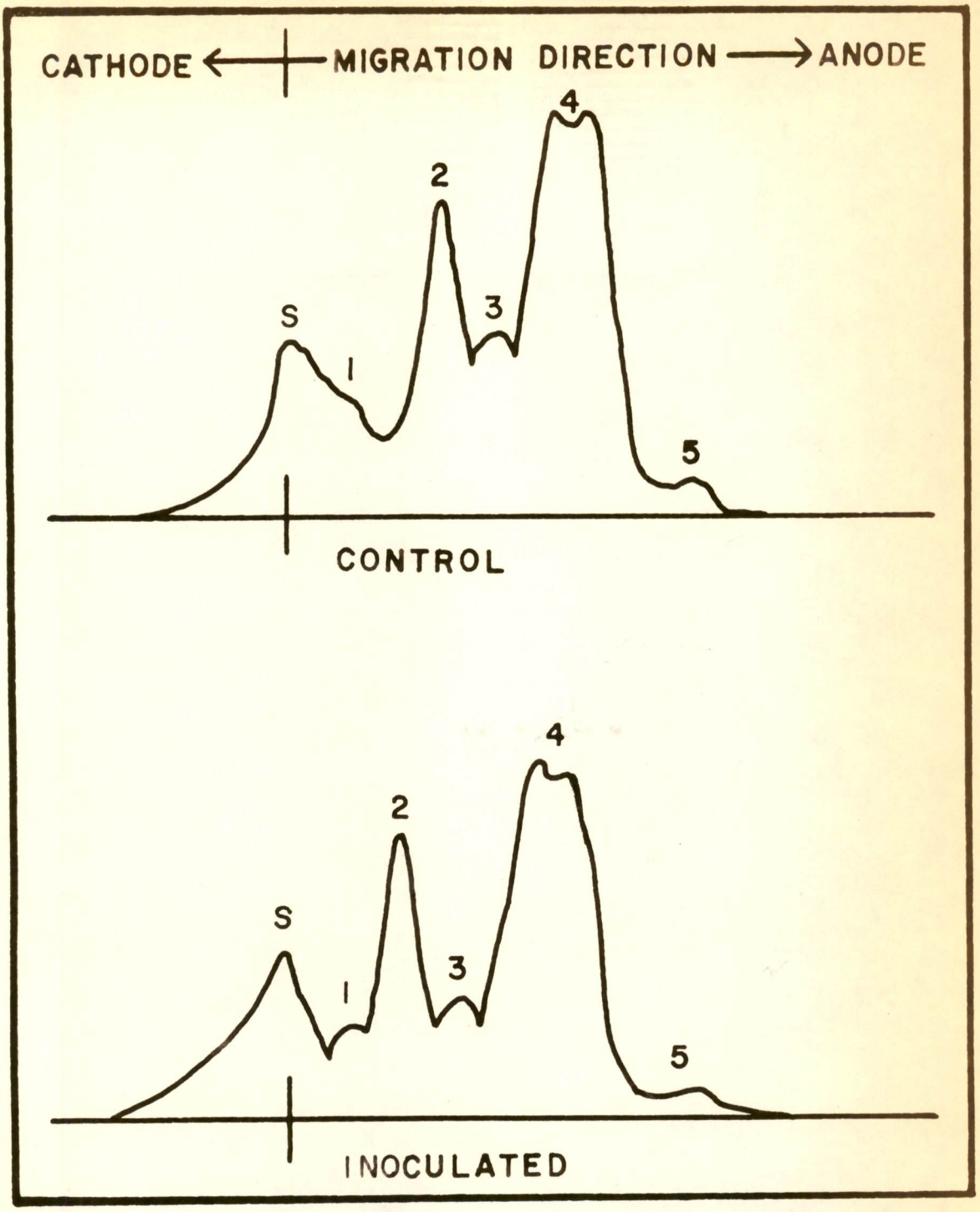


Figure 5. Representative paper electrophoretic patterns of whey proteins from samples of skimmilk at the end of the storage period in trials with P. fluorescens.

TABLE 10

AVERAGE DEVIATION FROM THE CONTROLS IN THE MEAN RELATIVE PER CENT OF EACH CASEIN FRACTION IN SKIMMILKS INOCULATED WITH PSEUDOMONAS PUTREFACIENS AND SUBSEQUENTLY STORED AT 3 TO 5° C.

Days of Storage	Casein Fractions		
	1	2	3
	(Per Cent[a])		
0	0.08	1.59	-1.67
14	1.35	-10.82	9.47
28	-5.02	-17.20	22.22
42	-7.43	-14.08	21.51

[a]Two trial average.

TABLE 11

AVERAGE DEVIATION FROM THE CONTROLS IN THE MEAN RELATIVE PER CENT OF EACH WHEY PROTEIN FRACTION IN SKIMMILKS INOCULATED WITH PSEUDOMONAS PUTREFACIENS AND SUBSEQUENTLY STORED AT 3 TO 5° C.

Days of Storage	Whey Protein Fractions						
	S	1	2	3	4	5	B
	(Per Cent[a])						
0	-1.11	-0.69	-1.70	-0.13	3.35	0.28	
14	6.77	0.54	-2.57	-4.59	-3.80	-0.24	3.88
28	1.02	1.06	-3.44	-6.83	4.11	0.42	3.65
42	0.44	1.72	-3.92	-5.86	4.90	0.56	4.59

[a]Two trial average.

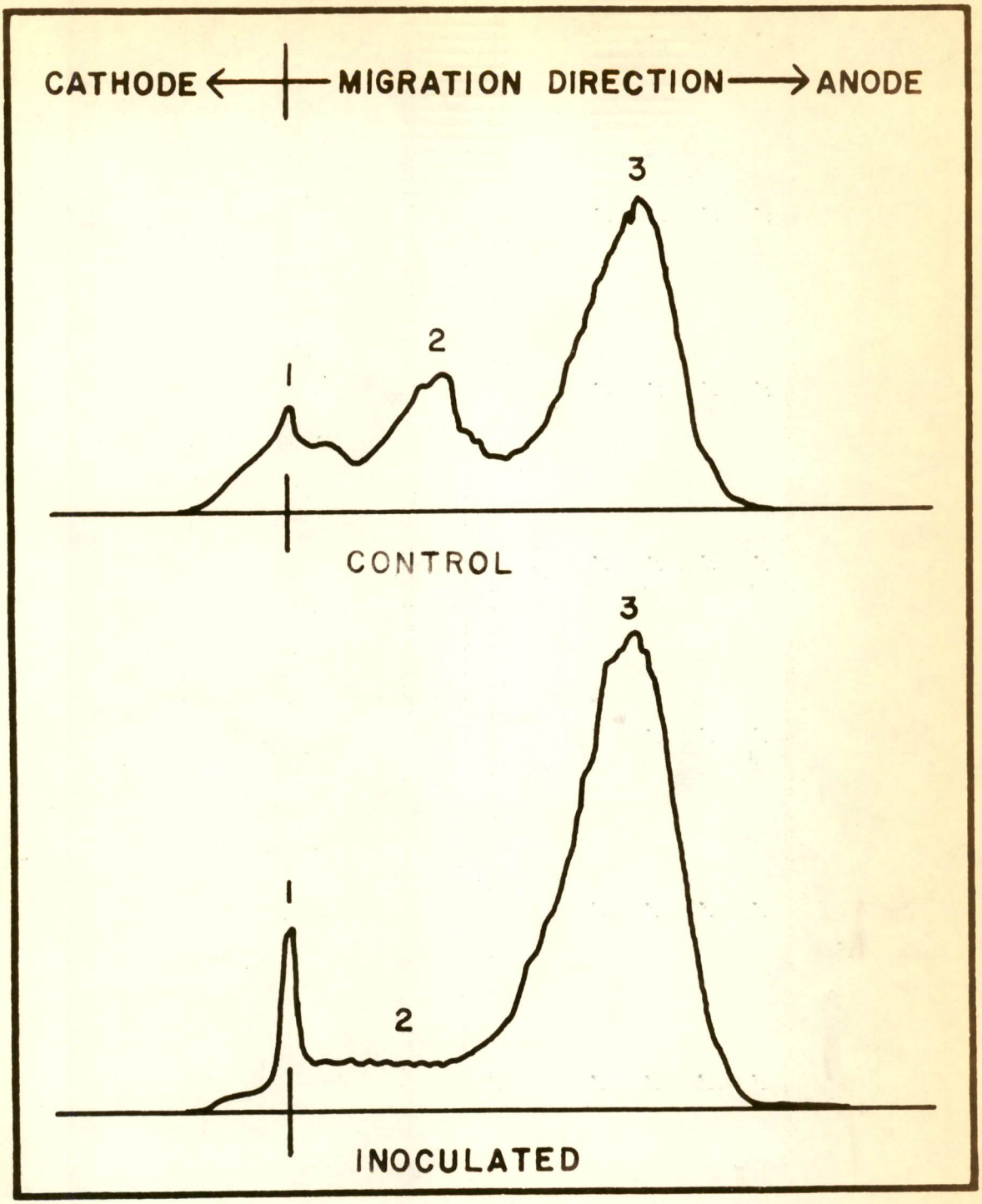


Figure 6. Representative paper electrophoretic patterns of casein from samples of skimmilk at the end of the storage period in trials with P. putrefaciens.

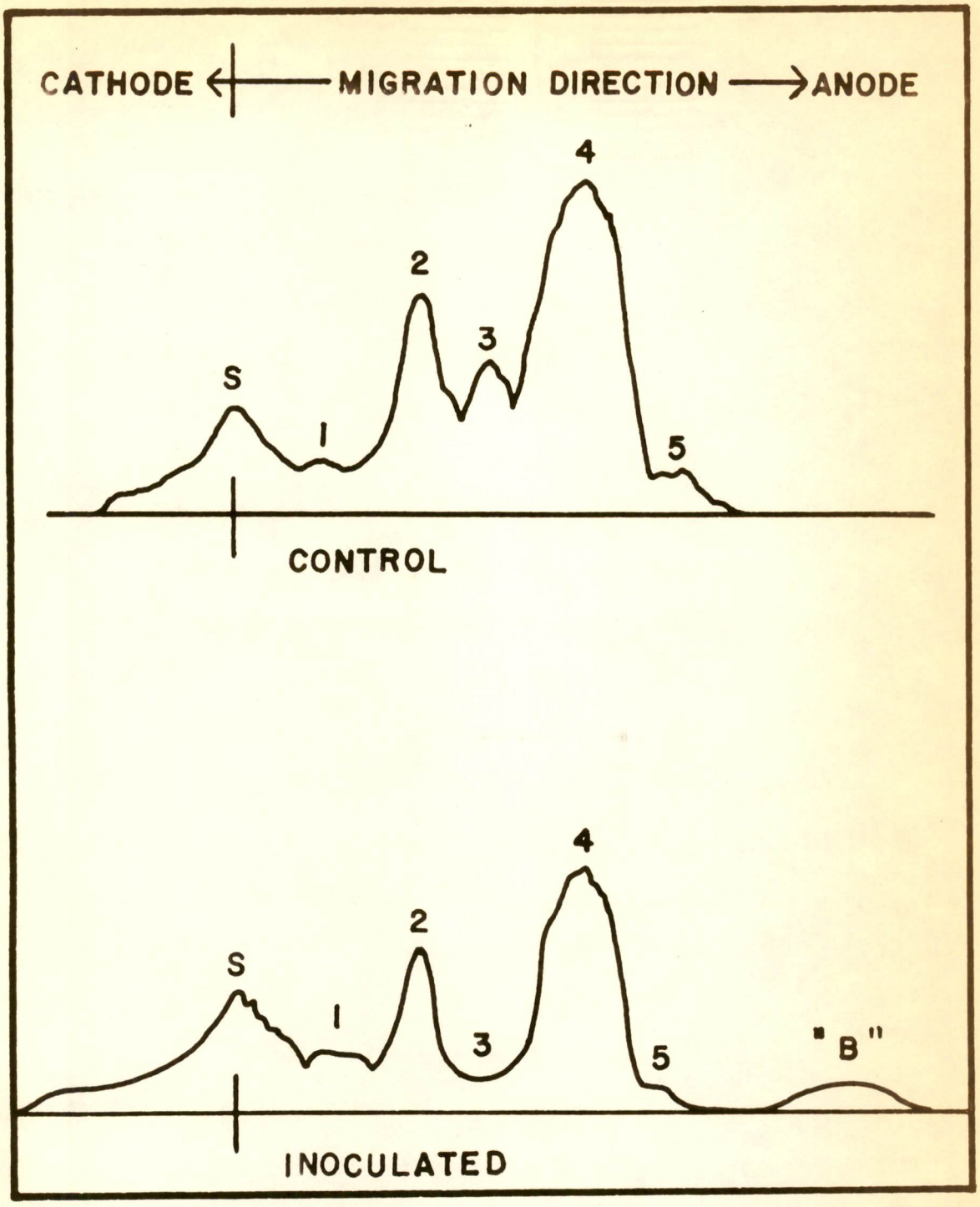


Figure 7. Representative paper electrophoretic patterns of whey proteins from samples of skimmilk at the end of the storage period in trials with P. putrefaciens.

There were changes in the milk proteins which were observed to be common to the growth of the three cultures studied. Perhaps the most outstanding of these was the reduction of casein fraction two which suggested a preferential degradation of that fraction on the part of these bacteria. This was not anticipated, but according to McMeekin (46) the different components of casein may be unequally susceptible to enzymatic hydrolysis.

The second change common to all cultures was the more or less marked decreases in whey protein fraction three. This particular electrophoretic component is a part of the "proteose-peptone" fraction of the whey proteins. The "proteose-peptone" fraction, being composed primarily of simpler protein groupings, might be expected to be utilized by the bacteria more readily than the more complex true protein fractions of the whey proteins.

Thirdly, a sharp peak was always evident in the electrophoretic patterns of casein from inoculated milk after considerable bacterial growth had occurred, usually by the end of the second week of storage. This peak appeared at the point of sample application. Similar peaks have been observed in the patterns of proteins from cheese during ripening (40). In both cases such peaks appeared after the proteins were subjected to bacterial action. It may be that a denaturation took place which caused some of the protein to be so strongly adsorbed to the paper that it underwent no electrophoretic migration.

The appearance of the atypical fractions "A" and "B" might be explained by the work of Pennington (63) who concluded that caseoses,

amino-acids and probably peptones are produced by bacterial action at the expense of the digested casein. It is not unlikely that the production of such materials may result in the formation of distinct electrophoretic fractions or contribute to existing electrophoretic fractions of similar composition.

The data in Tables 12, 13, and 14 summarize the flavors produced in the skimmilk with the growth of proteolytic psychrophiles. A bitter flavor was produced in the inoculated sample in all trials. With P. putrefaciens a putrid odor developed during the latter part of the storage period. The frequent detection of a rancid odor may be explained by the fact that these Pseudomonas species are also lipolytic and the small amount of fat remaining in commercially separated skimmilk, when hydrolyzed, is sufficient to give rancidity.

All inoculated samples were observed to change in color as compared to that of corresponding control samples. This was noticeable by the end of the fourth week of storage. Also, many of the inoculated samples showed a tendency for casein flocculation upon warming to room temperatures. This was inconsistent as to sample and interval of storage except for the two trials with P. putrefaciens. With this organism the casein flocculated by the end of the fourth week of storage without warming the milk to room temperature. These flocculations were not due to isoelectric precipitation of the casein as the pH of the milk was never lowered to that point. It is uncertain as to whether this flocculation resulted from bacterial coagulating enzymes or from denaturation of the casein through degradation to the extent that

TABLE 12

FLAVOR CRITICISMS ASSIGNED TO CONTROL SAMPLES AND SAMPLES OF SKIMMILK INOCULATED WITH *PSEUDOMONAS FRAGI* AND SUBSEQUENTLY STORED AT 3 TO 5° C.

Days of Storage	Control Samples[a]	Inoculated Samples	
		Trial 1	Trial 2
0	Cooked	Cooked	Cooked
14	Cooked	Cooked	Rancid Odor and Flavor
28	Cooked	Fruity Flavor, Rancid Odor	Fruity Flavor, Rancid Odor
42	Cooked	Bitter Flavor, Rancid Odor	Bitter Flavor

[a]Two trials.

TABLE 13

FLAVOR CRITICISMS ASSIGNED TO CONTROL SAMPLES AND SAMPLES OF SKIMMILK INOCULATED WITH PSEUDOMONAS FLUORESCENS AND SUBSEQUENTLY STORED AT 3 TO 5° C.

Days of Storage	Control Samples[a]	Inoculated Samples	
		Trial 1	Trial 2
0	Cooked	Cooked	Cooked
14	Cooked	Slightly Stale	Lacks Freshness
28	Cooked	Slightly Stale	Lacks Freshness
42	Cooked	Extremely Bitter Flavor, Rancid Odor	Bitter Flavor

[a]Two trials.

TABLE 14

FLAVOR CRITICISMS ASSIGNED TO CONTROL SAMPLES AND SAMPLES OF SKIMMILK INOCULATED WITH PSEUDOMONAS PUTREFACIENS AND SUBSEQUENTLY STORED AT 3 TO 5° C.

Days of Storage	Control Samples[a]	Inoculated Samples	
		Trial 1	Trial 2
0	Cooked	Cooked	Cooked
14	Cooked	Bitter Flavor, Rancid Odor	Slightly Bitter
28	Cooked	Extremely Bitter	Extremely Bitter
42	Cooked	Extremely Bitter Flavor, Putrid Odor	Extremely Bitter Flavor, Putrid Odor

[a]Two trials.

its structure no longer permitted it to remain in a completely dispersed phase.

SUMMARY

Samples of high quality pasteurized skimmilk were inoculated with pure cultures of Pseudomonas fragi, Pseudomonas fluorescens and Pseudomonas putrefaciens and subsequently stored at 3 to 5° C. for a period of 42 days. Bacterial growth, protein changes and flavor changes in the milk were studied, respectively, by agar plate counts, paper electrophoretic analysis and organoleptic examination made on the samples at 14-day intervals during the storage period.

Good growth with these bacteria was obtained throughout the storage period in all trials. This was indicated by both the total and psychrophilic counts made concurrently. Low total counts were maintained by the control samples throughout the storage period and a measurable increase in the psychrophilic count occurred in only one control sample during the entire study.

The changes in the milk proteins with the growth of these bacteria involved the total protein content, the relative percentage of the various protein factions and the appearance of electrophoretically different fractions. A preferential degradation of specific protein fractions by these bacteria appeared likely. This was particularly true of casein fraction two, or beta-casein, and whey protein fraction three, a part of the "proteose-peptone" component in milk. An atypical whey protein fraction appeared with the growth of P. fragi and P. putrefaciens. These fractions exhibited opposite electrophoretic migratory tendencies, which indicated they were of different composition.

Though these fractions likely were composed of the degradation products of more complex proteins, the exact sites of their origin were not known.

In most trials bacterial growth was accompanied by a flocculation of the casein which was not due to isoelectric precipitation. This was evident only very near the end of the storage period except with P. putrefaciens, with which it was evident always by the end of the fourth week.

Along with electrophorectically detectable changes in the milk proteins, the growth of all organisms studied was accompanied by the development of a bitter flavor in the milk. This flavor varied both in intensity and in time required for development by the different cultures. With P. putrefaciens putrefaction was evident by the end of the storage period. Also, it was demonstrated by the control samples of skimmilk that pasteurized milk may retain an acceptable organoleptic quality during relatively long periods of time if adequately refrigerated, provided there has been no post-pasteurization contamination with psychrophilic microorganisms.

REFERENCES

REFERENCES

1. American Public Health Association. Standard Methods for the Examination of Dairy Products. 10th ed. New York. 1953.

2. Association of Official Agricultural Chemists. Official Methods of Analysis. p. 745. 7th ed. Washington, D. C. 1950.

3. Atherton, H. F., Doan, F. J., and Watrous, G. H., Jr. Changes in Bacterial Population and Characteristics of Bottled Milk During Refrigerated Holding. Penn. State Coll. Agr. Expt. Sta., Bull 575. 1954.

4. Babel, F. J. Activity of Bacteria and Enzymes in Raw Milk Held at 4.4° C. J. Dairy Sci., 36:562. 1953.

5. Block, R. J., Durrum, E. L., and Zweig, G. A Manual of Paper Chromatography and Paper Electrophoresis. Academic Press Inc., New York. 1955.

6. Boyd, J. C. Sanitation Problems in the Manufacture of Cottage Cheese. J. Milk and Food Technol., 20:157. 1957.

7. Boyd, J. C., Smith, C. K., and Trout, G. M. The Role of Psychrophilic Bacteria in the Keeping Quality of Pasteurized and Homogenized Milk. J. Dairy Sci., 36:571. 1953.

8. Boyd, J. C., Smith, C. K., and Trout, G. M. The Role of Psychrophilic Bacteria in the Keeping Quality of Pasteurized and Homogenized Milk. J. Milk and Food Technol., 18:32. 1955.

9. Burgwald, L. H., and Josephson, D. V. The Effect of Refrigerator Storage on the Keeping Qualities of Pasteurized Milk. J. Dairy Sci., 30:371. 1947.

10. Claydon, T. J. and Hammer, B. W. A Skunk-Like Odor of Bacterial Origin in Butter. J. Bacteriol., 37:251. 1939.

11. Collins, E. B. Factors Involved in the Control of Gelatinous Curd Defects of Cottage Cheese. I. Storage Temperature and pH. J. Milk and Food Technol., 18:169. 1955.

12. Corley, R. T., Long, H. F., and Hammer, B. W. Water Supplies of Butter Manufacturing Plants. Iowa Agr. Expt. Sta., Res. Bull. 319. 1943.

13. Dahlberg, A. C. The Relationship of the Growth of All Bacteria and Coliform Bacteria in Pasteurized Milk Held at Refrigeration Temperatures. J. Dairy Sci., 29:651. 1946.

14. Davis, J. G. The Effect of Cold on Micro-Organisms in Relation to Dairying. Proc. Soc. for App. Bacteriol., 14:216. 1951.

15. Department of Dairy Husbandry. Fortieth Annual Report, Ind. Agr. Expt. Sta. p. 33. 1927.

16. Derby, H. A.,and Hammer, B. W. Bacteriology of Butter. IV. Bacteriological Studies on Surface-Taint Butter. Iowa Agr. Expt. Sta., Research Bull. 145. 1931.

17. Difco Laboratories. Difco Manual. 9th ed. Detroit. 1953.

18. Durrum, E. L. A Microelectrophoretic and Microionophoretic Technique. J. Am. Chem. Soc., 72:2943. 1950.

19. Eckles, C. H. A Bacteriological Study of the College Creamery Milk Supply. Iowa Agr. Expt. Sta. Bull. 59. 1901.

20. Elliker, P. R. Psychrophiles as Affecting Milk, Cream, and Cottage Cheese. Milk Industry Foundation Convention Proceedings. 4:53. 1953.

21. Erdman, I. E.,and Thornton, H. R. Psychrophilic Bacteria in Edmonton Milk and Cream. I. Numbers. Can. J. Technol., 29:238. 1951.

22. Foster, E. M., Nelson, F. E., Speck, M. L., Doetsch, R. N., Olson, J. C., Jr. Dairy Microbiology. Prentice-Hall, Inc., Englewood Cliffs. 1957.

23. Hadfield, W. A. Cold-Loving Bacteria. How to Control Them. Southern Dairy Prod. J., 59, 6:30. 1956.

24. Hammer, B. W., and Babel, F. J. Dairy Bacteriology. 4th ed. John Wiley and Sons, Inc., New York. 1957.

25. Hammer, B. W., and Olson, H. C. Phosphatase Production in Dairy Products by Microorganisms. J. Milk Technol., 4:83. 1941.

26. Harmon, L. G. Bacteriological Condition of Water Supplies in Dairy Plants. J. Milk and Food Technol., 20:196. 1957.

27. Hunziker, O. F., Spitzer, G., Mills, H. C., and Switzer, H. B. The Pasteurization of Sour, Farm-Skimmed Cream for Buttermaking. Ind. Agr. Expt. Sta. Bull. 208. 1917.

28. Hussong, R. V., Long, H. F., and Hammer, B. W. Classification of the Organisms Important in Dairy Products. II. Pseudomonas fragi. Iowa Agr. Expt. Sta. Res. Bull. 225. 1937.

29. Jenness, R., Larson, B. L., McMeekin, T. L., Swanson, A. M., Whitnah, C. H., and Whitney, R. McL. Nomenclature of the Proteins of Bovine Milk. J. Dairy Sci., 39:536. 1956.

30. Jezeski, J. J., and Macy, H. Cryophilic Organisms in Water and Butter. J. Dairy Sci., 29:439. 1946.

31. Kaufmann, O. W., and Andrews, R. H. The Destruction Rate of Psychrophilic Bacteria in Skimmilk. J. Dairy Sci., 37:317. 1954.

32. Kennedy, L., and Weiser, H. Some Observations on Bacteria Isolated from Milk That Grow Within a Psychrophilic Temperature Range. J. Milk and Food Technol., 13:353. 1950.

33. Larson, B. L., and Kendall, K. A. Protein Production in the Bovine. Daily Production of the Specific Milk Proteins During the Lactation Period. J. Dairy Sci., 40:377. 1957.

34. Larson, B. L., and Rolleri, G. D. Heat Denaturation of the Specific Serum Proteins in Milk. J. Dairy Sci., 38:351. 1955.

35. Lawton, W. C., and Nelson, F. E. The Effect of Storage Temperatures on the Growth of Psychrophilic Organisms in Sterile and Laboratory Pasteurized Skimmilks. J. Dairy Sci., 37:1164. 1954.

36. Lecce, J. G., and Legates, J. E. Changes in the Paper Electrophoretic Whey-Protein Pattern of Cows With Acute Mastitis. J. Dairy Sci., 42:698. 1959.

37. Lederer, M. An Introduction to Paper Electrophoresis and Related Methods. 2nd. impression. Elsevier Publishing Company, New York, 1957.

38. Libbey, L. M., and Ashworth, U. S. Paper Electrophoresis of Casein. J. Dairy Sci., 41:228. 1958.

39. Lindquist, B. Electrophoresis As an Aid in Cheese Investigations. Svenska Mejeritidn., 45:169. 1953. Chem. Abs., 48:13118. 1954.

40. Lindquist, B., Storgards, T., and Goransson, Maj.-Britt. Paper Electrophoresis for Studying the Ripening Process in Cheese. Proc. 13th Intern. Dairy Congr., 3:1261. 1953.

41. Long, H. F., and Hammer, B. W. Classification of the Organisms Important in Dairy Products. III. Pseudomonas putrefaciens. Iowa Agr. Expt. Sta. Res. Bull. 285. 1941.

42. MacRae, H. F., and Baker, B. E. Application of Electrophoresis on Paper to the Estimation of Alpha-, Beta-, and Gamma-Casein. J. Dairy Sci., 41:233. 1958.

43. Marth, E. H., and Frazier, W. C. Bacteriology of Milk Held at Farm Bulk Cooling Tank Temperatures. I. Effect of Time and Temperature During Storage, Farm Source of Milk and Season of Year. J. Milk and Food Technol., 20:72. 1957.

44. Marth, E. H., and Frazier, W. C. Bacteriology of Milk Held at Farm Bulk Cooling Tank Temperatures. II. Effect of Numbers of Bacteria in the Original Milk. J. Milk and Food Technol., 20:79. 1957.

45. Marth, E. H., and Frazier, W. C. Bacteriology of Milk Held at Farm Bulk Cooling Tank Temperatures. III. Psychrophiles Found and Their Growth. J. Milk and Food Technol., 20:93. 1957.

46. McMeekin, T. L. Milk Proteins. The Proteins, Vol. IIA, Chap. 16. Academic Press, Inc., New York. 1954.

47. Mikolajcik, E. M., and Burgwald, L. H. Effect of Incubation of Storage Temperature on the Growth of Psychrophilic Bacteria. J. Dairy Sci., 36:571. 1953.

48. Modi, V. V., and Owen, E. C. Riboflavin in Milk. Nature, 178, 4542:1120. 1956.

49. Moir, G. M., and Russell, R. R. Bacteriological Testing of Butter. New and Simplified Routine Methods. J. Dairy Res., 10:310. 1939.

50. Morrison, H. B., and Hammer, B. W. Distribution of Pseudomonas fragi. J. Dairy Sci., 24:9. 1941.

51. Naylor, H. B., and Smith, P. A. Factors Affecting the Viability of Serratia marcescens During Dehydration and Storage. J. Bacteriol., 52:567. 1946.

52. Nelson, F. E. Changes in Bacterial Counts of Stored Ice Cream Mix. J. Dairy Sci., 27:459. 1944.

53. Nelson, J. A. The Correlation Between the Organisms Found Microscopically and the Bacteriological Deterioration of Butter. Montana State Coll. Expt. Sta. Bull. 267. 1932.

54. Nelson, F. E., and Baker, M. P. The Influence of Time and Temperature of Incubation Upon Bacterial Counts of Market Milk and Related Products, Particularly after Holding Under Refrigeration. J. Milk and Food Technol., 17:95. 1954.

55. Olson, J. C., Jr., and Nielsen, A. J. Changes in Bacterial Counts and Flavor of Dry Milks After Recombination by Various Means and Storage at Low Temperatures. J. Dairy Sci., 38:361. 1955.

56. Olson, J. C., Jr., Nielsen, A. J., Thomas, E. L., and Morris, H. A. Changes in Bacterial Counts and Flavor of Concentrated and Recombined Milks During Storage at Low Temperatures. J. Dairy Sci., 36:817. 1953.

57. Olson, J. C., Jr., Parker, R. B., and Mueller, W. S. The Nature, Significance and Control of Psychrophilic Bacteria in Dairy Products. J. Milk and Food Technol., 18:200. 1955.

58. Olson, J. C., Jr., Willoughby, D. S., Thomas, E. L., and Morris, H. A. The Keeping Quality of Pasteurized Milk as Influenced by the Growth of Psychrophilic Bacteria and the Addition of Aureomycin. J. Milk and Food Technol., 16:213. 1953.

59. Parker, R. B., Coldwell, A. L., and Elliker, P. R. Psychrophilic Bacteria--A Sanitation Problem. J. Milk and Food Technol., 16:136. 1953.

60. Parker, R. B., and Elliker, P. R. Effect of Spoilage Bacteria on Biacetyl Content and Flavor of Cottage Cheese. J. Dairy Sci., 36:843. 1953.

61. Parker, R. B., Smith, V. N., and Elliker, P. R. Bacteria Associated With a Gelatinous or Slimy Curd Defect of Cottage Cheese. J. Dairy Sci., 34:887. 1951.

62. Pelczar, M. J., Jr., and Reid, R. D. Microbiology, McGraw-Hill Book Company, Inc., New York. 1958.

63. Pennington, M. E. Bacterial Growth and Chemical Changes in Milk Kept at Low Temperatures. J. Biol. Chem., 4:353. 1908.

64. Pennington, M. E., Hepburn, J. S., St. John, E. Q., Witmen, E., Stafford, M. O., and Burrell, J. I. Bacterial and Enzymic Changes in Milk and Cream at 0° C. J. Biol. Chem., 16:331. 1913-14.

65. Powell, M. E. Flavor and Bacterial Changes Occurring During Storage of Sweet Cream Which Has Been Flash Pasteurized at Various Temperatures. J. Dairy Sci., 21:219. 1938.

66. Prouty, C. C. Observations on the Presence of Facultative Psychrophilic Bacteria in Milk Produced Under the Farm Tank System. J. Milk and Food Technol., 18:250. 1955.

67. Rahn, O., Brown, C. W., and Smith, L. M. Keeping Qualities of Butter. III. The Decomposition of Proteins. Mich. Agr. Expt. Sta. Technical Bull. 2. 1909.

68. Robinton, E. D., Borman, E. K., and Mickle, F. L. Preliminary Bacteriological Study of Market Creams. J. Milk Technol., 4:253. 1941.

69. Rogick, F. A., and Burgwald, L. H. Some Factors Which Contribute to the Psychrophilic Bacterial Count in Market Milk. J. Milk and Food Technol., 15:181. 1952.

70. Rolleri, G. D., Larson, B. L., and Touchberry, R. W. Protein Production in the Bovine. Breed and Individual Variations in the Specific Protein Constituents of Milk. J. Dairy Sci., 39:1683. 1956.

71. Ruehle, G. L. A. Keeping Qualities of Butter. VII. The Microbic Flora of Off-Flavored Butter. Mich. Agr. Expt. Sta. Bull. 102. 1930.

72. Schultze, W. D., and Olson, J. C., Jr. The Distribution of Psychrophilic Bacteria in Commercial Dairy Products. J. Dairy Sci., 40:602. 1957.

73. Sherman, J. M., Cameron, G. M., and White, J. C. The Bacteriological Spoilage of Milk Held Near the Freezing Point. J. Dairy Sci., 24:526. 1941.

74. Seiberling, D. A., and Harper, W. J. HTST Pasteurization for the Control of Psychrophilic Organisms in Plant Water Supplies. J. Dairy Sci., 38:598. 1955.

75. Spinco Division, Beckman Instruments, Inc. Model R Paper Electrophoresis System Instruction Manual RIM-5. Palo Alto. 195_.

76. Spitzer, G., and Parfitt, E. H. A Study of the Proteolytic Action of Specific Organisms and Groups of Organisms in Butter Made From Graded Cream. J. Dairy Sci., 12:1, 1929.

77. Stark, C. N., and Scheib, B. J. A Study of Fat-Splitting and Casein Digesting Bacteria Isolated from Butter. J. Dairy Sci., 19:191. 1936.

78. Storgards, T., and Lindquist, B. A Comparison of the Ripening Process of Different Cheese Types, Based on Some New Methods of Investigation. Proc. 13th Intern. Dairy Congr., 2:625. 1953.

79. Thomas, S. B. Psychrophilic Micro-Organisms in Milk and Dairy Products--Part I. Dairy Sci. Abs., 20:356. 1958.

80. Thomas, S. B. Psychrophilic Micro-Organisms in Milk and Dairy Products--Part II. Dairy Sci. Abs., 20:447. 1958.

81. Van der Zant, W. C., and Moore, A. V. The Influence of Certain Factors on the Bacterial Counts and Proteolytic Activities of Several Psychrophilic Organisms. J. Dairy Sci., 38:743. 1955.

82. Wagenaar, R. O. The Bacteriology of Surface-Taint Butter: A Review. J. Dairy Sci., 35:403. 1952.

83. Watrous, G. H., Doan, F. J., and Josephson, D. V. Some Bacteriological Studies on Refrigerated Milk and Cream. Penn. State Coll. Agr. Expt. Sta. Bull. 551. 1952.

84. Watrous, G. H., Jr., Doan, F. J., and Josephson, D. V. The Significance of Psychrophilic Bacteria in Milk. 1953 Milk Industry Foundation Convention Proceedings, 4:49. 1953.

85. Weese, S. J., and Henderson, H. O. The Keeping Quality of Pasteurized Milk in Home Refrigerators. J. Dairy Sci., 32:945. 1949.

86. Weigt, V. U. Papierelektrophoretische Untersuchungen an normalen Rindermilchseren. Milchwissenschaft, 14:61. 1959.

87. Wolstenholme, G. E. W., and Millar, E. C. P. Ciba Foundation Symposium on Paper Electrophoresis. Little, Brown and Company, Boston. 1956.

88. Zweig, G., and Block, R. J. Filter Paper Electrophoresis of alpha-, beta-, and gamma-Casein. Fed. Proc., 12:296. 1953.