AN INVESTIGATION INTO THE USE OF STARCH-GEL-UREA ELECTROPHORESIS

AS A TECHNIQUE FOR STUDYING THE PROTEOLYSIS OCCURRING DURING CHEESE CURING

A thesis presented to the

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A man would do nothing if he waited until he could do it
so well that no one could find fault with what he has done -

Cardinal Newman.

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Supplementary Volume

    There is a Supplementary Volume containing the
original graphs that were obtained from individual
gel strips and were used in building up the composite
graphs appearing in the body of the main volume.

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INTRODUCTION

The protein of Cheddar Cheese makes up a quarter of its bulk, supplies its high biological value and is a major factor in regulating the characteristics of its body. Knowledge of the agents involved in converting milk casein into typical cheese protein must have value in indicating ways by which cheese quality can be improved, or alternatively indicate ways to accelerate or control the rather haphazard process of cheese curing.

Years of study into the subject of cheese protein degradation have shown the existence of a number of proteolytic agents present in cheese, viz:

1. The natural enzymes of milk.

2. The rennet enzymes.

3. Enzymes originating from the starter.

4. Enzymes originating from the adventitious flora of the cheese.

Enquiry as to the relative importance of each enzyme system has been a long and confusing process employing a variety of techniques.

Sherwood (1935) studied the changes in the various nitrogen fractions of cheeses in which bacterial numbers had been reduced by use of chloroform, but he was not able to completely eliminate the bacteria, neither distinguish between the activities of the various bacteria present in cheese, nor eliminate the effect of starter in the early period of manufacture.

Study of the characteristic enzyme systems of pure cultures of various organisms with comparison to the characteristics of enzyme systems of cheese was carried out by a number of workers (e.g. Peterson, 1948; Baribo & Foster, 1952; and Brandsaeter & Nelson, 1956), but this type of study did not yield reliable conclusions because of the variability of enzyme characteristics, even between strains of a bacterial species and further variability of results according to the conditions of the experiment. More recently, research into bitterness of cheese (Jago, 1962) has confirmed this inter-strain variability between starters and underlined the difficulty of approaching the problem, even through using pure cultures.

With the introduction of chromatographic techniques, the study of the order in which amino-acid release occurs during cheese ripening became possible. Results obtained by various workers (e.g. Dacre, 1953a; Kosikowski, 1951; Mabbitt, 1955, etc.) in different countries were not consistent with one another. However, it became apparent that different making and curing procedures were influencing the paths of proteolysis.

The tremendous complexity and variability of cheese as a medium for scientific study is apparent from the confusing and often conflicting results available in the literature. The desirability of simplifying and controlling the medium has become a necessity to basic study on this subject.

The complexity of casein itself indicates that a study of its components, rather than the protein as a whole, should provide a more fruitful approach to an understanding of the problems of proteolysis. Such an approach is possible, and has been demonstrated by Lindqvist & Storgårds (1959a) who used electrophoresis to follow the degradation of protein components during curing.

One aim of the present study is to extend the work of Lindqvist & Storgårds by the use of starch-gel-urea electrophoresis, which allows greater resolution of casein components than was achieved by these workers, Wake & Baldwin (1961).

Control of the cheese medium itself is also desirable and has been made possible by the introduction of methods of making cheeses under controlled bacteriological conditions by Mabbitt et al.(1959) and extended by McGillivray & Perry (1963). By application of this method, this study aims to manufacture cheeses with different but controlled bacteriological populations, and to compare the starch-gel-urea electrophoretic patterns obtained throughout their curing, in the hope that useful information may be obtained on this technique as a aid in the investigation on the role of the different proteolytic agents active in cheese curing.

REVIEW OF LITERATURE

The Heterogeneity of Casein

Interpretation of results obtained by use of starch-gel-urea electrophoresis on cheese requires an understanding of the structure of the associated phospho-protein called casein.

Linderstrom - Lang & Kodama (1928) were first to report that casein is not homogeneous, but it was Mellander (1939) who demonstrated the presence of three components by the use of moving-boundary electrophoresis. He named these components α β & γ in decreasing order of mobility.

Isolation of α and β -caseins was first achieved by Warner (1944) on the basis of solubility. It has been subsequently shown (Waugh, 1959) that the frequency of non-polar groups in β -casein is 0.49 as compared to 0.40 for the more soluble α -casein. γ -casein has a high solubility due to its high content of proline (Hipp et al. 1952). Using improved techniques, employing these different solubility characteristics, the three casein components were separated by Hipp et al. (1952). The same workers also pioneered the use of urea to fractionate the casein complex through its property of breaking up the hydrogen bonds that hold the complex together.

A further technique of separation was published by von Hippel & Waugh (1955) in which calcium was used to destabilize the casein micelle by destroying the action of a protective component which they later named κ -casein (Waugh & von Hippel, 1956). They suggested that α and κ -caseins exist as a complex in the micelle of casein. Their work also indicates that β -casein too must be associated with the micelle.

Later papers by Waugh (1958, 1959) have postulated a possible structure for the α_s / K complex in the micelle (α_s is used to distinguish "pure" α-casein from the α_s/K complex that had been previously isolated and referred to as α-casein). This concept may be summarized as follows.

The α_s-casein molecule appears to be a single coil 210A long and 16A in diameter. In the absence of calcium the protein is a highly soluble polymer which becomes quite insoluble in the presence of calcium. K-casein is also apparently a single coil about 150A long and forms "condition insensitive" monomers in the presence or absence of calcium. When mixtures are made containing molecular ratios of $3\alpha_s$ to $1K$, the original polymers disappear, and a stoichiometric α_s/K-casein complex forms. This complex can form in the absence of calcium and under conditions where both of the monomers carry a high net negative charge, so it is assumed that secondary valance forces are responsible for the association. Rennin action attacks the micelle and causes the caseins to become insoluble paracaseins. Attack on the protective component proceeds at the same rate as the clotting of milk and rennin also attacks α_s-casein converting it to para-α_s-casein but only $10^{-2.5}$ times as rapidly. β-casein conversion to para-β-casein occurs 10^{-3} times as rapidly as the formation of the clot.

Further proposals by Waugh, which give a detailed hypothetical structure for the casein miecelle, have been recently shown to be over simplified so will not be discussed here. Discovery of new fractions in the complex has been rapid.

Payens (1958) was first to clearly demonstrate a split in the α peak of the electrophoretic pattern, and after a certain amount of discussion (cf. McKenzie & Wake, 1959) the titles of α_1 and α_2 were given to two fractions isolated by McMeekin et al. (1959).

Tobias et al. (1953) had earlier reported a third component in the fraction and this, too, was finally isolated and characterized by Hipp et al. (1961). It was named α_3-casein and it contributes to the stability of the micelle, but unlike the κ-casein cannot be precipitated by rennin. A still further fraction isolated from the α-casein complex (Lang et al. (1958) has been called λ-casein.

The method of separating casein components by use of urea (Hipp et al. 1956) was applied to electrophoretic study by addition of urea to the electrophoresis buffer (Vlodavets & Zhdanova and Payens, 1960) with quite encouraging results. Some caution must be exercised in interpretation of results obtained by the use of urea, however, as the mobility of the κ component is influenced by urea concentration (Libbey & Ashworth, 1961) and it can appear in either the α or β peaks on paper electrophoresis, unless the urea concentration is held to 4.8 Molar.

Further confusion has arisen as to casein structure, because there is apparently a genetically controlled variation between the milks of individual cows. Thompson et al. (1962) have reported three components of α_s-casein which they refer to as α_s-A and α_s-B and α_s-C in order of decreasing electrophoretic mobility. More recent work by Waugh et al. (1962) has investigated two fractions of α_s-casein and notes the possibility that others exist.

Furthermore, Aschaffenburg (1961) has noted variability of the β-casein fraction which can be electrophoretically split into three separate bands (A, B and C) appearing in a limited number of apparently genetically controlled combinations (AC; A; AB or B). Two bands of casein were also detected and considerable variation in the strength of each band occurred between samples from individual cows. In his conclusion, Aschaffenburg states that it appears "that several independent genes" are involved in the elaboration of casein by the mammary gland, but "that the 'classical' subdivision of the complex into α, β, and γ caseins is justified and meaningful".

Many conflicting reports in the literature can be explained since the introduction of starch-gel-urea electrophoresis has detected seventeen components in casein, some of which remain unnamed and uncharacterized (Wake & Baldwin, 1961). Use of this method to investigate the purity of the casein fractions that have been isolated has shown that none are homogeneous (Neelin et al. 1962). This observation has been confirmed by the use of DEAE cellulose chromatography, agar-gel electrophoresis and immunoelectrophoretic analysis which showed heterogeneity in the α, α_s, α_2, κ, and β-casein fractions examined by all three methods (Garnier et al. 1962).

From this brief review of a most extensive and confused field of literature, it is apparent that little information is undeniably established with regard to the complex of casein components. Nomenclature of the various components has been disorderly and a number of components have been given more than one name by different workers. The use of starch-

gel-urea electrophoresis has been the means of obtaining the greatest resolution of components and, although these have not yet been fully characterized, the use of this method would appear to be desirable for all research into matters related to the basic composition of casein.

The use of Urea in Studies of Casein

The use of urea as a protein dispersant has become a standard technique in the study of the casein complex. This compound, however, is a protein denaturant and the possibility that a large number of components detected in casein through its use could be partly due to artifact formation must be considered.

The effect of urea on proteins has been generally ascribed to its very strong propensity to form hydrogen bonds with other organic molecules. It is assumed that urea forms hydrogen bonds with the side chains of protein, thereby destroying the effect of the natural hydrogen bonds that play a major role in holding the protein in its native configuration (Waugh,1954). To this extent urea could not be held responsible for artifact formation, for it merely acts as an agent that alters the configuration of the protein so as to free loosely associated components.

Other effects of urea have been noticed however, and the possibility of their being effective in studies of casein demands consideration before use of urea is acceptable. These possibilities are:-

(i)   The possibility that Urea Action might Split the Casein Molecule

A number of cases have been reported in which the Molecular-Weight of various proteins is severely reduced in concentrated urea solutions (Neurath & Bailey,1954). The Molecular-Weight of casein, as reported from ultracentrifugal studies at pH 6.8, was in the range of 75,000 -100,000 (Svedburg et al. 1930), yet the value generally accepted from study in urea solutions is about 33,000 (von Hippel & Waugh, 1955).

The polydisperse nature of the ultracentrifuge patterns obtained by Carpenter et al. and the more recent discovery that some casein fractions normally polymerise under even slightly acid conditions at room temperatures (Waugh, 1958) tends to throw doubt on the earlier estimation. Due to the complexity of the casein molecule, it would appear that study of the casein molecule as a whole is of little value, and recent studies have been confined to estimation of the Molecular-Weight of the individual casein components.

(ii)   The possibility of Carbamylation of Free Amino Groups

In concentrated solutions urea forms an equilibrium pair with ammonium cyanate which is the sole intermediate in the normal hydrolysis of urea to ammonium carbonate. Hydrolysis occurs on standing at room temperature. Under moderate conditions cyanate is able to react with amino or sulphydryl groups to yield carbamyl derivatives (Stork et al. 1960). The ability of this reaction to modify results of chromatographic procedures employing urea has been recently demonstrated (Cole, 1961).

In a study of casein, reactions involving sulphydryl groups may be disregarded however, as casein does not contain the amino acid cysteine (Gordon et al. 1948). The point is confirmed, as it is not possible to detect sulphydryl groups in casein, either in the native state, or when denatured by heat or urea (Neurath & Bailey, 1954). Urea solutions containing ammonium cyanate can effect carbamylation of amino groups of casein however, as a 29% reduction (approximately) in the number of free amino groups of α-$_s$ casein can be detected after treatment of casein with urea in equilibrium with cyanate (Manson, 1962).

As a check for the possible influence of this reaction under the conditions employed in starch-gel-urea electrophoresis, it has been shown that identical patterns are obtained with casein samples dissolved in urea a few minutes before use, and with samples dissolved and held for several days at 5°C (Neelin et al.1962). In the present study, urea solutions were made up fresh for each sample and stored at refrigerator temperatures for not longer than 12 hours.

(iii)   The possibility of the Formation of Urea Inclusion Compounds

It has been found that urea in concentrated solution can crystallize into a honey-comb-like structure. Inclusion of other ("guest") molecules within the lumena of this structure is possible (Schlenk, 1954). Due to the confined space within the structure it appears that only straight chain organic molecules of at least 4-6 carbon atoms in length may be included in such compounds. There appears to be no report of this occurring to the few straight chain amino-acids of proteins. The possibility of such adducts occurring in casein research is remote.

Published experience of the use of Urea for Casein Study

The dispersant action of high concentrations of urea on casein has been demonstrated by microscopic observation of the micelles (King,1960).

Hipp et al.(1952) have published a table giving a comparison of various properties of the α, β, and γ fractions prepared by urea, alcohol, and isoelectric precipitation techniques, and found them to be the same within the limits of experimental error.

Payens (1961) refers to "the apparently harmless influence
of urea on casein" and notes that electrophoretic properties of separate
components are completely restored after the removal of urea by dialysis.
Furthermore, after the removal of urea, the separated fractions showed
characteristic values for their sedimentation coefficients, and the
$\mathcal{K}$ -casein fraction was still sensitive to rennin action.

Wake & Baldwin (1961) in developing the starch-gel-urea
electrophoresis technique of analysis have tested their results to check
for artifact formation by re-running a number of components extracted from
previous gels. They found no cause to doubt their results,and point out
that the optical rotatory properties of casein and its vulnerability to
proteolytic enzymes indicate that casein, as it exists in milk already,
has the properties of a denatured protein. The use of this technique,
without the presence of urea, does not give a satisfactory resolution of
the complex (Neelin, 1962).

For the purposes of study of casein, the use of urea appears
to be essential if clear-cut and sensible results are to be obtained, and
it would appear to be idle to doubt the profusion of findings that support
its use.
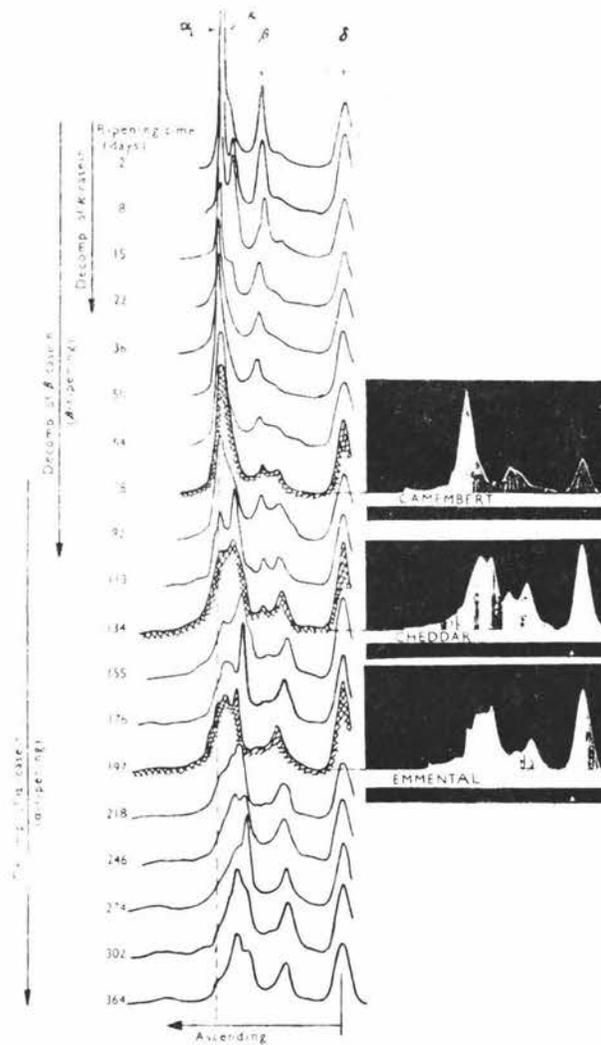
Fig. 1. The electrophoretic patterns from a single batch of Svecia cheese after different ripening times. Patterns from Camembert, Cheddar and Emmental cheese are included for comparison.

Figure 1: The electrophoretic patterns obtained by Lindqvist & Stårgårds.

The Application of Electrophoresis to variously modified Caseins

(1)   Electrophoretic Observations of Cheese Protein

Annabaldi (1959) has compared the free-boundary electrophoretic patterns obtained from proteins of various cheeses and noted the occurrence of quantitatively different amounts of each component in the different cheeses. Peaks referred to as α and β seem to be the main points of attack of proteolytic agents present in the cheeses. Either or both of these peaks appear to be converted to a residue which contributes to the rise in the so-called α_2 peak in many cases.

A more complete investigation has been reported by Lindqvist & Storgårds (1959a) in a paper which summarizes a number of their earlier papers. They have shown that the electrophoretic pattern, using free-boundary electrophoresis, of cheese protein alters through a definite sequence of events during the process of curing.

Three overlapping but separate stages of protein decomposition are noted as the κ , β ,   and α -casein fractions are successively attacked during the curing of Svecia cheese until, in the final state, all of the casein appears to have been modified.

The similarity of patterns obtained from mature cheeses of other types to some of the patterns obtained during the curing process of Svecia is noted. It has led the authors to suggest a relationship in the curing processes of superficially very different cheeses. The existence of such a relationship is not difficult to understand, as the cheeses mentioned all have rennet and acid-producing starters as the principal proteolytic agents.

The paper concludes with comment on the lack of knowledge about the role of the various proteolytic agents in cheese which would aid an interpretation of the above results.

(2)   Electrophoretic Observation of the Modification of Casein by Rennet.

The investigations of Lindqvist & Storgårds (1959a) have stimulated a certain amount of enquiry into the activity of the individual proteolytic agents of cheese.

Rennet action on casein proceeds through three stages, viz:

1.   Primary action is an enzymic alteration of the K-casein.

2.   Secondary action is the coagulation phase when a clot forms in the milk.

3.   Tertiary action is one of proteolytic degradation of the various casein components. It is this phase of rennet action which is of particular interest in studying the breakdown of cheese proteins during curing.

Yamauchi & Tsugo (1962) have reported that rennet action will split α-casein into two fractions in 1 hour. A split of α-casein into α_I and α_{II} after rennet action has also been reported (Payens, 1958; McKenzie & Wake, 1959). As rennet is not a pure enzyme, but a mixture consisting principally of rennin and pepsin, this type of work is of little value in trying to sort out the contribution of the individual proteolytic agents in cheese ripening. Though cheeses may be successfully produced by

use of crystalline rennin alone (Linklater & Ernstrom, 1961), it is probable
that the other enzymes present in commercial rennet will play some part in
the overall pattern of cheese protein degradation. To separate the effects
of rennet on casein it will be necessary to investigate the action of each
enzyme on each casein component. To date, pepsin and rennin action on
$\mathcal{K}$ - α - and β -caseins have been studied.

The action of rennin on $\mathcal{K}$ -casein has long been recognized as
the agent allowing milk to clot (Waugh & von Hippel, 1956). Wake (1959) has
reported that para - $\mathcal{K}$ -casein (i.e. rennin modified $\mathcal{K}$ -casein) moves as a
single component on paper electrophoresis, but with an increased extent of
'tailing". Starch-gel-urea electrophoresis showed that para- $\mathcal{K}$ -casein
concentrates at the starting slots after only 30 minutes of rennin action
(Wake & Baldwin, 1960).

The proteolytic action of the rennet enzymes on α- , β -caseins
has been more extensively investigated. Gonashvilli (1962) found that at
least 5 fractions are formed from α-casein by the action of rennet or pepsin
over a pH range of 5.1 - 5.8. At least 4 fractions were formed from β -casein.
The protein fractions obtained have been classified as casein A and casein B,
according to whether or not they precipitate from the reaction mixture at a
pH above or below 5.0. The electrophoresis patterns of the casein B group
after cleavage by rennet or crystalline pepsin differ from one another,
indicating that these enzymes act differently on casein.

Lindqvist & Storgårds (1959B; 1960A, B; 1962) have followed the
rennin proteolysis much more intensively. α -casein was rapidly split into
two fractions, as had previously been shown, and proteolytic action was
progressive until, by the 25th day, six components of widely differing

mobilities were detected. It was also found that variation in pH over the range of 4.21- 5.75 can markedly alter the path of degradation.

The course of degradation of β -casein by rennin was represented as a progressive reaction yielding, finally, five main degradation products. It is made clear that this protein is highly unstable to rennin action and a large number of different peptides can be detected by use of various conditions of reaction.

Pepsin, another enzyme present in commercial rennet, behaves similarly to rennin in its attack on α -casein in certain respects (Lindqvist & Storgårds, 1960b). The attack appears to be less specific than with rennin action and produces 4-6 electrophoretic peaks after 20 days of enzymic action on α -casein. Variation in activity is observed at various pH's in the range 4.6-5.7.

From this information it will be apparent that the action of rennet in cheese ripening is quite complex. Lindqvist & Storgårds (1962) comment that: "Certain products formed from β -casein coincide electrophoretically or lie very close to corresponding products from the α -casein. This makes it practically impossible to follow electrophoretically the degradation of the individual casein components in the total casein and to draw any conclusions regarding the resistance to proteolysis of the individual types of casein". Whether this conclusion will hold under the conditions of starch-gel-urea electrophoresis remains to be seen.

It is notable that the recent work has been carried out with paper electrophoresis, and further that the work of Lindqvist & Storgårds was carried out using the α - and β -casein fractions obtained by the urea

precipitation method of Hipp et al. (1952). This means that the fractions employed would not be homogeneous to study by starch-gel-urea electrophoresis. Possibly some of the new components appearing during the tertiary phase of rennet action could be due to the separation of various components already known, rather than the formation of new peptides.

The prevailing attitude to this objection may be detected from comments made by Lindqvist at the recent International Dairy Congress (Lindqvist, 1962). He says " . . . we venture now to use only the terms α -, β -, and γ -casein as the nomenclature of the groups. The heterogeneity within the groups is still very obscure and is probably great".

If the method of starch-gel-urea electrophoresis can be successfully applied to investigation of cheese curing it will be possible to investigate the process by observing changes in the most homogeneous fractions yet detected. By using the technique to compare one cheese with another, and by placing importance on the differences appearing, any objection about the lack of knowledge of the components detected loses its relevance.

(3)   Electrophoretic Observation of the Effect of Bacterial Enzyme in Cheese Ripening.

The bacterial enzymes, present in a ripening cheese, must provide a very complex system with which to work and this probably explains why little information has been published with regard to their activity.

Lindqvist & Storgårds (1959a) compared the electrophoretic pattern of rennin proteolysed casein to that of a mature Svecia cheese and noted that the cheese sample contained significantly more peaks. In view of their more recent papers on the tertiary action of rennin this may not be a legitimate comparison.

Use of a preparation referred to as "Bacterial Proteinase Novo" leads to a less differentiated electrophoresis diagram than that given by rennin. This indicates that the bacterial enzymes used can attack the casein molecule at many points (Lindqvist & Storgårds, 1962).

Annabaldi (1962) has studied the proteolytic action of <u>Bacillus</u> <u>subtilis</u>, <u>Streptococcus feacalis-lactis</u>, <u>Lactobacillus helveticus</u>, and bacteria of the Alcaligenes genus on ultracentrifuged sediments of phospho-protein. It is shown that <u>B.subtilis</u> can completely destroy the casein patterns within 72 hours under the experimental conditions. The other species used attack the casein less completely and it is apparent that species differences occur with regard to both the mode of action and to the extent of action.

The information available with regard to this phase of the investigation is, as yet, too meagre to critically evaluate.

The Proteolytic Breakdown during Ripening of Cheese

Investigation into the curing of cheese is an extensive field of research, but information about cheese protein breakdown has often been derived as an incidental to investigation into the source of cheddar cheese flavour. A distinction between the two studies can be drawn despite the certain amount of overlapping that occurs.

Cheese protein functions as a determinant of cheese structure and as a determinant of some components of the flavour of cheese. Published

reviews of literature in this field have either been incomplete or related specifically to cheddar cheese flavour investigation.

A general account of knowledge relating to cheese ripening has been presented by Kosikowsky & Mocquot (1958).

More specifically related to proteolysis is a section of a review by Mabbitt (1961) which summarizes papers published just prior to 1961. Part of a review by Ling (1958) is useful in relating chemically derived information to the cheese ripening process.

Information available in these reviews is not repeated in this work unless necessary to explain conclusions drawn.

The Relationship of Proteolysis to Cheddar Cheese Flavour

The reviews of both Mabbit (1961) and Ling (1958) comment on the appearance of free amino acids during ripening. Although most workers have detected about 18 free amino acids in cheese, the order in which these are released differs quite markedly (e.g. Mabbitt (1955) detected proline quite early in the process but no other workers found it until the cheese was nearly mature). The individual amino acids have distinctive flavour characteristics and much speculation about their contribution to cheese flavour has been published (e.g. Harper & Swanson, 1949). Such discussion was placed in perspective by Mabbitt & Zielinska (1956) who were unable to detect more than a brothy flavour in cheeses made from skimmed milk.

This flavour is found in mixtures of amino acids and probably acts as a base upon which the characteristic flavour of cheddar is superimposed.

A second influence of protein hydrolysis on the flavour of cheddar was shown by Raadsveld (1953). A polypeptide concentrate with a very bitter flavour was extracted from bitter and from non-bitter cheeses. It appears that the polypeptide fraction must always be present and contributing as a component to the overall flavour of cheddar cheese. Occasionally an imbalance of flavour components, resulting in excessive amounts of the bitter polypeptides, may lead to bitterness of the cheese.

The Path of Protein Hydrolysis

It has been established that the greater part of the phosphorus in casein occurs in the form of crosslinkages between adjacent polypeptide chains or as crosslinkages within the same chain or coil (Ling 1958). On the basis of this information it can be seen that the levels of phosphorus in the soluble nitrogen fraction in cheese will give information as to the extent of hydrolysis of the protein. Investigation of the phosphorus levels of the various protein fractions of cheese has been carried out (Klimovski 1959). From this paper it appears that, although much of the paracasein remains unchanged, some of the deep peptide bonds are attacked with release of phosphorus into the soluble protein fraction. As the levels of inorganic phosphorus remain constant there cannot be complete splitting of the molecule which would release the phosphate groups and many of the amino acids. This indicates that no phosphatase activity was occurring in the Edam cheeses investigated. Recent work by Aiyar (personal communication) has shown that rennin has phosphatase activity on casein under experimental conditions and this could possibly stand in opposition to conclusions drawn from Klimovsky's results.

The search for more basic information on the matter of cheese protein hydrolysis has been persued by use of electrophoresis (see appropriate section of this review) and by use of ion exchange resin chromatography. The latter method has been used to sort out the peptides appearing in cheese on the basis of molecular size (Tokita & Nakanishi, 1962a,b). Different cheese types are grouped into three modes of ripening, according to the patterns obtained.

Protein hydrolysis apparently occurs in two distinct stages.

1.   Primary Stage   - a hydrolysis of protein to give a proteose and peptone fraction.

2.   Secondary Stage - a hydrolysis of proteose and peptones to give lower-molecular-weight peptides and amino acids. In this step, certain low-molecular-weight peptides seem to resist the action of cheese proteinases. This conclusion is based on the appearance of a peak in the concentration graphs which increase in size during ripening.

The Relative Importance of the Various Proteolytic Enzyme Systems Present in Cheese.

Although literature relating to this question is extensive, it has not been reviewed recently. Because of the abundance of papers that can be related to this topic, reference will be made only to those papers definitely contributing to the discussion.

Very many proteolytic enzymes are present in cheddar cheese, but for purposes of comparison and discussion they may be separated into five groups as follows:-

1. The natural milk enzyme (s) ("galactase").

2. The enzymes of commercial rennet.

3. Bacterial enzymes originating from -

 (a) Streptococci of the starter.

 (b) Lactobacilli.

 (c) Other bacterial species that may appear in the cheese.

Each will be discussed in turn.

1. The Naturally Occurring Enzyme(s) of Milk

Harper et al. (1960) make reference to six earlier papers about this source of enzymes, but they find faults or limitations in all of them. These authors present results that indicate that raw milk contains a small and variable amount of protease enzyme. Tyrosine was the only free amino acid which increased in concentration during incubation of asceptically drawn milk.

The conclusion is drawn that this protease system is of little importance under practical conditions of cheese ripening. Another study of amino acids released in test tube experiments has given rise to a similar conclusion (Stadhouders, 1960).

This conclusion may need reconsideration in view of a paper by Kristofferson (1961). It is stated that "milk is constantly undergoing internal changes and cannot be considered an inert substance". Incubation of milk at 37°C for various periods up to 6 hours after milking showed that the longer incubation periods improved the flavour of cheeses made from the

pre-incubated milk samples. At first sight this would appear to be due to accelerated bacterial action. In the series of trials described, inoculation of cheese milk with micrococci and lactobacilli did not improve flavour development. Nevertheless, in every trial, regardless of the inoculation used, incubation of the milk for four to five hours prior to cheese manufacture was needed before a good "raw milk flavour character" developed in the cheese. Either the milk enzyme system or some, as yet unacknowledged, factor in the milk is influencing the flavour development in the cheese. This observation may have no connection with cheese proteolysis, but it is included to show that knowledge of the action of this enzyme actually in the cheese is as yet incomplete.

2.   The Enzymes of Commercial Rennet

Early recognition of the importance of this enzyme system was afforded by Sherwood (1935). By adding chloroform to cheeses, either at salting or one week after manufacture, he was able to reduce bacterial counts to about 10^5/gram. This caused a reduction in the amount of sub-peptone nitrogen appearing in the cheeses on curing, but did not influence the concentration curves for soluble nitrogen or non-protein nitrogen which were practically identical in shape with those in control cheeses. The author concluded that rennet must be the chief proteolytic agent in cheese ripening, but that bacterial action must, in some way, influence the further breakdown of protein fragments to sub-peptone levels.

Recent work using cheese made from aseptically drawn milk, under controlled bacteriological conditions, has shown that cheeses made with gluconic acid to replace starter show as marked proteolysis as normal cheeses made as controls (Lawrence - unpublished data).

Amundstat (1955) has noted that rennet action on casein solutions leads to an increase in soluble nitrogen, but did not increase the amino acid levels. Stadhouders (1959) agrees with other workers that addition of rennet to chalk milk cultures of lactic acid bacteria stimulates the production of amino acids by the bacteria. These observations support the conclusions drawn by Sherwood, and indicate that the major importance of rennet lies in its ability to modify the more complex protein fractions.

The action of rennet on casein has been **extensively** investigated in recent years (e.g. Wake, 1959; Waugh & von Hippel, 1956), and it appears that rennin acts on the $\mathcal{K}$-casein fraction to release a glycomacropeptide which will become part of the soluble nitrogen fraction. Electrophoretic investigation has shown that rennet enzymes cause further breakdown of the other casein components (Lindqvist & Storgårds, 1962). Both of these observations could readily explain the increase of soluble nitrogen observed by Sherwood.

3.   The Bacterial Enzymes in Cheese

During the period while a cheese is curing various bacterial species flourish. Starter streptococci grow rapidly in the young cheese, but as conditions in the maturing cheese alter, pediococci and lactobacilli may appear and even dominate the cheese flora for a period (e.g. Dacre, 1958; Davis, 1935). As the bacterial count of cheese generally reaches millions per gram, it will be apparent that a large number of bacterial enzymes of both extracellular and intracellular origin will exist in cheese as the bacteria flourish, then die, and eventually autolyse.

Investigation of the effect of bacterial numbers in milk on

the flavour score of resultant cheeses has high-lighted the many factors
which affect cheese during its curing and has lead to a number of discordant
conclusions (e.g. Smith et al. 1956). As in the field of flavour research,
it is dangerous to make categorical statements relating to the proteolytic
activities of the various bacteria present in cheese, because of this same
multiplicity of factors that influence the bacteria in the cheese.

Direct study of enzymes likely to appear in cheese during
curing has been attempted by a number of workers. By following the levels
of proteinase in cheese during ripening, it appears that the enzyme
concentration rises quite markedly during the later stages. This implies
that bacterial proteolytic enzymes are of major importance (Peterson et al.
1948). Quantitative work with enzymes is susceptible to criticism however,
and Baribo & Foster (1952) later pointed out that these results were obtained
by incubating the enzyme extracts at $40^{\circ}C$. This could provide a very false
picture if related to the conditions of cheese curing which is normally
carried out about $13^{\circ}C$. Baribo & Foster found a considerably lower increase
in proteinase levels of cheese when they repeated the work of Peterson et al.

Baribo & Foster (1952) have extended this form of study by
characterizing the enzyme systems extracted from strains of Streptococcus
lactis, Lactobacillus casei and Micrococcus freudenreichii. These were
compared to enzyme systems extracted from a one-year-old cheese. It is
apparent from their results that, although the enzymes from these organisms
could well be active under the conditions of cheese curing, the main
characteristics shown by the cheese proteinase extract have not been detected.
The authors are most cautious in discussing their results and do no more than
"suggest" that rennet may be the major source of proteolytic enzymes present

in cheese, but which are not detected or identified in these experiments.

In a similar investigation of the endocellular proteolytic system of S.lactis, van der Zaant & Nelson (1953) have found results broadly in agreement with those reported by Baribo & Foster. These authors are also cautious about interpreting their results and state that "the environment that exists in cheese cannot be duplicated in test tube experiments". This comment is very true and must limit the emphasis placed on the results obtained.

Brandsaeter & Nelson (1956) have enumerated further difficulties inherent in this type of investigation in explaining why their results on the characterisation of the enzyme system of L.casei differ from those found by Baribo & Foster. Apparently different strains of the same species may have different enzyme characteristics. Brandsaeter & Nelson have shown that enzymes extracted from L.casei have both proteinase and peptidase activity. This confirms earlier results published by Tarnanen(1930).

It appears from the foregoing discussion that this type of investigation is fraught with many inherent problems of technique, the cheese substrate cannot be duplicated in the test tube, and the many strains and species of organism present in cheese may release different enzyme systems. The proteolytic enzyme system present in cheese is too complex to usefully investigate in this manner, though the results have a common conclusion in that all enzyme systems investigated could be active in cheese. Enzymes having activity characteristics which will adequately explain those found in extracts of a one-year-old cheese have not been detected.

3(a).   Enzymes of the Starter Streptococci

A number of workers have shown that lactic streptococci do possess enzymes capable of proteolytic activity. For instance, Yates et al. (1955) have isolated from commercial cheeses 13 strains of S. lactis and S. cremoris that have proteolytic activity. It was shown further that these species had greater activity in rennetted milk than in skim milk. Proteinase activity, as distinct from peptidase activity, was evidenced by ability to utilise sodium caseinate (Husain & McDonald, 1957). A certain amount of interstrain variability was noted.

Incubation of starter in milk for 10 hours, prior to cheese making, will increase the breakdown of protein in the cheese (Stadhouders, 1959). This acceleration of proteolysis during ripening is achieved without alteration of the course of amino acid or peptide release as detected by chromatographic methods (Koning, 1962).

Variability of starter proteolytic activity, as previously mentioned, is confirmed by the now common observation that fast acid producing starters are more proteolytic than the slower ones (Stadhouders, 1959). An explanation of this interstrain variability has been tendered by Stadhouders (1961) as follows:- "As there are in milk no N compounds which can directly be assimilated by the Streptococci, the protein of the milk has to be hydrolysed before the bacteria can develop. The bacteria with the most proteolytic enzymes are, therefore, best capable of growing quickly. It is also clear that the fast acid producing streptococci are fast as they can hydrolyse the protein of the milk quite well and the slow acid producers are slow as they have lower proteolytic capacities."

Further evidence of the proteolytic activity of the starter streptococci has been found by investigation of bitterness in cheese. It will be recalled from earlier discussions that cheese bitterness has been related to the polypeptide fraction present in cheese. In investigating possible causes of bitterness, Emmons et al. (1962) quote 25 papers in which 14 different and often opposing views have been expressed. These authors have investigated the activity of 11 strains of S.cremoris starter and found that some gave bitterness to cheese and that there were highly significant differences in amino nitrogen levels between cheeses made with the various starter strains. Jago (1962) has extended these observations by showing that the "bitter" strains of S.cremoris do not have the ability to hydrolyse the peptides produced by rennet action to the amino acid level. "Non-bitter" strains appear to have this ability, presumably due to differences in their proteolytic enzyme systems. Work by Stadhouders (1962) supports this conclusion by showing that S.cremoris starters have variable but specific ability to break down rennet-produced peptides. This activity is detected through the molecular-weight of the peptides produced but, in contrast to Jago's work, it could not be correlated to production of amino nitrogen levels.

In cheeses made by addition of lactic acid to replace starter, it was shown that amino acid release was much less extensive than when starter was present in the cheese (Yamamoto & Yoshitake, 1962). As noted previously, Lawrence (unpublished data) found that cheeses made by addition of gluconic acid lactone, and under controlled bacteriological conditions, did not show any marked difference in proteolytic breakdown from cheeses made with starter. This work, however, is incomplete with regard to

amino acid analyses, and consequently cannot be used to dispute the conclusions to be drawn from the available literature.

It may be concluded from this review that proteolytic activity of the streptococci is established and that individual strains vary in their ability to degrade casein.

3(b). The Enzymes of the Lactobacilli

As has been mentioned previously, studies of the enzyme systems of this class of bacteria have shown that they possess both peptidase and proteinase activity (Tarnanen, 1930; and Brandsaeter & Nelson, 1956).

Deaminase activity with release of H_2S and ammonia by L.casei has been detected. However, variation in the ability of various species to carry out this activity is noted (Kristofferson & Nelson, 1955). Decarboxylase activity has also been detected through the action of L.brevis on tyrosine (Dacre, 1953).

Tokita & Nakanishi (1962) have found that L.casei is able to utilize peptide-like substances extracted from Edam cheese. The ability to grow on these extracts was found to increase as the cheeses matured.

A number of papers have been published on the various effects of lactobacilli inoculated into cheese. Davies et al. (1934) detected an increase in protein degradation at one month, but the effect of the inoculation was apparently lost at maturity, presumably due to growth of naturally occurring lactobacilli. Peters (1960) reports improved body and increased soluble tyrosine values in mature cheeses inoculated with L.casei

by comparison with controls. Bullock & Irvine (1956) found that cheeses inoculated with L.casei showed more rapid amino acid liberation at 8 months' old than uninoculated controls. This method of experimentation seems to give results, but it is open to criticism, as there is no control of the starter or adventitious flora of the experimental cheeses. Furthermore, techniques sufficiently selective to show that lactobacilli inoculated into experimental cheeses do actually grow and provide the mature cheese with a larger proportion of the inoculated organism, than in the control cheeses, did not exist at the time of writing most of these papers. Consequently, there can be no certainty that results achieved are actually due to the lactobacilli inoculated.

A recent paper (Stadhouders, 1960) has employed Rogosa's medium for enumerating lactobacilli and a graph is given to show the effect of the inoculation. After an initial high level of lactobacilli, the population falls and is little greater than in the control after 6 weeks. No detectable difference in protein hydrolysis is attributable to inoculation of various varieties of L.casei which could well be due to the high numbers of lactobacilli in the controls.

There can be no doubt that lactobacilli find in cheese a suitable growth medium during the latter stages of ripening and presumably they will utilize some part of the protein available. Test tube experiments show that this class of bacteria possess proteolytic activity, but it is difficult to show their activity in cheese where they appear after the protein has been modified by the enzyme systems of both rennet and starter.

3(c).   Enzyme systems of other Bacterial Species

Very little work has been carried out in relating other
adventitious flora present in cheese to proteolytic breakdown. The
regular appearance of pediococci (Dacre, 1958) has been shown. Selected
micrococci, although never dominant in the cheese population, have been
shown to exert some influence on cheese flavour (Robertson & Perry, 1961).
Both of these types of bacteria could exert some influence on the
proteolysis occurring in cheese, but there has been no critical study
published giving information on their proteolytic activity in cheese.

In pure culture many bacterial species have proteolytic
properties, as has been shown by electrophoretic investigation (Annabaldi,
1962). The cheese medium is very different to that in pure culture studies
and this type of information is not necessarily applicable to cheese curing.

Stadhouders (1960) has reported work with cheeses inoculated
with various bacterial species and these cheeses were checked during curing
to detect the continued presence of the inoculated organism. The bacteria
used included gram negative rods (Pseudomonas sp., Alcaligenes sp.,
Achromobacter sp., Serratia sp., Flavobacterium sp., and Escherichia coli);
Micrococci (Staphylococcus lactis); and Enterococci (Streptococcus feacalis &
Streptococcus liquifaciens). In no case could proteolysis in the inoculated
cheese be considered significantly greater than in the control cheeses.

The Effect of Conditions in the Cheese upon the Proteolytic Breakdown

During cheese ripening there is a succession of bacteria which dominate the flora. The reason for this has never been fully elucidated, but there are a number of factors which could have an influence on the bacteria growing in the cheese. These are:-

pH

lactate concentration

Oxidation/Reduction potential of the cheese

Nitrogen metabolism of the flora

Carbon source

Osmotic pressure

Salt in moisture levels

Because of the effect that these factors could exercise on the bacterial flora, there could well be a consequent effect on the rapidity of protein breakdown. Furthermore, the factors of pH, oxidation/ reduction potential and saline concentration could well influence the activity of any enzyme systems present in the cheese.

Little is known about the influence of the cheese medium on the path of proteolysis, although a certain amount of speculation has been published (e.g. Kristofferson, 1961).

The levels of saline concentration in the moisture fraction of the cheese have been observed to influence the protein breakdown quite markedly (Davies et al., 1937). This author gives six suggestions as to why this should be so. Stadhouders (1962) has shown that saline concentration markedly influences rennet activity and is, therefore, liable to consquently influence proteolysis. Monib (1962) has critically reviewed literature relating to this question and concludes that salt seems to act through a salting out effect on the protein and through liberation of part of the bound water. Bacterial activity is apparently not considered in this paper, but release of bound water could well influence it through reducing the saline concentration in the free moisture of the cheese.

These brief observations are made to indicate the importance of studying the curing process in cheese rather than in artificial substrates. Furthermore, experimental cheeses should be comparable in all aspects of composition. In practice this may be difficult to achieve, but every effort must be made to produce cheeses of even quality and they should also be acceptable with regard to normal grading criteria.

THE THESIS STUDY

AIM:

To manufacture cheeses under controlled bacteriological conditions, so as to obtain cheeses with different bacterial contents. To follow the protein degradation of these cheeses using starch-gel-urea electrophoresis and, from this information, to assess the feasibility of using these methods to elucidate the role played by various agents in the proteolytic breakdown of cheese protein.

OVERALL PLAN OF EXPERIMENTAL WORK:

Cheeses were manufactured from aseptically drawn milk using aseptic techniques and sterile cheesemaking equipment. The experimental cheeses were in the form of 10 lb.loaves and were inoculated during the making process with the following proteolytic agents.

- 2 cheeses with rennet, but no bacteria.
- 3 cheeses with rennet, plus

    STREPTOCOCCUS CREMORIS (strain HP)

- 2 cheeses with rennet, plus S.cremoris HP, plus

    LACTOBACILLUS CASEI-PLANTARUM (strain 25.2)

All cheeses were made over a period of 15 days during December and milk was drawn from the same cows on each occasion, so that the possibility of compositional variation of the milk was minimized.

The natural enzymes of milk will be common to all the
cheeses, so their activity (if significant) will not be distinguishable.
To check whether experimental cheeses do mirror the proteolytic
breakdown occurring in commercial cheese, a 40lb. rindless cheese made
with S.cremoris HP as starter, under normal factory procedure, has also
been sampled. This cheese was made within two months of the experimental
cheeses, but could differ slightly from them through variations in milk
composition.

Samples for bacteriological analysis were taken at 1 day,
14 days, 1 month and 4 months after manufacture. These were used to
estimate the effectiveness of the control measures taken during
manufacture to ensure asepsis on controlled inoculation.

Samples for protein study were taken from all cheeses after
1 day, 1,2,3,4,6,10 and 16 weeks of curing. Samples were prepared for
storage by the "Acetone Powder Method" and were stored in a deep freeze
until a convenient time for analysis by starch-gel-urea electrophoresis.

A sample for chemical analysis was taken after 14 days.

METHODS:

  (1) ASEPTIC CHEESEMAKING

  (2) CHEMICAL ANALYSIS OF CHEESE

  (3) BACTERIOLOGICAL METHODS

    (a) With aseptically drawn milk samples

    (b) With cheese samples

  (4) PROTEIN ANALYSIS

    (a) Protein extraction from cheese

    (b) Starch-gel-urea electrophoresis

      - Starch extraction from potato

(1) THE ASEPTIC MANUFACTURE OF CHEESES

The possibility of manufacturing cheeses under controlled bacteriological conditions was first reported in work by Mabbit, Chapman & Sharpe (1959) and the technique has recently been improved by McGillivray & Perry (1963).

Equipment

The equipment in use was that described by McGillivray & Perry, with the slight difference that industrial rubber gloves were used in place of surgical rubber gloves, and these were sealed in place with cotton wool pads to prevent bacteria from entering the vat.

Sterilization of Equipment

After scrupulous cleaning the vat was filled with a solution of hypochlorite. The gauntlets and the thermometer bulb were sunk into this solution which remained in the vat for about 40 hours, during which time the content of free chlorine was periodically tested to ensure it did not fall below 100 p.p.m. concentration. Just before use, the vat was emptied and rinsed with practically sterile bore water to remove traces of residual chlorine. All autoclavable equipment (viz: 2 testing buckets, weighed salt in wide mouth bottles, curd knife, milling knife, drain gate, cotton wool, rubber stoppers, sample bottles, pyrex funnel, and a dressed hoop) was wrapped in parchment and autoclaved at 20-25 p.s.i. for 30 minutes.

A plastic bag used to hold the curd prior to hooping could not be autoclaved, so was "sterilized" by boiling for 20 minutes within an hour of cheesemaking.

Rennet was sterilized by Seitz filtration and sterility checked by plate counts.

Gluconic acid lactone could not be sterilized, but bottles containing it were rinsed in chlorine solution and flamed with alcohol prior to placing them on the vat shelf.

Source of Milk

Milk was aseptically drawn from selected cows of the No.2 dairy herd of the Massey College dairy farm. Six cows were used to supply the necessary 83 - 93 lbs. of milk. The technique of aseptic collection has been described by McGillivray & Perry.

Aseptic Procedure during Cheesemaking

Once the vat was emptied and rinsed out, the curd knife, milling knife and drain gate were placed on the floor of the vat. The vat lid was

quickly closed, then with rubber gauntlets and thermometer in position the assembly was steamed for 30 minutes. The temperature rose to 212°F within the vat in 5 minutes. The articles placed within the vat were big and awkward but covered little floor space, so should not have adversely affected the value of the steaming process. The reason for storing this equipment in the vat was to eliminate as much as possible the necessity of opening the vat lid after sterilization.

After steaming, the making equipment in the vat was placed on the shelf and other necessary sterile equipment (viz: salt, plastic bag, gluconic acid lactone, sample bottle) was placed in the vat by lifting the lid and handling it with the rubber gloves after removal of the protective parchment by an assistant. Further steam was then blown through the vat to purge any air that had entered. All outlets were plugged with cotton wool and a slight positive steam pressure maintained. The milk was then transferred from the containers to the vat by lifting the vat lid and pouring it in through the cloud of steam that escapes.

A raw milk sample for bacteriological purposes was taken from the drainings that remained in the buckets. This was not good sampling technique but was unavoidable. Counts derived from these samples were probably higher than the actual count of the milk.

Steam was again blown through the vat for 5 minutes to purge the non-sterile air which had entered above the milk level. Following this steaming, air, filtered through a previously sterilized cotton wool plug, was blown into the vat. All outlets were plugged with sterile cotton wool so that a positive air pressure was created within the vat. This will cause a strong outward blast of air from any leaks in the equipment. Once heat

sterilization was completed hypochlorite was added to the solution which forms a water seal for the lid of the vat.

The milk was heated and cooled by admitting steam or water to the jacket of the vat. The milk temperature was raised to $155^{\circ}F$ and held for 3 minutes. Warm-up and cool-down times were about 20 minutes each.

A sample for bacteriological examination was taken after pasteurization and left within the vat throughout the following processes. This treatment, in effect, provided a pre-incubation period for the sample which meant high plate count results.

The cheesemaking technique was essentially the rapid method described by Whitehead & Harkness (1959).

The single strain starter used was, in all cases, S.cremoris HP and the lactobacillus inoculated belonged to the strain L. casei-plantarum 25.2. All cultures were supplied from the Dairy Research Institute Bacteriology Laboratory. 1 litre of starter (about 2% level in the vat) and 500 mls. of lactobacillus culture were used for inoculation.

Aseptic precautions taken during the rest of the cheesemaking process were as described by McGillivray & Perry. Manufacturing records for individual cheeses are given in Appendix No.1.

After pressing, cheeses were cured for the 14 days at $55^{\circ}F$ with daily turning, then waxed and curing completed at $45^{\circ}F$ for a further $3\frac{1}{2}$ months.

Gluconic Acid Lactone Cheeses

The use of gluconic acid lactone to provide acid breakdown in the curd in the absence of starter was originally devised by Mabbitt et al. (1955). The technique has been modified by workers at the Dairy Research Institute (N.Z.) and the author. Details of the method employed in manufacturing cheeses without starter were as follows:-

1. Aseptic precautions as previously detailed.

2. Heat treatment at 155°F for 3 minutes, then cool to 95°F.

3. Add 1000 ml. of 0.2 N HCl (HCl diluted with sterile water).

   250 gm. of gluconic acid lactone.

   20 ml. of Seitz-filtered rennet.

4. Cut after 10-15 minutes and raise temperature to 100°F.

5. After 50 minutes raise temperature to 110°F and hold for 5 minutes.

6. Run, cheddar for 20-30 minutes.

7. Mill and salt immediately afterwards ($\frac{1}{3}$ lb. of salt).

8. After 20 minutes, scatter 125 gm. gluconic acid lactone over the curd.

9. After 50 minutes, add 1 gm. nisin in sterile water.

10. Allow water to drain off for 5 minutes, then hoop up inside the vat with same precautions as previously stated.

    Time set to press, about $3\frac{1}{2}$ hours.

11. Curing conditions as for the inoculated cheeses, but a double waxing to prevent over drying and cracking of the rinds.

Sampling the cheeses

Samples were taken at intervals for bacteriological, protein
and chemical analyses. The procedure was:-

1. Clean, then sterilize a standard commercial cheese trier with
    burning alcohol.

2. The cheese surface at the point of sampling was also
    sterilized with burning alcohol.

3. Take a plug of cheese, break off and discard the $\frac{1}{2}$" nearest
    to the surface of the cheese.

4. Place sample in a sterile bottle.

5. Immediately fill the hole with molten wax.

Plugs were taken from points at both ends of the cheese and care
was taken to ensure the holes were at least one inch apart.

Discussion of the Cheesemaking Technique

The ideal case where no bacterial contamination of the cheese
will occur is very difficult, if not impossible to achieve, and extreme
care at all stages of manufacture is essential to ensure asepsis.

The heat treatment at $155^{o}F$ for 3 minutes is greater than the
equivalent pasteurization standard which would be $155^{o}F$ for 1 minute (Kastli,
1957). These treatment conditions are designed to kill a maximum number of
bacteria without weakening the rennet clot or the effect of syneresis.

The single strain starter, S. cremoris HP, was employed for all
inoculated cheeses for the following reasons:

(1)   It is readily available and in common use.

(2)   It does not produce nisin.

(3)   There is a low incidence of phage resistant forms appearing under normal culture conditions. This is necessary for satisfactory application of the phage plating technique (see notes on bacteriology).

It will be further noted that HP starter has been implicated in producing bitterness in cheeses. Emmons et al.(1962) and Jago (1962) have shown the lack of ability of this strain to produce amino nitrogen in milk cultures, although proteolytic activity is definitely present.

It will be noted that levels of acidity are not recorded in the manufacturing sheets after drying the vat because very little whey is lost from the small quantity of curd in the cheddaring blocks. Any whey collected does not give an accurate picture of the curd pH because of the time factor between expulsion and collection. Consequently, the stage for milling and salting were judged on a time basis only.

The "feel" of the curd is not readily detectable while handling with rubber gloves, so dry stirring was not practised and moisture control was achieved by regulating cheddaring time and stirring of the milled curd. Salting levels could not be used as a means of controlling moisture, as the salt had to be weighed and sterilized prior to manufacture. 100 grams were used in each case.

"Hooping up" the curd within the confines of the vat was tricky and badly dressed cheeses sometimes eventuated. Furthermore, the pressing arrangements, and the shortage of curd in a number of hoops causing "homers", led to a certain amount of mechanical openness in the mature cheese.

With the gluconic acid lactone cheeses the whey acidity gives no indication of the curd pH and the process is run on a time basis only. Curd was taken from the vat just after cutting and a regular check of its pH was made during making. The breakdown rate was obviously different in the two curd samples and, if the result may be translated to the cheese, could indicate differences in the cheese medium and, hence, explain any differences noted between the two cheeses.

Addition of the antibiotic, nisin, to the curd of the gluconic acid lactone cheeses was carried out in an attempt to reduce bacterial numbers in the cheese. Nisin is a naturally occurring inhibitory substance produced by a number of streptococci and was considered unlikely to influence the non-bacterial proteolysis of these cheeses. It was added, according to the manufacturers' instructions, as a solution. Application of nisin to processed cheese has proved successful and, as it is able to withstand the high cooking temperature of $80-90^{\circ}C$ used in processing, it should remain effective even when added to the milk as in the case of one cheese in the present study. It must be noted, however, that nisin is destroyed by a number of micro-organisms including certain yeasts, lactobacilli and streptococci, which could well reduce the value of this application.

The order in which cheeses were made was as follows:

1st	Starter inoculated cheeses	- Code RS-
2nd	Starter plus lactobacilli inoculated cheeses	- Code RSL
3rd	Gluconic acid lactone cheeses	- Code R --

Cheeses have been coded for simplicity of reference by using the initial letter of the various proteolytic systems known to be present in them. Numbers following the code letters signify the order in which they were made (viz: RS-1 was made before RS-2). The order of manufacture was chosen so that the most practised technique would be available for the gluconic acid lactone cheeses. As a check on the efficiency of asepsis is not possible with the RSL series they were not placed first.

It can be seen from the manufacturing schedules that some difficulties were experienced with handling the equipment for the first two cheeses, but thereafter manufacture was reasonably satisfactory.

(2)   CHEMICAL ANALYSIS OF CHEESES

Testing cheese samples for moisture, salt and fat (Gerber test) were carried out by the standard methods taught at this College.

Cheese pH was measured with cheese probe electrodes on a Radiometer pH meter.

(3)   BACTERIOLOGICAL METHODS

All bacterial counting was done by standard plate counting technique. The chief medium used was G.L.Y.P.A. (Glucose lactose yeast phosphate agar) as described by Robertson (1960). This has been shown to give more satisfactory results in counting bacterial populations in cheese than other common media (Robertson, personal communication).

Samples were handled as follows:-

(a) <u>Bacteriological check on quality of cows' milk</u>

Quarter samples were drawn with aseptic precautions and held in an ice box until plated, immediately after milking was completed, as follows:

1st check   - quarter samples plated separately on G.L.Y.P.A. and on L.T.I.A. (Lactose tryptone indicator agar) at $30^{\circ}C$ for $2\frac{1}{2}$ days.

2nd check   - quarter samples bulked to give one sample per cow. Plated on G.L.Y.P.A. at $30^{\circ}C$ for 3 days.

3rd check   - Plated as above at $45^{\circ}C$.

(b) <u>Analysis of Cheese Samples</u>

Cheese plugs were brought into emulsion according to the following procedure:

1. Weigh out 10 gms. of cheese on to sterile parchment.
2. Transfer to sterile Waring Blendor jar.
3. Add a sterile solution of 2% sodium citrate.
4. Emulsify by running blendor at slow speed for 1 minute and high speed for 2 minutes.
5. Allow to stand for 2 minutes.
6. Withdraw 1 ml. and serially dilute with sterile water.

Plate counts were made from plates using G. L. Y. P. A. medium and 3 days' incubation according to the following routine.

For the gluconic acid lactone cheeses (i.e. R -- series) -

Total Count at 30°C

Total Count at 45°C

For all cheeses having bacterial inoculations -

Total Count at 30°C

Total Count at 45°C

Phage Count at 30°C (for technique see Robertson, 1960).

After incubation, further information as to the types present was obtained, whenever required, as follows: 20 Colonies were randomly picked (using the disc described by Harrison, 1938) from a suitable plate and grown in Y. D. L. M. (Yeast dextrose litmus milk) at 30°C. Reactions were noted from daily observations and morphology observed on the freshly clotted culture by use of the methylene blue staining technique.

Further observations were carried out, where necessary, to determine more specific information.

Equipment and media were sterilized by autoclaving at 15 p.s.i. for 20 minutes. Petri dishes and pipettes were sterilized in a hot air oven at 350°F for 4 hours.

Discussion of Techniques Employed

In studying the aseptically drawn milk samples, the use of L.T.I.A. medium was employed to gain information on the extent of proteolytic activity of the organisms present. This information aided in the rejection of samples, since the bases used were undesirable flora as well as high total count.

The bacteriological study of the cheeses is purely supplementary to the thesis, as it functions only to determine the extent of contamination, and was not designed to provide a study of the flora present. In the perfect cheese no contaminating bacteria will exist. If any contaminating organisms are present it is sufficient to detect their presence.

The choice of which selective technique to use for enumerating the streptococci in the starter-inoculated cheeses was made, bearing in mind the following considerations:

1. Selective media, such as acetate agar, are prepared specifically to show numbers of non-starter organisms in cheese and will inhibit a number of species that could be contaminating.

2. The use of $\propto$ -bromopropionic acid (Robertson, 1960) inhibits the growth of a number of streptococci that could be contaminants and, furthermore, only a limited number of bacterial species have been characterised with respect to inhibition by this substance.

3. The chosen technique (Phage plating - Robertson, 1960) has the disadvantage that it will not destroy phage-resistant mutants of the starter strain. However, the use of phage plating cannot

affect other flora of the cheese and it provides the best available solution to the problem.

4. Phage resistant forms generally show poor acid producing activity, so can be separated from most other possible contaminant streptococci on this basis. This activity was checked, if thought necessary, by inoculation of colonies from the plates into yeast dextrose litmus milk. Observation of the morphology of isolates grown in Y.D.L.M. has also provided information in some cases.

5. Incubation of plates at 45°C provides a simple method of eliminating the inoculated organisms and also gives information about species that could have survived the heat treatment applied to the milk.

The cheeses inoculated with both starter and lactobacilli are impossible to check for contamination with existing techniques, and the 45°C incubation is the only attempt to check on adventitious flora. Colony shape and characteristics on the total count plates were also observed closely, and gross contamination with any of the distinctive organisms (e.g. moulds, pigmented types, etc.) would have been detected.

(4)   PROTEIN ANALYSIS

(a) Extraction of Protein from the Cheese Samples

This was accomplished by use of a modified form of the "Acetone Powder Method" of enzyme extraction (Morton, 1957). As far as is known, this method has not previously been used to extract cheese protein. It has been employed largely because of its simplicity and because of its non-denaturing effect on protein.

The procedure followed was:-

1.   Place about 3 grams of shredded cheese in a Waring Blendor jar.

2.   Add about 30 mls.of acetone at -15°C.

3.   Run blendor at low speed for 1 minute and top speed for a further 1-2 minutes.

4.   Filter immediately through Whatman No.40 filter paper on a Büchner funnel.

5.   Using the residue on the filter paper repeat 1-4 using   n-butanol at -15°C.

6.   Repeat as for 5 using acetone at -15°C.

7.   Place the dry protein powder in a vacuum desiccator at room temperature for at least 24 hours to ensure removal of all acetone.

8.   Store in a dark place at -15°C in an air tight bottle.

Discussion

As this technique is used in the preparation of enzymes there can be little danger of denaturing the cheese protein.

Acetone and butanol denaturation of proteins are temperature dependant reactions that become insignificant at temperatures below $-14^{\circ}C$. Butanol is employed because of its ability to break lipo-proteins bonds that exist in the fat globule membrane material.

To check that the technique is harmless to the present study, casein has been precipitated at pH 4.6 from bulk whole milk and the precipitate has been dried by the above technique. Starch-gel-urea electrophoretic patterns of the precipitated casein and the acetone powdered casein have been compared (see Figure 5).

(b) Starch-gel-urea electrophoresis

The technique of starch-gel electrophoresis, as originally described by Smithies (1955, 1959), has been modified by Wake & Baldwin (1961) to meet the requirements of casein study. This technique is based on the use of starch-gel as a support and urea as a protein dispersant. A discontinuous buffer system of Tris and Sodium borate is employed.

The apparatus used for this study was basically as described by Smithies (1955). It was constructed by the author and K.R. Aiyar. Two removable perspex trays were made to hold gels and they had internal dimensions of 260 mm. in length, 93 mm. in width and 9.0 mm. in depth. These were fitted into a unit constructed of perspex, which acted as a support, and had compartments for buffer and electrolytes. Electrodes were of platinum wire, 11.5 cms. in length, 4.4 sq. cms. in surface area, and of 18 S W G gauge.
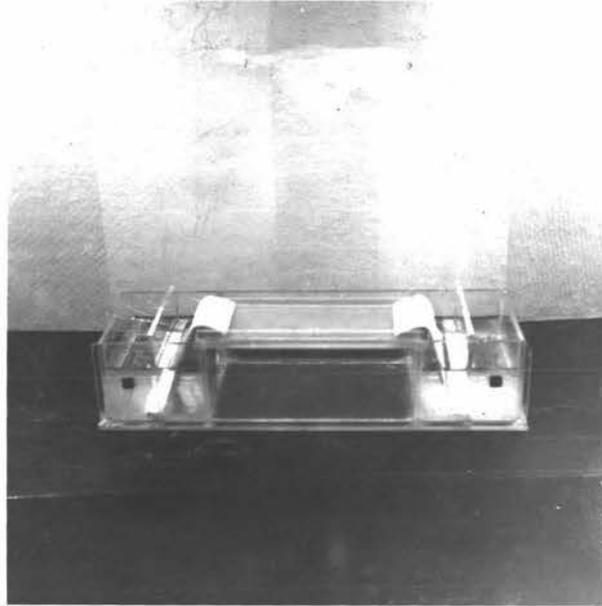
The Horizontal Electrophoresis Apparatus

The gel tray is supported between the two buffer compartments
and contact is made via. two strips of "Wettex" cloth. Contact
between buffer compartment and electrode compartment is made
via. a "Wettex" strip, placed beneath the perspex dividing wall.

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The filter paper bridges to connect the electrolyte and buffer compartments, as described by Smithies (1955), were replaced by a Wettex cloth which rested under the slotted partition between the two compartments (Dr.Esam Moustafa, personal communication). This arrangement permitted a compact, tidy and efficient contact.

The power supply for the electrophoresis apparatus was a Baird and Tatlock Stabilized Power Unit connected to the mains supply.

Slicing of the gels was carried out by use of a nichrome resistance wire (33 gauge), stretched taut between the supports of a fret-saw frame, and modified to allow different heights to which the wire may be set for slicing.

Details of the starch-gel-urea electrophoresis technique employed are:-

Gel preparation

The method of gel preparation was as detailed by Wake & Baldwin (1961), except that the step of degassing the boiling solution was omitted.

The gel was left to set in a humid atmosphere, maintained under a parafilm tent for 24 hours, and the gel was then placed in a refrigerator for 5 hours to reach equilibrium at the temperature of electrophoresis.

Sample Preparation and Application

0.2 gm. of the dry sample of cheese protein was wetted with a

little water with the aid of a glass mortar and pestle. The suspension
was then poured into a test tube containing 4.2 gm. of urea crystals and
0.2 gm. of starch grains. Distilled water was added to make up 10 mls.
The samples were then shaken for half an hour or longer, until permanent
suspension of protein and starch was achieved.

Three protein samples at 2% concentration and one casein standard
solution at 1% concentration were prepared for each gel.

Samples were held at refrigerator temperature until used, not more
than 12 hrs. later, to allow further hydration to occur. The casein used
for preparation of the standard sample was prepared in bulk from 5 gallons
of milk by K. R. Aiyar, by the method of iso-electric precipitation. Casein
was stored at -15°C until required for use.

At the appropriate time the gel tray was removed from the
refrigerator, and the slot-former and tray sides were removed. The surface
layer of the gel was sliced off and discarded to leave a gel of 6mm. in
depth. Samples were then filled into the slots using narrow nozzled pipettes.
The slots were covered by small squares of film and these were, in turn,
covered by a liquid petroleum jelly. A sheet of white polyethylene film
was then placed over the gel surface leaving only a few centimeters bare at
each end to allow electrical contact. Tray sides were replaced, so that all
surfaces of the gel were covered to minimize moisture evaporation from the
gel. The gel was then placed in position and a tent of parafilm was folded
over the unit.

Electrophoresis

Electrophoresis was carried out at 7-12°C. Care had to be taken to ensure firm and even contact of the Wettex cloth onto the gel, or an uneven boundary line could result.

Electrophoresis conditions were 160 Volts for $16\frac{1}{2}$ hours.

The borate buffer can be used for three electrophoretic runs, provided the direction of electricity flow is reversed for each run (Esam Moustafa, personal communication).

Slicing and Staining

After electrophoresis, the gel was uncovered and sliced twice to obtain a 2 mm thick strip from the centre of the gel, where effects of water evaporation from the surface and uneven movement, due to contact with the perspex tray, were negligible. Use of a strip of polyethylene film as backing for this central strip during handling was necessary, for it would rip easily if handled with bare hands.

The gel was stained for 7 minutes in a solution of the following composition:-

2 gm. Napthalene Black

50 ml. Methanol

50 ml. Distilled water

10 ml. Acetic acid

Staining & Washing Trays

(Cheese grater used as a gel
support can be seen in the
staining tray).
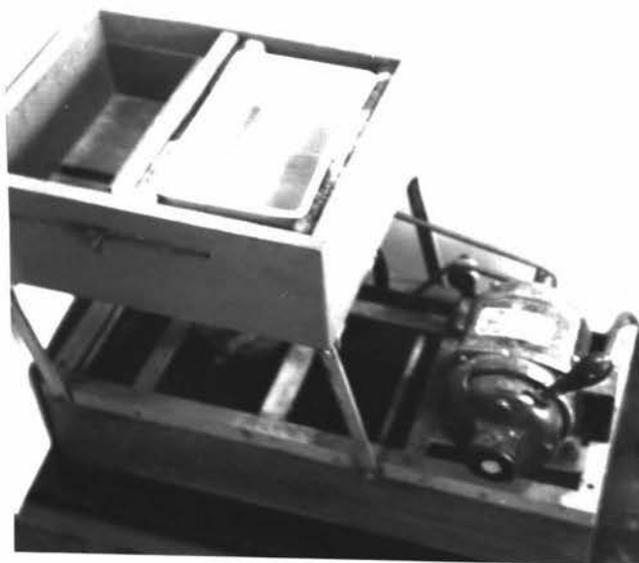
Shaker

(used for washing of stained gels)

Washing

After staining, the gel was washed in a solvent which removed all the dye, except that which was protein bound. The washing solvent used had the composition of: 50 parts methanol; 50 parts water; 10 parts acetic acid, and 10 parts glycerol.

The stained gel was transferred to a covered dish containing the solvent and placed in a reciprocating shaker. As the gel almost floated, the slow movement of the shaker caused the solvent to move lazily over the surfaces of the gel.

Superficial stain adhering to the gel was readily rinsed off, and when the solvent became blue it was poured away and replaced. In total, four washes were employed. The washing routine was standardized so that it was repeatable, as follows:-

1st wash (to remove superficial stain)	- 15 minutes
2nd wash	- 30 minutes
3rd wash	- 30 minutes
4th wash	- 60 minutes

This was a minimum treatment for each gel and it usually left the gel quite white with dark bands where the protein material was located. Owing to unavoidable variability in the thickness of the gel slices, it was occasionally necessary to continue the washing for a short while longer until the white background became apparent. A total washing period of longer than three hours indicated a very thick gel slice which was discarded, as it was unlikely to provide a clear pattern for scanning.

The Scanning Device

(a) The Platform in place.

The Scanning Device

(b) An exploded view showing
     position of gel strip
     during passage past the
     light source.

After use the solvent was decolourized, by drawing it through an activated charcoal pad on a Büchner funnel, and then reused. Solvent decolourized in this manner was used for the initial washes of the gels, but for the final wash, only a freshly made up solvent was used. Decolourizing of the solvent can not be repeated more than twice because the vacuum applied would draw off some of the volatile components and some glycerol appeared to be lost (presumably it was held up on the charcoal pad).

Up to this stage, the gel was particularly tender and could not be handled more than necessary. To allow it to toughen up by drying it was laid out on a glass surface and covered with filter paper. After two days the gel had dehydrated sufficiently for further, and more rigorous, handling.

Scanning the Gel Patterns

The semi-dry gel was cut into narrow strips following the path of migration of each sample. Each strip was covered on both sides by clear cellotape. These mounted strips were then gradually drawn through a Beckman D.U. Spectrophotometer and a graph drawn from the readings of the percentage transmission of light through many points along the gel.

This was made possible by the use of a wooden platform that could be fitted onto the spectrophotometer chasis. A sliding wooden square served to draw the gel strip past the light source. A 100 mm.rule of clear plastic was attached to the arm of the square and served as a measure to plot the percentage transmission readings onto graph paper ruled in millimeter squares.

This procedure produced a graph showing peaks where the dyed protein components reduced the amount of light transmitted through the gel strip.

Operating conditions were:- (a) A slit width of 0.05 mm.was used to provide clear definition.

(b) A wave length selector was set at 610 mμ.

Discussion of S.G.U.E. technique

The technique of starch-gel electrophoresis has found useful application in the study of blood serum proteins, and recently investigations aimed at improving it to provide quantitative assessment in this field have been published (Pert et al. 1957; 1959, and Rubinstein et al. 1960). It was found that variation of only $\pm$ 0.01 units in pH of the gel or variation of only $\pm$ 0.0005 M boric acid concentration in the buffer could cause a variation in the patterns of protein components observed. Assuming that these findings apply with equal force to starch-gel-urea electrophoresis, it is apparent that quantitative accuracy is not obtainable in this study using reagent grade chemicals. Quantitative accuracy is not really necessary where the electrophoretic patterns obtained are to be used for comparative purposes only. It will be equally apparent that rigid standardization of technique is necessary if electrophoretic patterns obtained from over 30 gels are to be comparable.

To permit the necessary standardization, the published method of starch-gel-urea electrophoresis (Wake & Baldwin, 1961) has been modified to fit the dictates of the following considerations.

(a) Voltage gradient

For the voltage gradient to be repeatable, both the voltage applied and the distance between the electrical contacts must be constant from gel to gel. The former condition was met by use of a stabilized power unit and the latter by use of a length of film that covered all, except a few centimeters at either end, of the gel. As this piece of film was 20 cms.in length,the Wettex contacts were always placed at this distance apart.

This means that the voltage gradient applied was invariably 8 Volts/cm.

(b) Period of Electrophoresis

The period allowed for electrophoresis may be standardized by either time or distance of migration of the boundary line. The former method was employed for the following reasons:-

(i) It was impossible to exactly measure the distance of migration without switching off the current.

(ii) It was more convenient for purposes of routine to have a set time to switch off the power.

(iii) If migration was slow for any reason it will be readily seen. Samples that had not migrated at least 100 mms. were discarded as abnormal.

(iv) The time of 16 hours was convenient, as it allowed use of the refrigerator over-night when it would not normally be in use, thereby minimising temperature fluctuations or the possibility of loosened contact.

Various electrophoretic conditions were employed. Wake and Baldwin preferred the use of 170 Volts for 16 hours, but little difference in resolution obtainable was noticed within the limits of 160-175 Volts.

Extreme variation did cause abnormal patterns to be obtained. A gel run at 200 Volts for 13 hours gave an indistinct pattern with the most mobile component actually running in front of the boundary line.

Use of low voltages caused slow migration, thereby introducing difficulties into the routine of daily analyses. Two gels were run using 120 Volts for 22 hours, but the results obtained were no better than those obtained under the conditions finally chosen.

(c) Preparation of Samples

It will be noted that urea solutions were made up freshly with each sample, so that the possibility of cyanate formation is minimised or eliminated. (ref. to Review of Literature for discussion on this point).

Mechanical shaking of the samples was necessary to get the dry cheese protein into proper dispersion. Shaking was also desirable, even with casein samples, to get a proper dispersion of the starch present in the solution. The starch would otherwise settle out and not perform its function of preventing electrodecantation.

(d) Degassing of the Molten Gel

Both Smithies (1955) and Wake & Baldwin (1961) advocated the use of vacuum to cause vigorous ebullition of the hot starch suspension to remove the air bubbles present in it. Pert (1959) commented on the

desirability of standardizing the de-aeration procedure because of the loss
of moisture which must occur. It is difficult to see how this could be done.
It was observed that, provided the starch solution was poured into the gel
tray while reasonably hot (about $50^{\circ}C$), any air bubbles floated to the top
of the solution before gelation set in. When the top slice of the gel is
sliced off, prior to sample application, all bubbles that had not burst were
removed. The technique described by Smithies (1955) required that a parafilm
sheet should lie upon the gel surface while it set. In this study, gelation
occurred under a parafilm tent which permitted any air bubbles present to
break out of the gel surface. Recently, Elton & Ewart (1962) reported a
similar experience with starch gels.

(e) Edge effects

The apparatus described by Smithies (1955) had provision for six
sample slots. It was found that samples which ran within 1 cm. of the edge
did not produce clear patterns and were liable to confuse other samples
running beside them. Consequently, it was decided to use only the inner
four sample slots.

(f) Length of Staining Period

A staining period of 30 seconds was employed by Smithies (1955) and
Wake & Baldwin (1961), but this was found to be inadequate for clear and
repeatable results in the present study. Pert (1959) used a 5-minute staining
period. Rubinstein et al. (1960) showed that the uptake of napthalene black
stain by proteins continues for 4 hours. In view of these different practices,
many staining periods were employed, and eventually 7 minutes was accepted as
sufficient to allow adequate penetration and staining.

(g)   Decolourization of Solvent

Pert (1959) described a method of circulating solvent from the washing tray, through activated charcoal, and back to the washing tray. This technique has the advantage of leaving the gel continually in contact with clear solvent. Apparatus employing this principle was set up and used. Difficulties arose because finely divided charcoal remained in the solvent after decolourizing and settled out onto the gel, thereby leaving a black smear over the gel surface. This and further difficulties of ensuring an even contact of the solvent with all the surfaces of the gel caused the idea to be discarded.

Use of a charcol pad over a filter-paper filtered out much of the charcoal in the decolourized solvent, and use of a final wash with fresh solvent, as finally employed in this study, gave good and quite reproducible conditions.

(h)   Period of Washing Gel

This was restricted because it appeared that the protein bound dye of the minor bands may be partially removed if washing was continued too long.

This is illustrated by the fact that, in some gels, the colour in the protein area of the gel was actually less than the background colour of the gel. Apparently some sort of differential washing occurred. Insufficient washing of the gel will not remove all of the free dye present in the pores of the gel, so that the presence of minor peaks will not be detectable (see Figure 2). Irregularities in the thickness of the gel slice would probably explain the difficulties noted and the use of an improved slicing technique could improve the method.

(i)   Use of Cellotape to Strengthen Gel Strip while Scanning

The white colour of the gel, soon after washing, reverted slowly to a blue colour as it dried.   Presumably this blue background colour was due to the background protein levels observed by Gordon (1960). As the gel dried it became clearer, but the drying also concentrated the background blue colour which became progressively deeper.   Concomitant with this phenomenon, the sharpness of the dark blue protein bands was reduced.   It would be an ideal practice to scan the gel strips immediately after they are washed and clear, but this was not possible until they toughened on dehydrating.   The best practicable procedure was to scan the gel strips as soon as possible after washing and to invariably allow a standard period for dehydration.

Two days of drying proved to be the minimum period practicable.   Even after two days the gel strips were tender and liable to stick to the chasis of the Beckman spectrophotometer.   The tensile strain necessary to draw the strips through the Beckman was often sufficient to rip the strips if they became caught up on projections, etc.   To permit regular and unhindered scanning of two-day gel strips, it was necessary to apply cellotape to both sides of each strip.   This provided strength, smoothness and flexibility to the strip, and prevented the sticky gel from picking up dust and dirt which would confuse the patterns obtained.

Imperfections in the clarity of the cellotape could have caused difficulties in scanning.   With the main peaks on the final graphs, it was always possible to join the plotted points by a series of straight
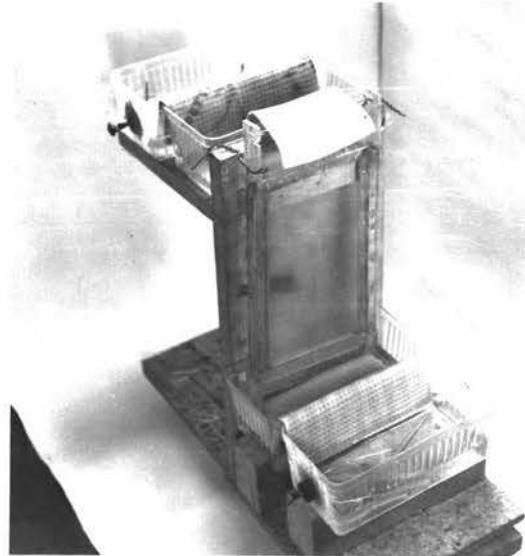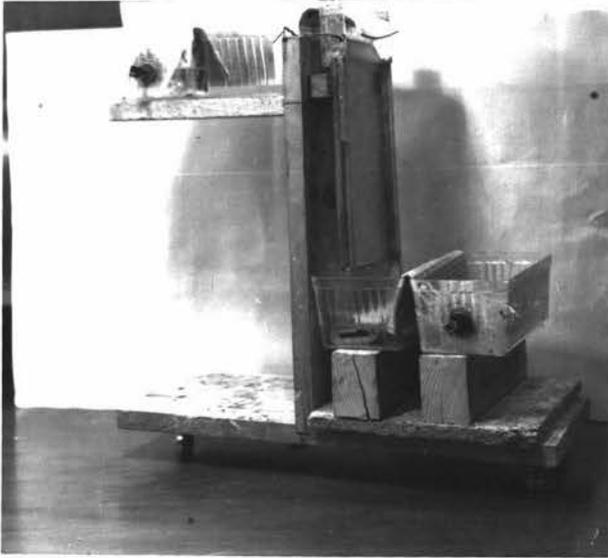
The Vertical Electrophoresis Apparatus

The gel tray is supported on a wooden frame. The foot
of the gel stands in the buffer of the buffer compartment
and is borne by a wad of "Wettex" cloth. Contact to the
upper part of the gel is made via "Wettex" cloth.
Electrode compartments are quite separate from the buffer
compartments.

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lines, so that minor changes in the form of peaks were sharply defined. Cellotape was not found to contribute irregularities in any way and, consequently, minor changes in peak contours were considered to be significant. Possibly some of the very minor variations near the starting slots could be blamed on this source of error, but certainly no major error could have been introduced. The advantage of clarity gained by scanning at two days far outweighs the possible risk of clarity imperfections in the cellotape.

(j) Use of Vertical Starch-Gel Electrophoresis

Electrophoresis carried out in the vertical plane has been described by Smithies (1959) and employed in casein study by Neelin (1962). Apparatus necessary for this technique was made to the plans given by Smithies. Difficulties in handling the larger gels were eventually overcome, but it was felt that the patterns obtained were much more difficult to achieve than with the horizontal technique and superior quality was never obtained. Because of this, pressure of time, and the fact that vertical gels required twice the volume of materials necessary for horizontal gels, it was decided to economize. Use of vertical starch-gel-urea electrophoresis was consequently abandoned.

(k) Cyanate formation

It is known that a degradation product of urea in solution, ammonium cyanate, can form artifacts by complexing with $\propto_s$-casein (see Review of Literature). The appearance of cyanate in urea solutions is slow, but is accelerated by heating. Boiling of a urea solution for 5 minutes

will give rise to a positive test for presence of cyanate (Werner, 1923). In the manufacture of gels for electrophoresis, the urea is added to the hot starchy suspension and it was thought possible that cyanate could be present in the urea starch-gels. Urea goes into a solution by an endothermic process however, and it was found that the addition of urea to the starch suspension reduces the temperature. Even after continued heating, following the addition of urea which was normal to gel manufacture, the temperature of the suspension did not rise above 65°C. Tests for the presence of cyanate in pieces of gel, and in the sample solutions using the method of Werner, gave negative results in all cases.

An attempt to Extract Starch from Potatoes

In view of the expense of imported hydrolysed potato starch, made by the Connaught Laboratories in Toronto, an attempt was made to extract starch from locally grown potatoes and modify it so that it could be used for the experimental work.

Commercially unusable potatoes were obtained from the property of Mr.H.T.Wenham near Palmerston North. The following methods of extracting and modifying the starch were carried out.

Extraction of Starch

1. Wash, peel, and slice the potatoes.
2. Grind in a Waring Blendor for 5 minutes with 2-3 volumes of distilled water.
3. Screen the slurry through a nylon cloth. Continuously scrape the cloth with a spoon and rinse the starch through it with a jet of distilled water.
4. Return the pulp to the blendor and grind for an additional 5 minutes with sufficient water to give a thin slurry.
5. Rescreen this slurry and discard the pulp.
6. The combined starch suspensions are then rescreened through a cloth of finer mesh into a tall pyrex beaker.
7. Allow to settle for approximately 1 hour or until the starch deposits as a firm dense cake.
8. The best starch settles out rapidly, so when fibre begins to settle on top of the starch cake discard the supernatant.
9. Rescreen through a fine mesh cloth, then allow the starch to settle in a tall beaker. Discard supernatant.

10. Continue the resuspension procedure with distilled water until the starch settles as a dense firm cake, with no evidence of a cloudy supernatant or a soft top surface.

11. The starch cake is then suspended in methanol, filtered on a Büchner, washed with methanol, and dried at 40°C to 50°C.

Defatting of the Starch

1. Suspend 1 Kg. of the dry starch in 5 litres of 85% methanol in a 5 litre flask equipped with reflux condensor and a motor driven stirrer.

2. Heat at gentle reflux temperature for 1 hour in a water bath.

3. Filter the starch hot on a Büchner and wash with 85% methanol.

4. Wash the starch cake twice further with 85% methanol.

5. Dry at 50°C.

Hydrolysis of Starch

1. Take the prepared starch and double its volume of acetone/ concentrated HCl mixture (100/1; v/v) and hold at 38.5°C overnight to equilibrate to that temperature.

2. Mix the components of the reaction at 38.5°C.

3. Stand for 40 minutes (this time of hydrolysis will have to be predetermined by small scale trial and error experiments).

4. Add 150 ml. of aqueous, molar sodium acetate to stop the reaction.

5. Filter on a Büchner.

6. Wash with distilled water.

7. Stand in distilled water overnight to remove all traces of acetate.

8. Filter on a Büchner and wash the cake with distilled water.

9.   Dehydrate with acetone.

10.   Dry at $45^{\circ}C$ - $50^{\circ}C$.

(These details were developed from comments given by Schoch (1955) and Smithies(1955)).

Discussion Related to an Attempt to Manufacture Hydrolysed Starch

A batch of hydrolysed starch which was prepared by the author and K.R. Aiyar proved unsatisfactory for use. The starch-urea gels formed from it were weak, unless prepared at a concentration of 13% starch in gel, instead of the normal 9.4% starch in gel (Wake & Baldwin, 1961). At the higher concentration gels set satisfactorily, but on electrophoresis protein patterns obtained were poorly separated and showed heavy tailing. Poulik & Smithies (1958) stated that over hydrolysis of starch caused a progressive weakening of gels and this apparently occurred in the present cake.

As noted previously, a correct time of hydrolysis must be selected to suit each batch of starch that is made up. In the present case this was done by hydrolysing starch on a small scale and withdrawing aliquots from the hydrolysis mixture at time intervals of 30, 45, 60, 75 and 90 minutes of hydrolysis. These samples were washed, dried and submitted to Dr. Esam Moustafa of the D.S.I.R., who had equipment suitable for small scale gels, and also had experience of using starch-gel electrophoresis with blood serum proteins. He obtained satisfactory separation by comparison to commercially available hydrolysis starch (Connaught Medical Laboratories, Toronto) by using the samples of starch that had been hydrolysed for 30 and 45 minutes. He recommended that we employ 40 minutes as hydrolysis time for preparation of our bulk supplies of starch. The bulk of the starch ($1\frac{3}{4}$ Kg.) was hydrolysed according to this recommendation. It was not realized that Dr. Moustafa was using starch-gels in which the concentration of starch in the gel is 13%,

whereas in the present study urea was incorporated in the gel to give a gel with only 9.4% of starch in gel. For our purposes the starch had apparently been over hydrolysed.

Hydrolysis of starch influences the gelation by dividing the starch granules into smaller parts, so that gel structure is formed from small units (Hofstee & Willigen, 1953). This improves the extent of retrogradation of the starch (i.e. the reorientation of the dispersed starch after boiling to a more or less organized structure) and thereby raises the strength of the resultant gel. However, there is an optimum level of hydrolysis. Over hydrolysis will reduce the particle size to the point where dispersion of the particles is too great so that, on heating, the grains cannot swell sufficiently to immobilize the available water and, consequently, a strong gel will not form.

Consideration of this information will explain why differences in gelling behaviour occur between the conditions of the test in Dr.Moustafa's laboratory and in our laboratory where smaller concentrations were in use. The extent of hydrolysis cannot be the only reason for this problem however, because the commercially available hydrolysed starch is satisfactory for making both starch-gels and starch-urea gels. There are other factors that influence the gelation of starch suspensions and some of these must have bearing on the present discussion. These are as follows:-

(1) It was necessary to use a large quantity of potatoes (3 sugar sacks full) to get the required 2 Kg. of starch. The scale of equipment available was too small to allow quick extraction of the starch. About 2 days was required for this process and this allowed time for microbiological action to occur during the settling period. Natural potato starch contains

adsorbed potassium which promotes the associative forces between the micellea of the starch-gel. Lowering of the pH (viz: through microbiological activity) can remove the potassium to some extent and, thereby, detrimentally influence gelation.

(2)   Starches extracted from potatoes grown in different localities appear to have differing properties of retrogradation (Hofstee & Willigen, 1953). Starch consists of a mixture of the straight chain molecules, amylose and the branched chain molecules, amylopectin. It is apparently easier for the straight chain amylose molecules to associate and the extent of retrogradation in starch-gels will be dependent on the ratio of amylose to amylopectin in the starch. Presumably various varieties of potato could have varying ratios of these two components, and hence show different properties of retrogradation and association. Furthermore, the potatoes freely available to us were not sound and were of reject quality. These considerations may help explain the differences in the characteristics of the commercially available starch and the starch that we have extracted.

(3)   Close control of the hydrolysis temperature was not possible with the equipment available. Pert (1959) found that hydrolysis temperature variations of only $\pm$ 0.2°C were equivalent to a variation of $\pm$ 5 minutes in time of hydrolysis. This consideration could even indicate that differences in treatment between the trial samples and the final bulk extracted have occurred.

From the above mentioned considerations, it can be seen that it is difficult, or even impossible, to obtain starch of comparable characteristics to that in use overseas by extraction from New Zealand potatoes, as we have tried to do.

Other alternatives suggest themselves however.

Firstly, it may be possible to purchase commercially available potato starch and modify it to suit the conditions of experiment. No knowledge would be available, however, as to the manufacturing history of the starch. It is known that microbiological action often occurs during commercial extraction of starch. Enquiries revealed, furthermore, that no potato starch is commercially available in this country.

Corn and wheat starches were also investigated. They were defatted and made up into gels, but gave weak and opaque granular gels, not at all suitable for electrophoresis. Potato starch is known to vary in its chemical and physical characteristics from grain starch. Potato starch contains higher amounts of phosphorus that is bound as a monoester to the starch. Furthermore, potato starch contains little or no free fatty acids, whereas corn starches contain 0.84% and wheat starches have 0.50%. Even after defatting treatment with methanol the fat content cannot be completely removed (Schoch & Elder, 1955). These authors stated that fatty acids (and other compounds too) form insoluble complexes with the linear fraction of the starch leading to precipitation within gels. This reaction "resulted in increased opacity, a 'short' thick paste consistency, and a complete loss of gel strength". This was confirmed by our experience.

In the face of all the preceding information, it was considered wise to purchase an internationally reputable brand of hydrolysed starch and be sure of a reliable technique.

<u>Grading of the Experimental Cheeses</u>

   All experimental cheeses were graded after 4 months of curing.
The commercial cheese used as a comparison was included for grading, although
it was only 10 weeks' old at the time. A panel of three members of the Dairy
Research Institute staff graded the plugs independently and without knowledge
of the origins of the individual plugs. A summary of the comments made is
given below.

Cheese Code	Inoculation	Body	Colour	Closeness	Flavour
R — 1	-	Weak Slightly mealy	Slightly mottled	$17\frac{1}{2}/20$	Bitter, unpleasant Slightly rancid
R — 2	-	Firm Mealy	Slightly mottled	18/20	Very bitter Cardboard, Metallic
RS - 1	HP	Normal	Normal	18/20	Bitter Metallic after taste
RS - 2	HP	Normal	Normal	$18\frac{1}{2}/20$	Sharp, slightly bitter No agreement
RS - 3	HP	Crumbly Firm	Normal	18/20	Flat, slight defects No agreement
RSL1	HP 25.2	Normal	Normal	$18\frac{1}{2}/20$	Slightly bitter
RSL2	HP 25.2	Normal	Normal	18/20	Bitter Metallic after taste
Commercial	HP	Normal	Normal	$18\frac{1}{2}/20$	Mild Cheddar flavour

   From this summary of grading comments, it may be seen that the
cheeses were of acceptable to good quality, but that the typical cheddar flavour
was lacking. The 10-week-old commercial cheese gave the only indication of

typical flavour development.   This lack of distinctive flavour accentuated those off-flavours that were present and incidentally it led to a disagreement within the grading panel on a number of points.

Bitterness was noticeable in most of the cheeses and it was particularly distinct in the cheeses made with gluconic acid lactone.   This was to be expected in the inoculated series, as the HP starter strain has been implicated in contributing to bitterness in commercial cheeses (Emmons et al. 1962).   The more distinct bitterness of the gluconic acid lactone cheeses can probably be ascribed to the formation of bitter peptides by rennet action. Such bitter peptides are presumably broken down to unobjectionable forms by the bacteria inoculated in the other experimental cheeses.   One of the grading panel noted cardboard and metallic elements in the flavour of the gluconic acid lactone cheeses which indicated that fat hydrolysis was not proceeding normally. Conceivably, the extra bitterness of the lactone cheeses could be due to an imbalance of lipases forming tributyrin, rather than a wholly proteolytic defect.

In the inoculated series the off-flavours noted were within the range normally experienced and the main point for comment is the lack of characteristic cheddar flavour.   This finding agrees with Perry & McGillivray (1963) and Mabbit et al. (1955) who suggested that rennet starter and lacto-bacilli do not provide all of the components necessary to characteristic cheddar flavour.

The colour of the cheese plugs was generally acceptable, except that a mottled defect appeared in the gluconic acid lactone cheeses.   This was apparently due to the curd particles not knitting together properly and so destroying the evenness of the colour.

Closeness was generally acceptable, although some mechanical openess was noted. This could well be due to the small press that was used.

In a study of protein breakdown the body of the experimental cheeses is of prime importance. The inoculated cheeses were good in this repsect with the exception of RS-3 for which the curd was cut a little too fine and dried out in the vat leading to a final body that was a little too firm. The gluconic acid lactone cheeses (R - - 1 and R - - 2) were not normal. Cheddaring in the vat was only partially successful and the cheese plugs drawn during the first month of curing were particularly crumbly. At the time of grading the body had improved considerably, but was still mealy in character.

It may be concluded that the inoculated cheeses were all sound and of reasonable to good quality, but lacked in flavour after 4 months of curing. Those cheeses made with gluconic acid lactone were intensely bitter and had a mealy body. Both of these facts indicate that the proteolysis was not normal.

Chemical Analyses of Asceptically made Cheeses (14 days' old).

Results of chemical analyses are as follows:-

Cheese No.	pH	Fat %	Salt %	Moisture %	M.N.F.S. %	F.W.F.S. %	Salt/ Moisture
RS - 1	5.1	30.0	1.25	40.61	58.0	50.5	3.08
RS - 2	5.25-5.3	34.0	1.32	36.39	55.4	54.7	3.63
RS - 3	5.3	32.5	1.33	35.23	52.2	50.2	3.78
RSL1	4.95-5.0	31	1.34	37.50	54.4	49.6	3.58
RSL2	5.2	32.5	1.4	38.31	56.7	52.7	3.66
R - -1	5.5	34.7	2.16	33.11	50.7	51.9	6.5
R - -2	5.6-5.7	34.3	1.91	32.67	49.7	51.0	5.85
Commercial	5.01	38.21	1.64	33.30	53.71	57.4	4.92

From the above table it can be seen that the inoculated cheeses make up a fairly even line. The values for M.N.F.S. are related to body of the cheese and the reason for the overfirm body of the RS-3 cheese can be readily seen. The first cheese made, RS -1, carried a little much moisture, but this was not commented on by the grading panel so was not a severe defect. Salt and fat levels were not controllable but were, nevertheless, reasonably even. The levels of salt in moisture were rather lower than normal and these may influence both rennet and bacterial action in the cheeses. Comparability between cheeses should be reasonable however.

The gluconic acid lactone cheeses (R - -1 and R - -2) differ from the others in a number of respects that could influence the proteolytic breakdown to some extent, viz: high pH, low M.N.F.S., and high salt in moisture levels. Considering the difficulties of manufacturing cheeses without starter the moisture expulsion has been most successful. It is strange that these two cheeses, both with low M.N.F.S., should be graded, one with a weak body and one with a firm body. Apparently the correct moisture level has almost been achieved, but at a lower level than for cheeses containing starter. On the basis of chemical composition the gluconic acid lactone cheeses approximated the inoculated series, but might be sufficiently different to influence the direction of proteolysis in the two series. As a duplicate pair the two cheeses are closely similar in composition.

The commercially made cheese that has been included in the thesis for comparison is of excellent quality on both chemical and grading criteria, and had developed a good cheddar cheese flavour after 4 months of curing.

Bacteriological Results and Discussion

The Aseptically Drawn Milk Supply

The bacteriological quality of the milk drawn from the eight highest yielding cows of the No.2 dairy herd at the Massey College Farm was assessed. The results are given in Table I.

TABLE I

BACTERIOLOGICAL QUALITY OF ASEPTICALLY DRAWN MILK

1st CHECK: Selection of Cows to Supply Milk

Quarter Sampled	Total	Alkali Reaction (Count per ml.)	Acid Reaction (Count per ml.)	Proteolytic Reaction (Count per ml.)
Cow No.6				
L. F.	100	0	0	0
L. B.	6	0	0	0
R. F.	66	0	0	0
R. B.	45	0	1	0
Cow No.85				
L. F.	0	0	0	0
L. B.	1008	0	High	High
R.F.	0	0	0	0
R. B.	468	0	91	91
Cow No.76				
L. F.	High	0	206	0
L. B.	High	0	High	0
R. F.	456	?	0	1
R. B.	10	0	0	0
Cow No.79				
L. F.	121	0	2	3
L. B.	0	0	0	0
R.F.	0	0	0	0
R. B.	0	0	4	0

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Table I continued....

Quarter Sampled	Total	Alkali Reaction	Acid Reaction	Proteolytic Reaction
		(Count per ml.)	(Count per ml.)	(Count per ml.)
Cow No. 88				
L.F.	30	0	2	2
L.B.	22	0	11	11
R.F.	594	0	35	35
R.B.	144	0	43	43
Cow No. 28				
L.F.	16	1	3	0
L.B.	1248	0	232	232
R.F.	135	0	11	11
R.B.	2640	0	High	High
Cow No. 59				
L.F.	High	0	0	0
L.B.	352	0	High	High
R.F.	High	High	High	High
R.B.	High	High	High	High
Cow No.M61				
L.F.	3	0	2	2
L.B.	27	0	0	0
R.F.	37	0	8	8
R.B.	42	0	2	2

NOTE:

Designation of quarters - L.F. = Left Front
L.B. = Left Back
R.F. = Right Front
R.B. = Right Back

Total Counts from G.L.Y.P.A. Plates

Other Counts from L.I.T.A. Plates

From these results, four cows were selected to provide milk of high bacteriological quality for the experimental cheesemaking. The cows selected were numbers M61, 79, 6 and 88, but because these four did not produce enough milk to fulfil requirements, it was necessary also to include cow number 85.

It will be noted from the results given above that the counts made from the L.T.I.A. plates (Lactose Tryptone Indicator Agar) were of only limited value and often gave counts much below those obtained from the G.L.Y.P.A. plates. This was largely due to colonies that were of pin-point size on the G.L.Y.P.A. plates, but did not appear on the L.T.I.A. plates. Presumably this medium was less suitable for the growth of these types. Because of this finding, L.T.I.A. has not been employed for subsequent work.

Despite the inclusion of milk from cow number 85, which had one quarter producing milk with a high count, the milk received at the factory was of quite acceptable quality, viz:

1st day - 86 lbs of milk with a total count of 250/ml.

2nd day - 88 lbs of milk with a total count of 200/ml.

3rd day - 86 lbs of milk with a total count of 2000/ml.

During these three days milk quantities were lower than desired and insufficient curd was made to adequately fill the cheese hoops. The prospect of a further fall in milk quantity enforced a second check to select more cows to bolster the supply.

Because of the lack of time and materials, quarter samples were bulked and well mixed to give one sample per cow. Counts were derived

on G. L.Y.P.A. medium at 30°C. It was possible to allow only $2\frac{1}{2}$ days for incubation. Results were as follows:-

TABLE II

2nd CHECK: Selection of Cows to Supply Milk

Cow No.	Count per ml.
48	185
57	644
37	4700
47	326
81	19
2	High
65	High
68	High

From these figures, cow numbers 81 and 48 were chosen for inclusion in the milk supply herd and cow number 85 was withdrawn.

From this time onwards the milk received at the factory was unsatisfactory. The fall in milk production continued and, despite the extra cow included in the supplying herd, an adequate quantity of milk was not received. This led to difficulties in pressing the cheeses. Furthermore, counts of the milk, both raw and after heat treatment, were high which indicated that a number of heat resistant organisms were present.

TABLE III

Day	Weight Received	Raw Milk Count	Pasteurized Milk Count
4th	93 lbs.	10^4/ml.	200/ml.
5th	83 lbs.	10^3/ml.	1000/ml.
6th	83 lbs.	10^5/ml.	-
7th	88 lbs.	10^3/ml.	-

(Counts of the pasteurized milk on the 6th and 7th day
were not made until after the addition of gluconic acid
lactone to the vat so are not included, as they do not
accurately indicate the effectiveness of pasteurization).

When these high results were obtained a concentrated effort to
sterilize the equipment was undertaken. Milking equipment was scrubbed even
more scrupulously clean and later soaked in boiling water, in addition to the
normal treatment in Zepharin solution. This apparently had no effect on
counts of the milk supplied. At that time, analyses of the cheeses were
showing the presence of heat tolerant organisms in larger numbers than
anticipated. From these observations, it would appear that the origin of
these heat tolerant types must be at the udder of the cow. To check this
inference, a third check on all cows was carried out with incubation of
plates at 45°C for three days. This gave the following results:-

TABLE IV

3rd CHECK: Detection of Origin of Heat Tolerant Organisms

Cow No.	Count per ml.
M61	4
6	70
88	45
79	6
48	540
85	125
81	2500

These figures clearly show that the major source of heat tolerant organisms was in the milk of the two cows which were included in the supplying herd after the third day of cheese manufacture. Apparently these organisms were of only pin-point size on the plates incubated at $30^{o}C$ in the second check. Furthermore, these plates were only given $2\frac{1}{2}$ days' incubation, and it is possible that the pin-point colonies may have been overlooked at this stage.

It may be concluded that the milk supply over the first three days was of excellent quality. Thereafter, the bacteriological quality was lower than desired, because of the appearance of heat tolerant bacteria which were not detected on plates incubated at $30^{o}C$ for $2\frac{1}{2}$ days. These organisms were able to survive the heat treatment applied and were, consequently, able to appear in the cheeses. The raw milk received at the factory was, nevertheless, of very high bacteriological quality and was never more than 3 hours' old, so that the possibility of bacterial proteolytic activity prior to cheese manufacture has been minimised.

Bacteriological Check on the Asepsis of Cheese Manufacture

TABLE V

Results: Bacteriological Examinations of Aseptically Made Cheeses

Cheese No.	Age	G.L.Y.P.A.Plate Counts of Cheese					Plate Counts of Milk	
		30° Total	Phage	$45^{\circ}C$	Mould		Raw Milk	Pasteurized
			(Count per gram)				(Count per ml.)	
RS-1	1 day	1×10^8	2×10^6	$< 10^3$	-		250	.5
	14 days	3×10^7	-	$> 10^4$	-			
	1 month	2×10^6	-	$< 10^3$	-			
	4 months	3×10^6	3×10^6	0	0			
RS-2	1 day	2×10^9	1×10^7	1×10^3	-		200	-
	14 days	4×10^7	-	6×10^6	-			
	1 month	6×10^6	-	$< 10^4$	0			
	4 months	2×10^4	2×10^4	0	0			
RS-3	1 day	3×10^{11}	4×10^9	1×10^5	-		4×10^3	8×10^5
	14 days	1×10^8	4×10^7	6×10^6	-			
	1 month	10^6	-	$< 10^4$	0			
	4 months	8×10^4	4×10^4	300	0			
RSL1	1 day	4×10^9	1×10^7	0	-		2×10^3	-
	14 days	4×10^7	-	$< 10^4$	-			
	1 month	1×10^7	-	3×10^4	-			
	4 months	3×10^6	-	800	0			
RSL2	1 day	2×10^8	2×10^7	1×10^5	-		1×10^4	200
	14 days	1×10^{10}	3×10^9	3×10^4	-			
	1 month	1×10^6	-	$< 10^3$	0			
	4 months	1×10^6	-	100	0			

Table V continued....

Cheese No.	Age	Plate Count			Milk Count	
		Total	45°C	Mould	Raw	After heat treatment and G. A. L. addition
		(Count per gram)			(Count per ml.)	
R--1	1 day	1×10^3	1×10^6	0	10^5	10^4
	14 days	2×10^5	-	0		
	1 month	2×10^2	2×10^2	0		
	4 months	2×10^4	0	30		
R--2	1 day	100	-	1×10^2	10^3	1×10^4
	14 days	4×10^4	4×10^5	1×10^3		
	1 month	4×10^5	-	7×10^4		
	4 months	1×10^5	0	10		

Discussion of Bacteriological Results

(a) Starter Inoculated Series

In commercially manufactured cheese the numbers of streptococci rapidly reach a peak and, thereafter, decline steadily over a period of months. This behaviour was exhibited quite clearly by the starter inoculated cheeses. The total count levels fell from a peak of 10^8 to 10^{11} /gm., to a level of 10^4 to 10^6 /gm within 4 months. This is a similar level to that reported for aseptically made, S. cremoris inoculated, cheeses by McGillivray and Perry (1963) and could indicate that satisfactory asepsis has been achieved.

The phage plating technique, as employed to detect, by difference from total count results, the numbers of contaminating bacteria, was not successful. The results at 1 day showed that the activity of the phage reduced the counts by only $\frac{1}{100}$ of the total count figures. This indicated contamination in the order of 10^6 to 10^9 per gram. To investigate the types growing on the phage treated plates, 25 colonies were picked from the phage plates of RS-1 and RS-2 and inoculated into Y. D. L. M. (Yeast Dextrose Litmus Milk) tubes. Random picking of colonies, using the method of Harrison (1938), was employed. After incubation of the isolates at 30°C for 3 days the following results were obtained.

Cheese Code	RS-1	RS-2
Morphology	24 chains 1 No growth	22 chains 1 rod 2 No growth
Y. D. L. M. Reaction		
1 day	No reaction	No reaction
3 days	10 acid,clot,reduction	4 acid,clot, reduction
7 days	21 acid,clot,reduction	20 acid,clot, reduction

It would appear from this table that in all except one case streptococci were present, but the activity in milk was much slower than with normal starter.(Tubes showing no growth are assumed to have missed inoculation). It is improbable that contaminant streptococci could have entered the aseptically made cheeses in such high numbers, so it would seem that the majority of organisms appearing on the phage plates were probably phage resistant mutants of the inoculated S.cremoris (HP). Being mutant forms these organisms appeared to have lost much of their activity in milk.

Phage plates prepared from the 14-day and 4-week samples were unsuccessful. In the former case, the phage obtained from the D.R.I. bacteriology laboratory was heavily contaminated with penicillium mould. This phage was then Seitz filtered and its sterility checked by plating 1 ml. of it on G.L.Y.P.A. for 3 days at 30°C. This phage was then employed in preparing the phage plates for the 1-month cheese samples. In this case, the phage plate counts were equal to those on the total count plates and 20 colonies picked from both plates were all streptococci that clotted milk within two days. This indicated that the phage had been inactivated in the Seitz filtering process and it was confirmed by a titre which was carried out. Plaques did not appear with any of the dilutions indicating that the phage had completely lost its activity, either on Seitz filtering, or on subsequent storage.

Phage used for the 4-month samples was adequately tested before use and results were practically the same as those obtained from the total count plates. Ten isolates were randomly picked from each of the total count and phage count plates of the RS-2 cheese and inoculated into Y.D.L.M. tubes. Observations are summarized as follows:-

Total Count Plates	Phage Count Plates
2 Streptococci; Clotted in 7 days	3 Streptococci; Clotted in 7 days
8 Yeast; No reaction	7 Yeasts; No reaction

These observations show that, although some streptococci survived in a weakened form, the major proportion of the cheese flora consisted of yeasts that developed over the later months of curing. It is known (Lenney, 1956 and Cook, 1958) that yeasts exhibit a certain amount of proteolytic activity, so electrophoretic analysis of the 4-month samples of RS-2 has not been made. The close similarity of phage and plate counts suggests that none of these cheeses were satisfactory as indicators of streptococcal activity. A 4-month electrophoretic examination was not employed at all.

For each cheese, plates were poured and incubated at 45°C. Growth on these plates should have indicated organisms able to survive the heat treatment that was employed. Certainly the starter strain could not grow above 40°C. (Bergey), so this provided a further means of selectively investigating the flora present. The results showed a very unsatisfactory condition of the cheeses. The high counts reflected the high counts found in the milk supply. The first three cheeses made (viz: RS-1, RS-2, RSL1) were made from excellent milk and showed low counts in the early stages, but even these climbed to a peak of millions per gram after 14 days of curing. The third cheese of the starter series was made from unsatisfactory milk, yet counts at 45°C were similar in all three cheeses. This fact might be taken to indicate that the source of the heat tolerant bacteria has not been

established, or that even the small numbers of heat tolerant organisms in the original milk supply were ample to cause severe contamination of the cheese. Further work would be necessary to establish the validity of either alternative.

It should be noted that the colonies growing at 45°C were very small and liable to be confused with the precipitation within the medium that occurred on drying out over the 3-day period.

Fifteen of these heat tolerant contaminants were randomly isolated from plates of RS-1 and an investigation of these isolates yielded the following information.

1.   The time to clot Y.D.L.M. was - 1 day at 45°C.
                                    2 days at 30°C.
                                    6 days at 20°C.

2.   In all cases reduction occurred prior to clotting.

3.   Morphologically, all isolates were short chains or cocci pairs.

4.   All isolates grew with acid production in McConkey broth at 30°C.

5.   All isolates clotted Y.D.L.M., plus 6.5% salt in 3 days at 30°C.

All 15 isolates cultured gave identical reactions. From this information the contaminant may be tentatively classified as <u>Streptococcus feacalis</u>. The organism does not survive well in the cheeses and numbers were declining after 4 weeks' ripening.

As noted in the literature review, Stadhouders (1960) has not been able to detect any influence of <u>S. feacalis</u> on protein breakdown in

inoculated cheeses by comparison with controls. Dahlberg and Kosikowsky (1948) suggested that this organism plays a part in cheddar flavour development, but their results do not indicate that it has any marked proteolytic behaviour. The influence that this contaminant would have on the present study, if any, is therefore open to question.

Mould contamination was not detected in any of the cheeses in this series.

(b) The Starter and Lactobacilli Inoculated Cheeses

Little information as to the number of contaminating bacteria in these two cheeses is available (see previous discussion). However, the 45°C count does show the presence of heat tolerant types, which again show high counts over the first fortnight of curing.

No mould contamination was detected.

(c) The Gluconic Acid Lactone Cheeses

The total count figures for these two cheeses were initially low. Addition of non-sterile gluconic acid lactone undoubtedly provided a major source of entry of those organisms that were initially present.

The influence of the nisin added to the milk and the curd of the second cheese is apparent, in that the counts of both the raw milk and of the cheese in the early stages were lower than in the first cheese made.

Peak values for total counts were much higher than desired. It should be realized, however, that in the peak count of 10^{5}/gm these cheeses had a bacterial content that was $\frac{1}{1000}$ of the peak counts of the inoculated

cheeses and this must be a significant reduction in numbers. Furthermore, the elimination of starter must have altered the nature of the range of proteolytic enzymes that were present in the cheeses. At the very least these cheeses should have provided more reliable information than has been obtained from the inoculated cheese type of experiment that so often appears in the literature.

Plate counts derived from $45^{\circ}C$ incubation were again high and unsatisfactory.

Mould was detected in R--2. The mould appearing from the 1-day sample was a black slow growing member of the order Mucorales, which was found in high concentrations in the air of the factory. This particular mould did not survive in the cheese and later mould counts were all of Penicillium sp., which were also present in the air of the factory. As this cheese was the last one manufactured it should have received the most experienced care of handling. Furthermore, as none of the other cheeses (including R--1) have mould contamination, it could be concluded that some structural defect in the equipment had occurred (e.g. a leak in the sealing of the perspex window), and air-borne contamination had been able to enter the vat.

The presence of yeast had also been detected in the 4-month sample of R--1 in the following manner. Sixteen colonies were randomly isolated from the total count plates and were grown in Y.D.L.M. for 7 days. In 15 tubes, yeasts having no reaction in the milk were noted and in two cases a streptococcus, the colonies of which had red pigmentation, were found.

General Conclusion

    It may be concluded that the desired degree of asepsis in cheesemaking has not been achieved. In all cheeses a heat-tolerant contaminant, that was tentatively identified as S. feacalis in one cheese and could well be the same organism in the other cheeses, has been detected. Yeasts have developed after 4 months of curing in two of the cheeses and may also be present in the other cheeses of this age. Mould was present in R--2, but was not detected in any of the other cheeses.

    It should be recognised, however, that the bacterial populations of the various cheeses will have differed widely. Although it will not be possible to definitely ascribe differences between cheeses in proteolytic breakdown to the inoculated species, these organisms must predominate in the flora in at least the early stages of curing and should, therefore, provide the major cause for any differences that are noted.

    In both lactone cheeses the initial plate counts were low but rose rapidly. This would indicate that the use of gluconic acid lactone provided a method of eliminating starter from cheesemaking experiments, but even light contamination of the fresh curd led to rather high counts in the ripened cheese.

FIGURE 1.
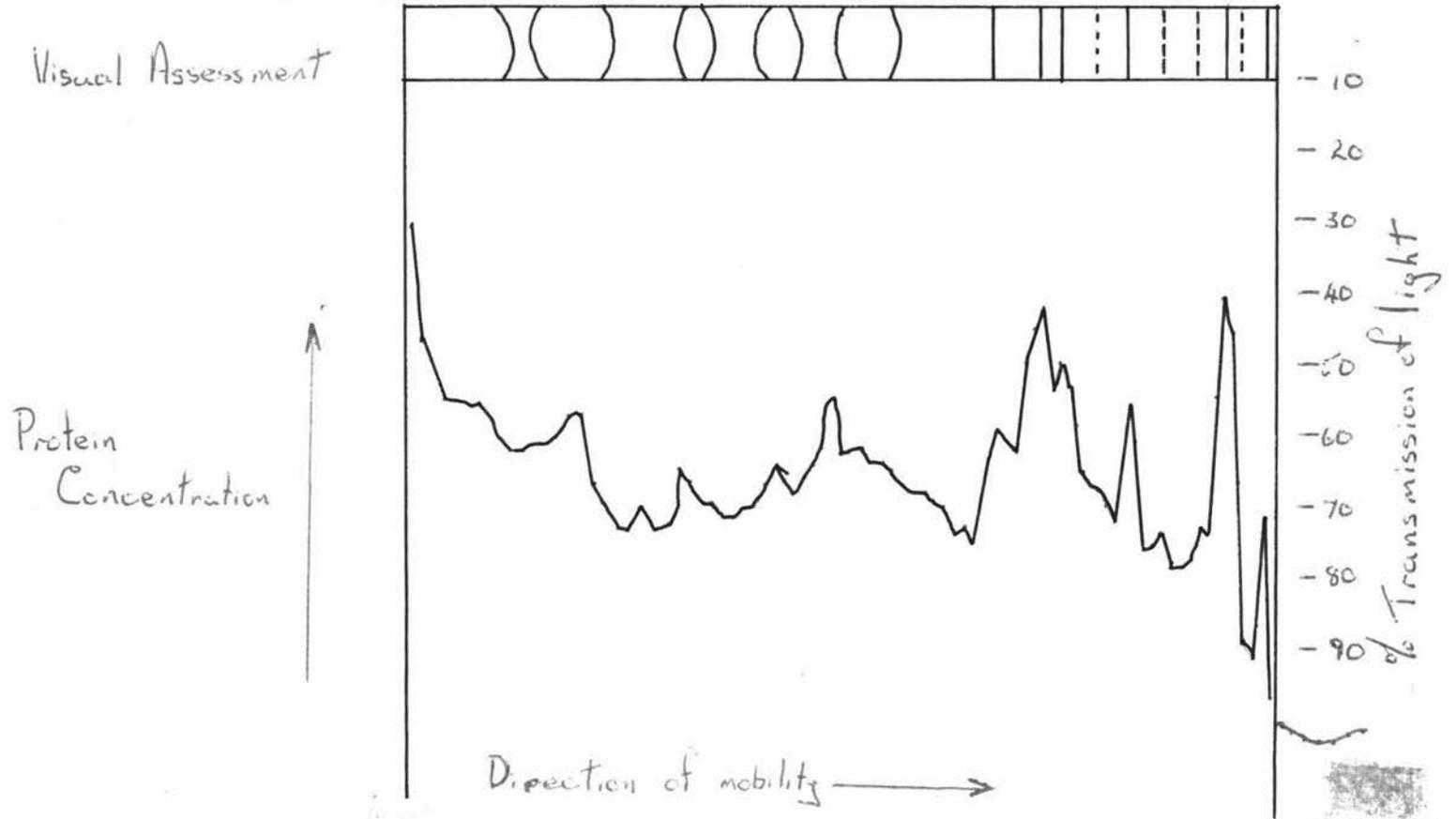
Visual Assessment

Protein Concentration

% Transmission of light

Direction of mobility ⟶

Origin ⟶

Boundary

RS-1   Cheese Code

6 weeks   Age at Date of Sampling.

GELL   24

Results and Discussion of Electrophoretic Study

Interpretation of Electrophoresis Graphs

The protein samples from the cheeses were separated into their component fractions by starch-gel-urea electrophoresis and a graph of the protein concentrations at various points along the gel was obtained by passage through a spectrophotometer, as previously explained.

For the purpose of completeness in the recording of results, the original gel strip was placed beneath each graph so that the position of the various bands of protein can be seen and related to the protein concentration graphs given (see Figure 1).

The information given in red ink on Figure 1 is not repeated on the graphs that make up the raw data of the thesis (see Supplementary Booklet). This has been done to simplify the layout of the graphs.

The X abscissa of the graph measures the distance of migration of the protein, which moves from the origin at the left of the graph towards the boundary line at the right of the graph. Beyond the boundary line, a short red line is drawn to show the transmission readings obtained from the background blueness of the starch gel.

The Y ordinate of the graph measures the percentage transmission of light passing through points along the gel. In every graph the 100% transmission level is to be found at the point where the line of concentration graph meets the boundary line. Every millimeter division along the Y ordinate is equal to a change of 1% transmission.

Transmission levels have not been recorded on each graph for the following reasons:-

(a) The level of background colour in the gel strips varied, depending mainly on the efficacy of washing, between gels and, consequently, the 100% transmission level will not be the same in every case.

(b) Protein concentration and percentage transmission of light are not necessarily directly related. Different protein components will probably vary in the extent to which they bind the napthalene black dye. Comparison of the concentrations of any one component in any two samples will be possible, but accuracy of estimation of protein concentration for all components should not be inferred by placing a scale on the axis of the graph.

(c) The electrophoresis technique is not quantitative and recording of results should not indicate impressive accuracy.

The value of the graphs obtained in this work is to allow comparative study to show the shift in concentration of the protein components during ripening of the cheeses. From this point of view, knowledge of the level of percentage transmission of light is neither necessary nor desirable.

Beneath each gel is recorded the code name of the cheese from which the sample was taken, viz:

RS-1 is the first cheese made with starter inoculation

RSL1 is the first cheese made with starter and lactobacilli inoculation

R--1 is the first cheese made with gluconic acid lactone

C is the commercially manufactured cheese.

Beneath the code name is recorded the stage of curing at which each sample was taken and the number of the gel from which the electrophoresis strip was removed. This is recorded so that it will be possible to check back to the casein standard which is taken from each gel to ensure that there is nothing grossly wrong with the handling of the gel.

Above the concentration graph a representation of the visual appearance of the stained gel, one day after it was stained, is presented. It has been previously pointed out that the clarity of the positions of the protein bands became less distinct as the gel strip dried and aged. For this reason a visual assessment of the position of the various bands of the protein components was made and recorded, so that a comparison with the graphed results can be made. This record has been made by making the position of distinct bands by a continuous vertical line (viz: (|)), the position of less distinct bands by a vertical dotted line (viz: ⁞); and the position of an area of protein concentration by a bracket (viz: ( )).

It will be seen that the distinct bands are to be found in the area of the gel farthest from the origin. Near to the origin the protein is dispersed more evenly along the gel, so that less distinct bands can be seen. This confirms the experience reported by Wake & Baldwin (1961) and Neelin (1962).

Consider the most mobile peak in Figure 1. It can be clearly seen in the gel; its position is marked clearly on the graph and it is recorded as a distinct band in the visual assessment record. The second fastest peak consists of two bands according to the visual assessment record, but the graph records one of these bands as only a change in gradient in the slope of one combined peak. Indistinct bands are recorded, but only as very small peaks on the concentration graphs. This demonstrates that care in observation of the graphs is necessary to accurately determine the position of bands in the original gel. Nearer to the origin the variations in protein concentration are more gradual and are not so easily seen, so are normally represented by brackets. The patterns of the graphs, as detected by the spectrophotometer in this zone, are irregular and it is difficult to comment reasonably about them.

One point worthy of note is that, in the great majority of gels, the protein does not appear as a series of distinct protein bands. The background colour of the gel was generally well below the level of colour found behind the boundary line. This must mean that quite a lot of protein resided at all points along the gel strip and was not merely concentrated at the points where heavily stained bands of protein can be readily seen.

Because of this finding the use of photography to get a true indication of the pattern of protein concentration in any gel is limited. Published photographs of patterns obtained by use of starch-gel electrophoresis show narrow bands where protein was concentrated, but do not indicate the level at which protein occurred throughout the length of the gel.

Figure 2
Influence of Incomplete Washing
of Gells

Incompletely
Washed
Casein Sample
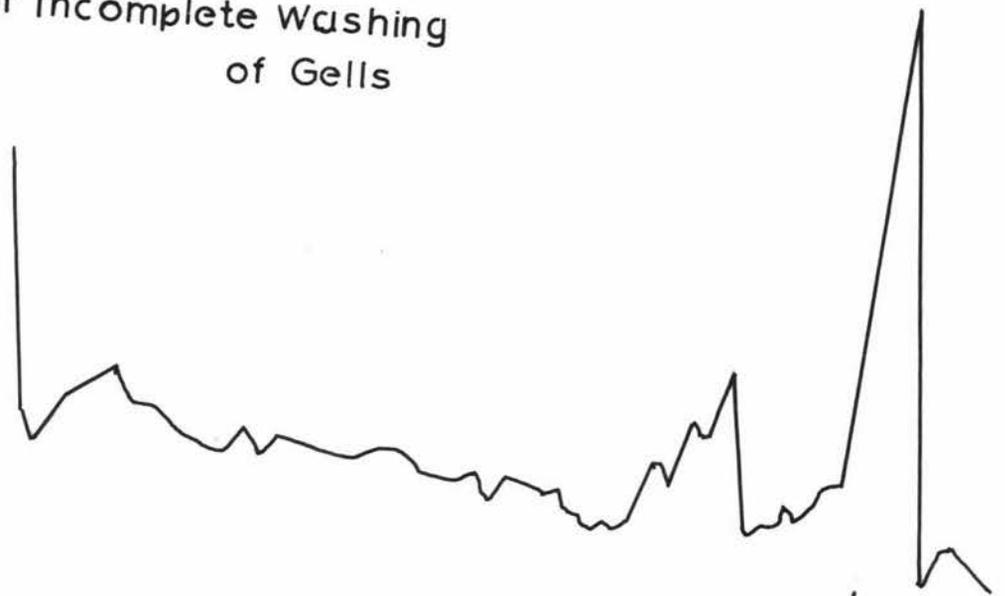
Completely Washed :-
(Same Casein Sample)
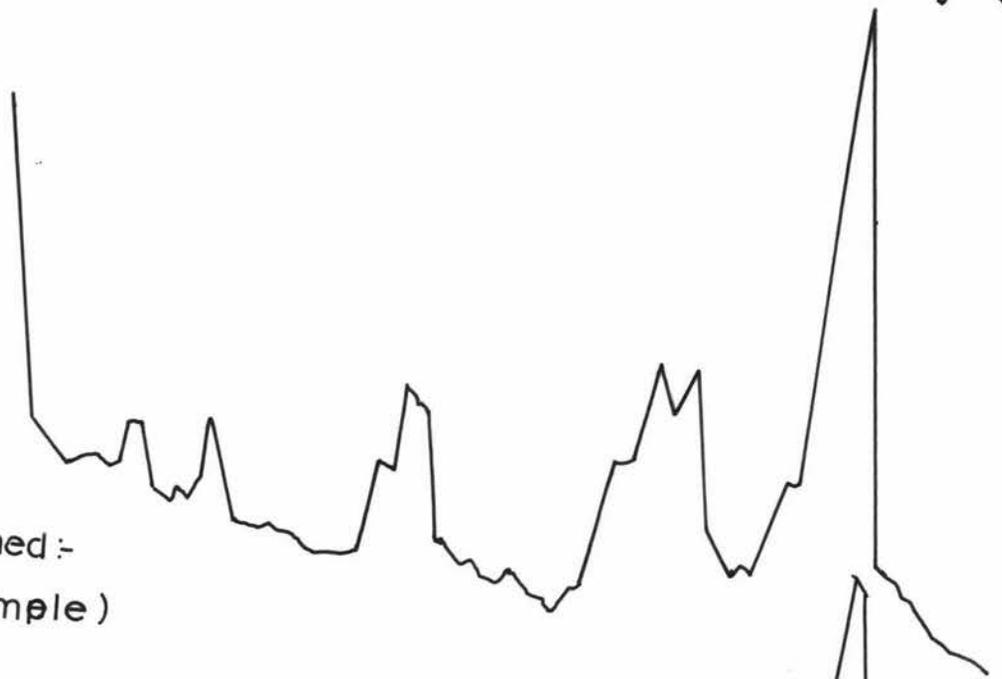
Peculiarity of Washing :-
(Part of Gell Shows Transmission
Greater Than That of Background
Colour of Gell)
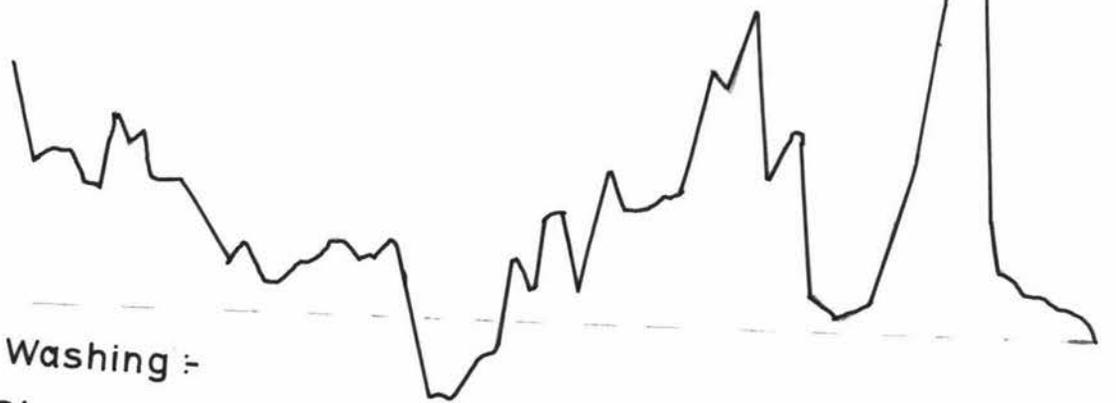
Reliability of Electrophoresis Results

(a) Influence of Washing of Gels (Figure 2).

It has been previously noted that variations in electrophoresis graphs have occurred through inadequate washing of the stained gels. This may have occurred because of variations in the thickness of the gel slice, or variations in the efficiency of the washing procedure. The effect of such variations is apparent from the graphs presented in Figure 2. The lowest graph presented in this figure is one in which some irregularity has occurred, causing an excessive effect of the washing procedure at one point of the gel, so that the level of colour actually decreased to a level below that of the background colour of the gel. The most probable explanation for such an irregularity would be variation in the thickness of the gel slice, leading to local variations in effectiveness of the washing procedure.

FIG. 3

ELECTROPHORETIC PATTERNS OF CASEIN

FROM VARIOUS GELLS.

GELL 13.

GELL 12.

GELL 17.

GELL 10.

GELL 9.

GELL 11.

GELL 15.

GELL 18.
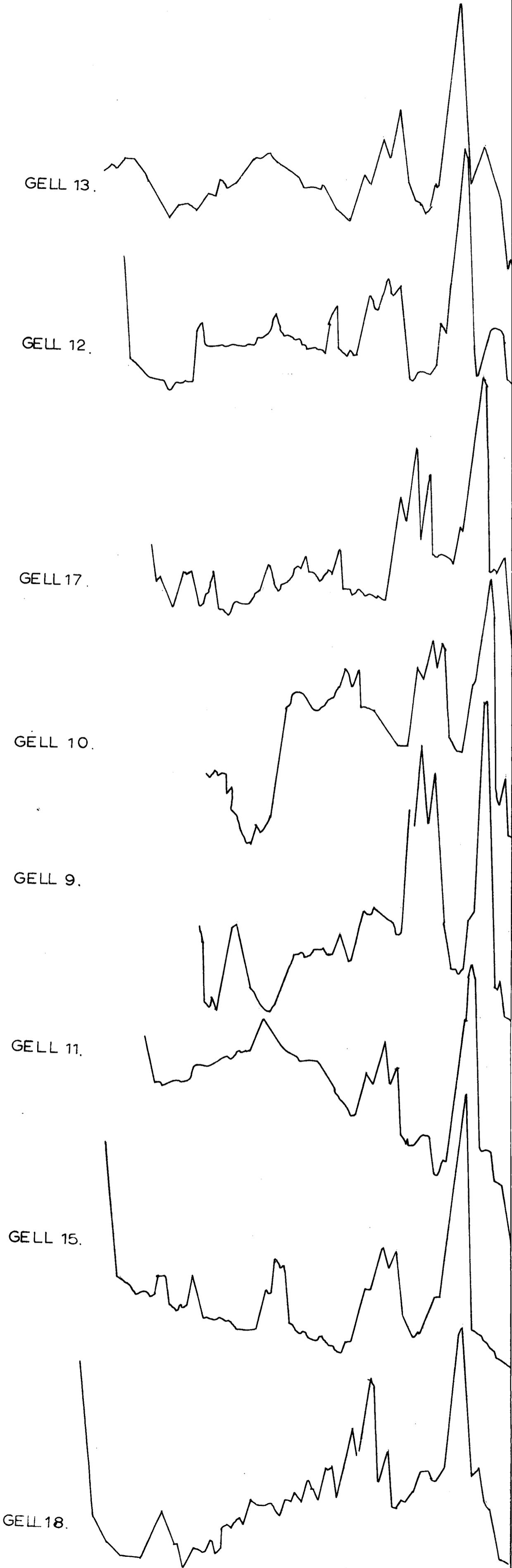
FIG. 4

ELECTROPHORETIC PATTERNS OF CASEIN

FROM VARIOUS GELLS.

Page 100A

Gell 23.

Gell 28.

Gell 24.

Gell 30.

Gell 27.

Gell 29.
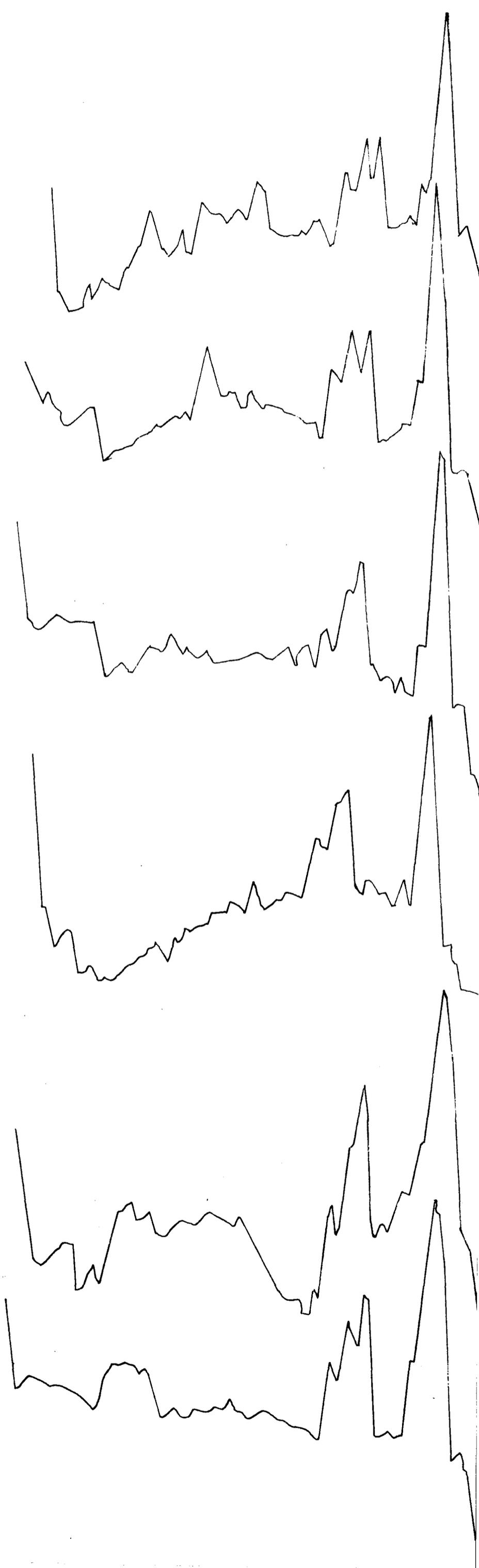
(b)   Influence of Unknown Factors (Figures 3-4).

Despite the precautions observed in rigid adherence to a
standardized procedure of electrophoresis, it was found that results
obtained from the gels were not consistent. A common casein sample
was run with every gel that was made. This was done to give a standard
pattern which could be observed to check on the comparability of one
gel with another. Numerous gels were discarded because of gross errors
in handling but, nevertheless, large variations in the results obtained
from apparently satisfactory gels occurred. Figures 3 and 4 show
tracings made from the graphs of the casein samples run in various gels
and placed beneath one another, so that the variation that occurs can be
readily seen. It will be noted that, although the positions of the major
peaks of protein concentration are always present, there is some variation
in the shape of these peaks due to variations in the amount of protein in
any one peak. Considerable variation in the patterns of the less mobile
components will be noted and it is apparent that the technique, as used
in this study, cannot be used to demonstrate any changes that may occur
in this zone.

(c)   The Influence of the Protein Extraction Technique(Figure 5).

The use of the "acetone powder method" for extracting protein from cheese has been tested. Casein was precipitated at pH 4.6 from a sample of bulked whole milk and the whey was poured off. A sample from the resulting wet casein was washed in warm distilled water and then prepared for electrophoresis. The bulk of the wet precipitated casein was dried following the procedure outlined for the extraction of protein from cheese. A fine white powder was prepared for electrophoresis.

After electrophoresis had been carried out, visual examination of the gel could detect no variation between the two samples that were run together on one gel. The resulting graphs appear in Figure 5. Slight variations may be detected in the two graphs, but it can be seen that no major alteration of the two electrophoresis patterns occurred through the use of acetone and butanol at $-15^{\circ}C$.

It is considered that these two graphs justify the use of the "acetone powder method" for extracting protein from cheese preparatory to electrophoresis study.

The possibility of extracting protein from cheese by conventional methods and using the resultant extract as a standard for comparison with protein extracted by the acetone powder method was considered, but it was felt that there is no certainty that conventional procedures do not alter the protein in some way and, consequently, might not provide a suitable control method.

FIGURE 6
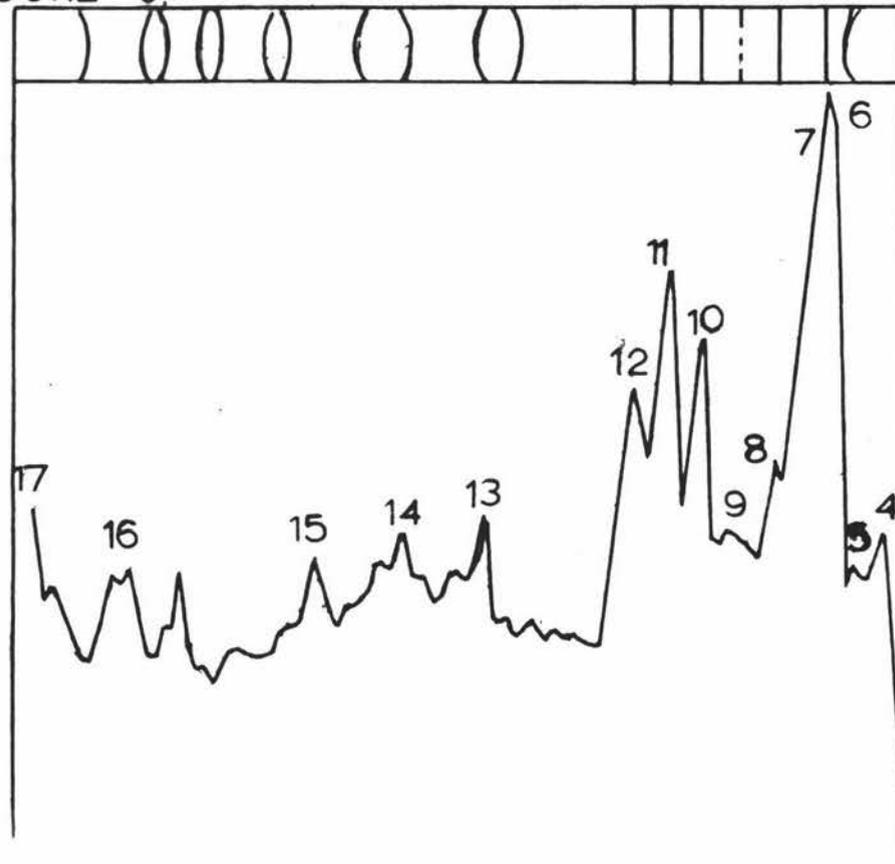
GELL 7

CASEIN

Identification of Casein Components(Figure 6).

Bearing in mind the lack of reliability inherent in the technique of starch-gel-urea electrophoresis it is, nevertheless, possible to tentatively indicate the identity of some of the components that have been separated. Wake & Baldwin (1961) have detected 17 components in casein by the use of a range of protein concentrations, as applied to the sample slots. Neelin et al. (1962) have also detected 17 components by the use of two dimensional vertical starch-gel-urea electrophoresis and using different electrophoresis conditions than those employed in the present study. Such an extent of resolution is not to be expected when using a 1% casein concentration with one dimensional horizontal electrophoresis. Using the work of Neelin et al. (1962) it is possible to provide a tentative nomenclature for the various peaks of the graphs that have been drawn. Figure 6 shows an electrophoresis graph in which the numbered peaks may correspond to the following identities as given by Neelin et al.

Zone No.	Component
4	Unknown
5 - 8	Major components of calcium-sensitive α -casein (α_s casein)
9 - 10	Unknown (contaminants of both α_s and β -casein)
11 - 12	Major components of β -casein
13 - 15	K -casein
16	γ -casein (occasionally not distinct from zone 17)
17	Unknown (probably a denatured fraction)

<u>Study of the Electrophoresis graphs obtained from the Experimental Cheeses</u>(Figures 7.

Individual protein samples taken from the eight cheeses made for this study have been analysed by starch-gel-urea electrophoresis, and the graphs that record the individual results have been collected and bound into the supplementary volume that accompanies this thesis. So that these graphs may be used to follow the changes that occur during the ripening of a cheese, they have been traced and placed beneath one another to make up Figures 7-14.

In most cases, the first four graphs that are given (i.e. graphs of casein - 1, 7, and 14 day samples) have been derived from one gel; the following three graphs (i.e. 3,4 and 6 week samples) have been derived from another gel; and the final sample (or samples) have been derived from a further gel (i.e. 10 week and 4 month samples). It has been previously stressed that large variations occur in the electrophoresis patterns derived from different gels, but these variations are reduced considerably when comparing samples derived from any one gel (cf. Figure 5). Study of the changes occurring in the protein of the cheeses must be carried out bearing these considerations in mind.

A pattern of a casein sample is given in every case and it will be realised that the cheese protein will be largely made up of proteins of the casein complex, although there may possibly be other milk proteins contributing in a small way to the cheese protein graph.

In every case it will be noted that the most mobile group of peaks (that have been tentatively referred to as the α_s-casein complex)

were split into two major fractions within the first fortnight of curing and they were susceptible to continued proteolysis until the end of curing. It appears that the peaks tentatively referred to as β -casein were less susceptible to proteolysis and seem to have more or less retained their characteristic shape throughout the curing period under study. Both of these findings confirm those of Lindqvist & Storgårds (1959).

It is of interest to note that the five or six peaks observed in the one-day-old cheese sample had increased to eight or nine peaks before the end of the curing period in all cheeses. It is impossible to suggest the origin of any of the new peaks that have appeared. The time of appearance of the various new peaks was irregular. Consider the very mobile peak that developed very close to the boundary line. This was a very distinct, narrow, band that could not possibly have been mised when it appeared. It appeared after 1 day in RS-1 and R--1; after 14 days in R--2 and C; after three weeks in RS-2; after 10 weeks in RS-3, and not at all in RSL1 (RSL2 is a doubtful case and difficult to interpret). Similarly, a definite peak that arose between the α - and β -casein zones appeared at widely different stages of ripening. This behaviour is not easy to understand. The variations that occur from gel to gel cannot adequately explain the irregularity of appearance date for such distinct protein bands and the problem is apparently not related to the treatments given to the cheeses. It is conceivable that slight differences in the chemical composition of the cheese medium have influenced the proteolysis. It is common knowledge that the cheese medium is a very complex one and in this study attempts to control the major variables have been carried out. Such variations, as mentioned above, point to the necessity for even greater control of the medium.

Discussion of the patterns appearing in the low mobility zone of the gels is pointless. This is the area that has been named "The K - zone" (Neelin, 1962) and contains an indistinct area in which K -casein collects. It is in this zone that the greatest variations occur in Figures 3 and 4. The action of rennet in cheesemaking will cause immediate alteration of the K -casein and may tend to confuse the picture even more.

Neelin et al. (1962) considered that their band number 17 was probably a denatured fraction. The author's experience with cheese protein tended to confirm this, as the amount of deeply staining material that appeared at both edges of the starting slots was much greater with cheese samples than with casein samples.

Possibly the most significant observation that may be made from all cheeses is that, as curing proceeds, the level of protein distributed along the gel strip tended to increase to a level well above that of the background blue colour of the starch gel. The distinct peaks present in the early stages tended to disappear as this occurred. This could be interpreted as suggesting that the protein degraded to a large collection of peptide forms which, when separated by starch-gel electrophoresis on the basis of molecular shape, molecular size and electrical charge, tended to spread themselves throughout the length of the gel. If this is the case, there can be little hope of detecting the individual activity of the various proteolytic enzymes by the method employed in this study, unless individual enzymes were to specifically attack a degraded form residing at any one point in the gel. This would be unlikely to occur.

Despite the great differences in body, flavour and bacterial flora of the lactone cheeses, by comparison with all of the inoculated cheeses, there is nothing particularly distinctive about the graphs that have been produced from them. Indeed the patterns obtained from the 10-week samples are more notable for their similarities than for their differences. This is all the more remarkable considering the variations that can occur between gels. The action of rennet as the only source of proteolytic enzymes common to all cheeses (ignoring the natural proteases of milk), could have led to this observed similarity, but there are too many variables not adequately controlled in this study to allow firm conclusions to be drawn.

Useful conclusions about the role of the various other proteolytic enzyme systems in the different series of cheeses is not possible, because of the inadequately controlled bacteriological flora of the cheeses and the lack of reliability of the starch-gel-urea electrophoresis technique.

Summary and Conclusions

The technique of starch-gel-urea electrophoresis has been applied to cheese protein and found to be of only limited value in following the course of proteolytic breakdown in cheese. Seven cheeses have been made under controlled bacteriological conditions with varying degrees of success in controlling their flora. Protein breakdown in all cheeses is shown, within the limits of the technique employed, to be similar, despite the differences in bacterial populations of the experimental cheeses. This is taken to indicate that rennet is the major proteolytic agent in the proteolysis of cheese to the levels observable by starch-gel-urea electrophoresis. No firm conclusions can be drawn however.

Recommendations for Further Study

The aim of the present study was to investigate the practicability of applying starch-gel-urea electrophoresis to the investigation of the process of proteolysis in cheese curing. It is considered that experience in handling of the various techniques used would eliminate many of the difficulties encountered by the present author.

The feasibility of manufacturing truly bacteria free cheese must be considered. One of the gluconic acid lactone cheeses in this study showed an initial total count at $30^{\circ}C$ of only 100 per gram (one millionth of the total count of the inoculated cheeses), but this figure had risen to 40,000 per gram within a fortnight. It appears that the incorporation of some agent, more effective than nisin, with the cheese curd to prevent this natural increase in numbers, is a necessity if successful reduction in bacterial count is to be achieved.

The use of starch-gel-urea electrophoresis in this study has been of limited value, largely because of the variations of patterns that occur between gels. By the use of a wider gel, to analyse ten samples at a time, it would be possible to eliminate this problem. This modification would allow all the samples from any one cheese to be analysed together, or, alternatively, samples drawn from every cheese could be analysed together to give comparisons between treatments, since the patterns within any one gel are reasonably consistent. Furthermore, use of eluate from sections of the gel strips to improve the quantity aspects of the electrophoresis should be investigated.

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APPENDIX NO. 1

Manufacturing Details for Aseptically Made Cheeses

Cheese Code	Milk Weight	Pasteurization	Inocu-lation	Cutting Acidity	Cooking Temp.	Run Time Hrs.	Run Time Mins.	Run Acidity	Dry Acidity	Mill Time Hrs.	Mill Time Mins.	Salt Time Hrs.	Salt Time Mins.	Stirs	Comment
RS-1	86	153°F/7 mins.	HP	.105%	102°F	2	0	0.15%	0.16%	3	37	4	10	3	Cook too soon, Slowed the starter.
RS-2	88	155°F/3 mins.	HP	.11%	99/105°F	1	48	0.14%	0.16%	3	32	3	53	3	Cook high
RS-3	83	155°F/3 mins.	HP	.11%	99/101.5°F	2	25	0.15%	0.16%	3	33	4	13	1	Starter slow, hence low cook. Cut a little fine, hence a dry curd.
RSL1	86	155°F/3 mins.	HP + Lacto-bacillus	.12%	98/103°F	1	17	0.15%	0.16%	2	25	2	55	4	
RSL2	93	155°F/3 mins.	HP + Lacto-bacillus	.13%	99/102°F	1	40	0.15%	0.16%	2	45	3	10	2	

R--1   Gluconic Acid Lactone added (made according to schedule given previously).

R--2   Gluconic Acid Lactone added.

Note:
Rennet addition   =   20 ml. in all cases.
Starter addition   =   1000 ml. in all cases (about 2½%).
Salt addition   =   100 gms. in all cases (about 2%).
Cooking temperatures refer firstly to holding temperature, and secondly to shock cook temperature.
Times given are from setting time.

- - - -

FIG. 7.

ELECTROPHORETIC PATTERNS

FOR RS-1.

Casein

1 day

7 days

14 days

21 days

4 weeks

6 weeks

10 weeks

4 months

FIG. 8.

ELECTROPHORETIC PATTERNS

FOR RS-2
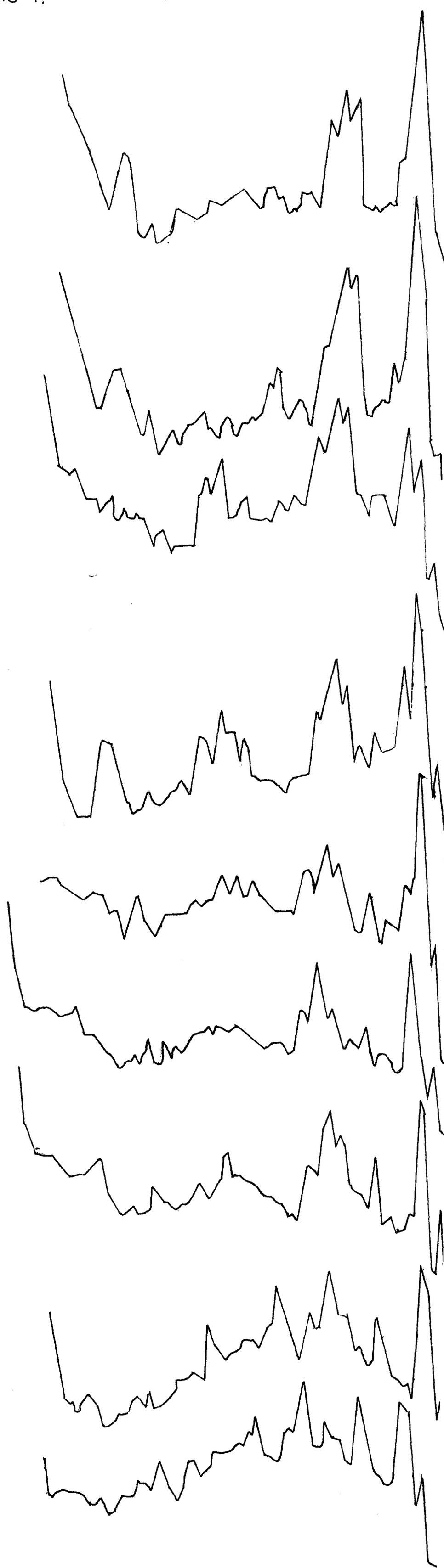
Casein

1 day

7 days

14 days

3 weeks

4 weeks
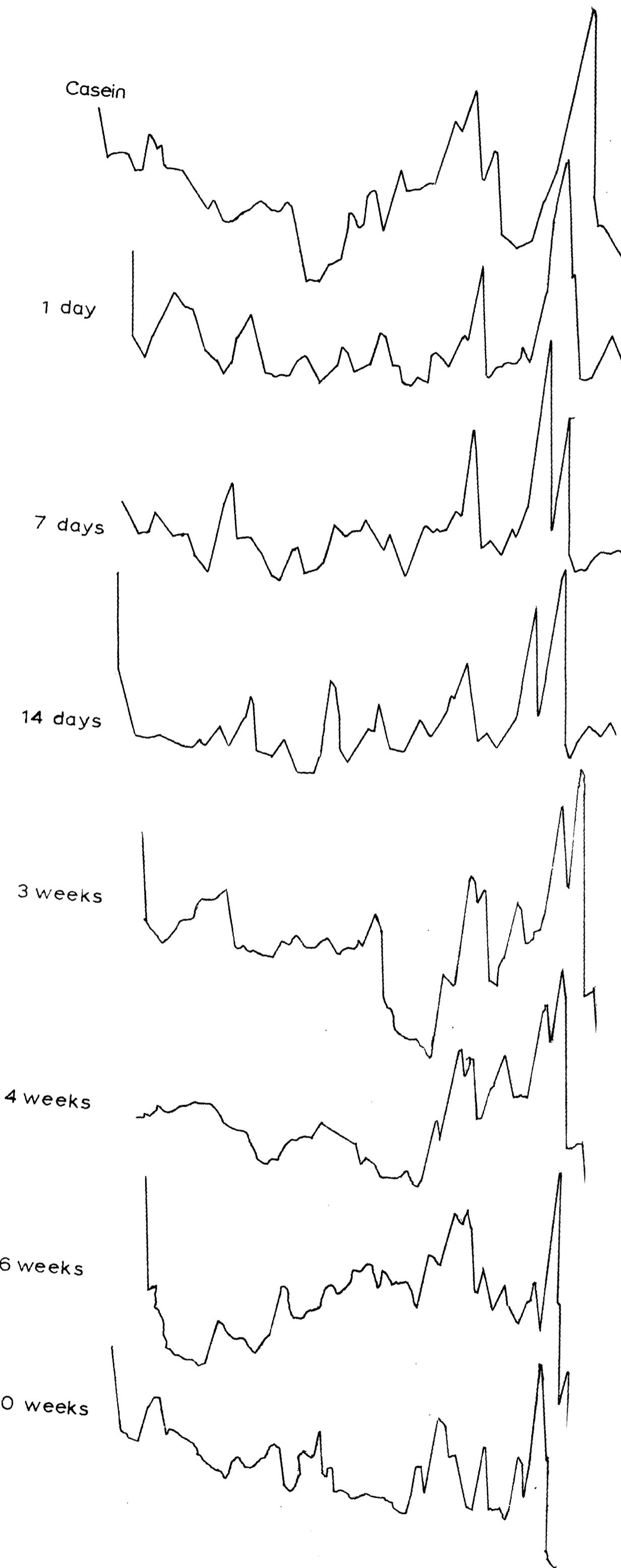
6 weeks

0 weeks

FIG. 9

ELECTROPHORETIC PATTERNS

FOR RS-3

Casein

1 day

7 days

14 days

3 weeks

4 weeks
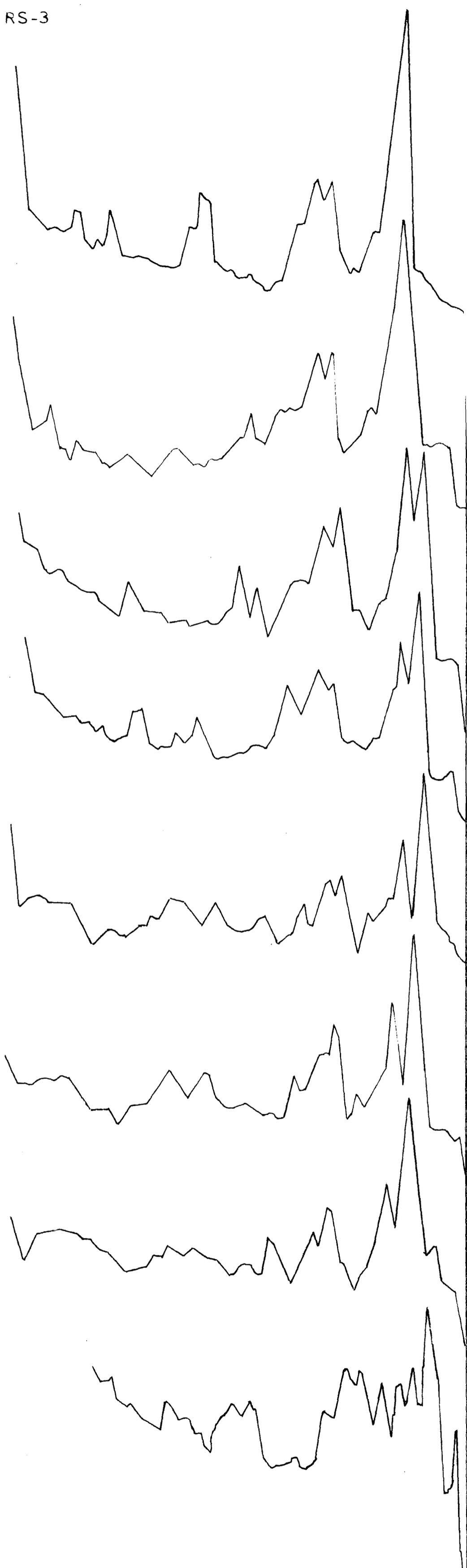
6 weeks

10 weeks

FIG. 10.

ELECTROPHORETIC PATTERNS

FOR RSL1

Casein

1 day

7 days

14 days

3 weeks

4 weeks
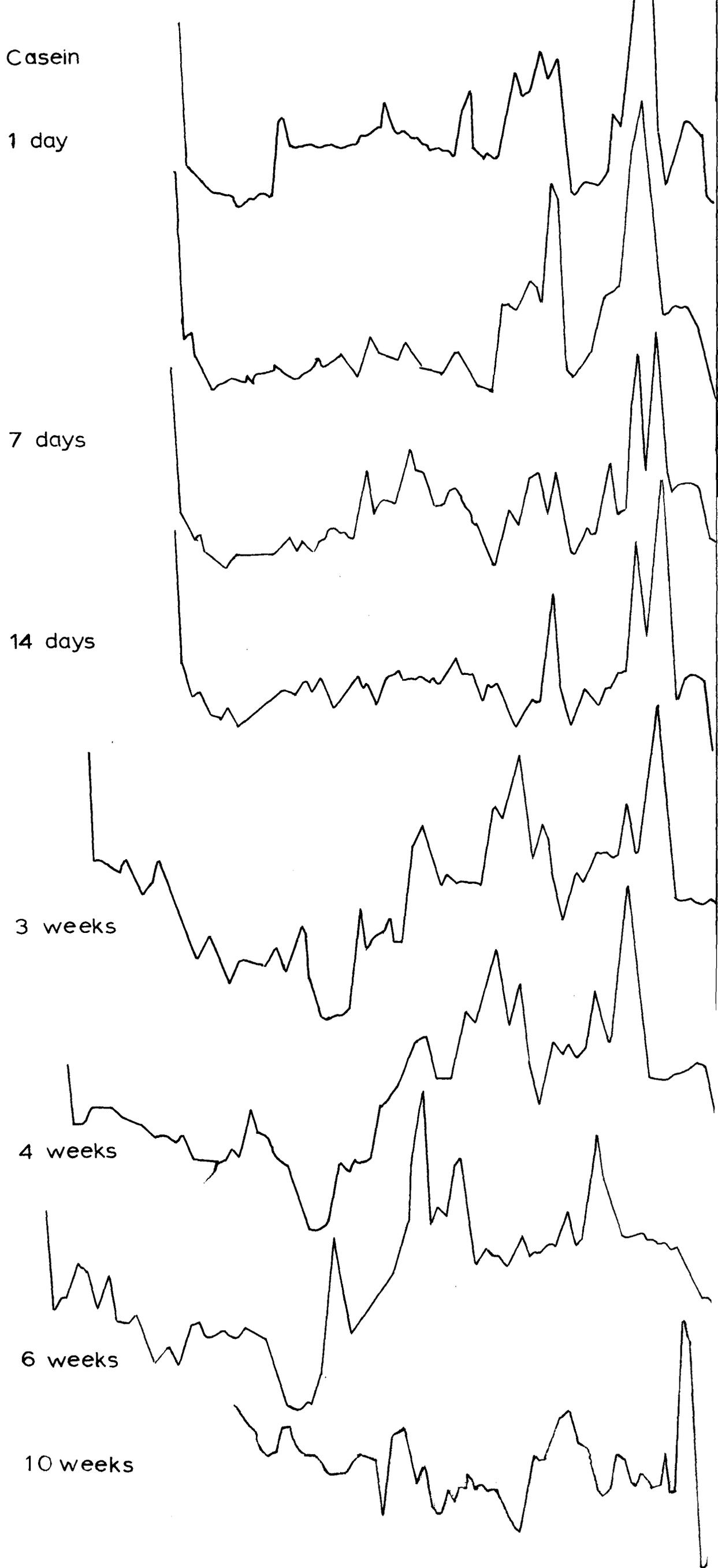
6 weeks

10 weeks

G. 11.

LECTROPHORETIC PATTERNS

   FOR   RSL 2

Casein

1 day

7 days

14 days

3 weeks

4 weeks

6 weeks

10 weeks

FIG. 12

ELECTROPHORETIC PATTERNS
FOR R--1
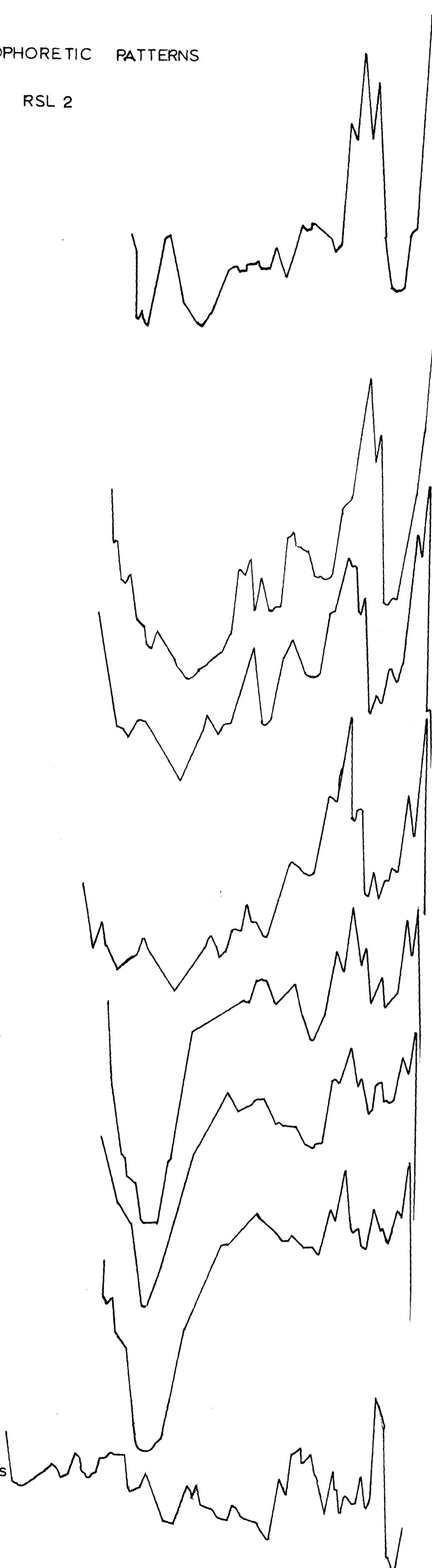
Casein

1 day

7 days

14 days

3 weeks

4 weeks
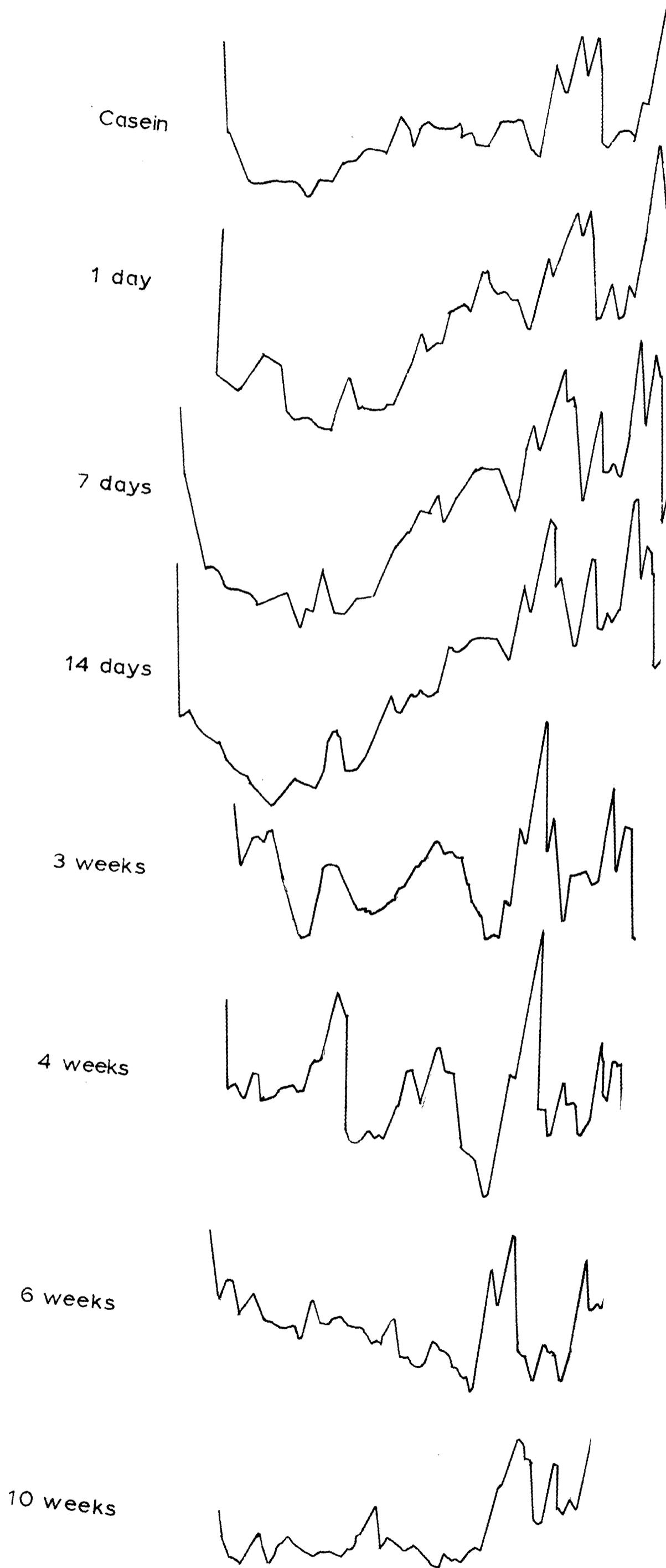
6 weeks

10 weeks

FIG. 13

ELECTROPHORETIC PATTERNS
FOR R--2

Casein

1 day

7 days

14 days

3 weeks

4 weeks

6 weeks

10 weeks

4 months
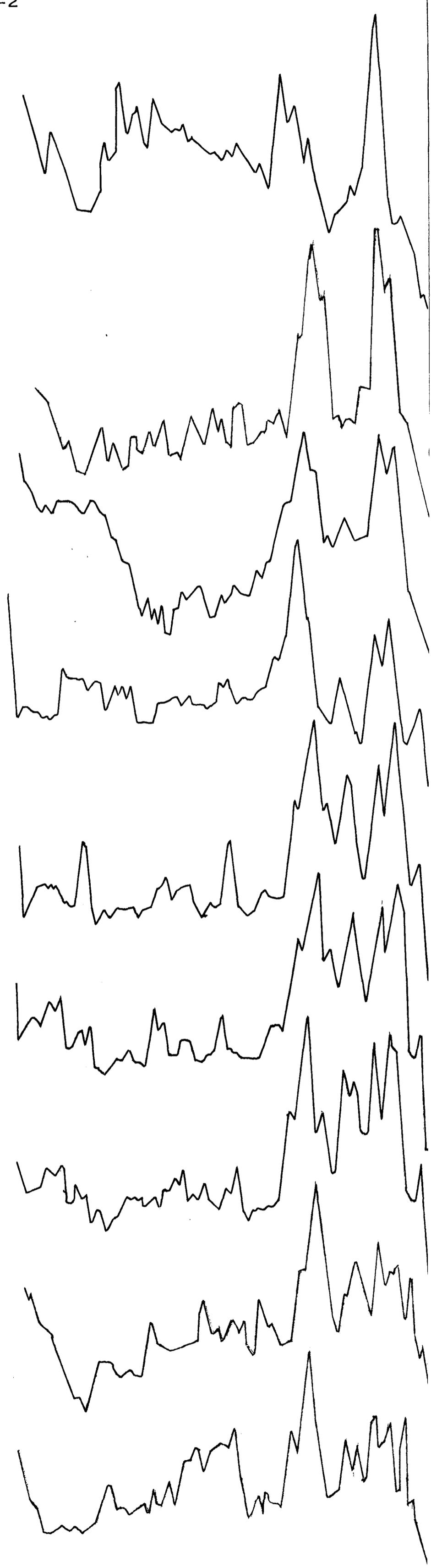
FIG. 14

ELCTROPHORETIC PATTERNS
FOR THE COMMERCIALLY MADE CHEESE

1 day

7 days

14 days

3 weeks

4 weeks

6 weeks

10 weeks

4 months