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THE INFLUENCE OF STARTER STREPTOCOCCI  
AND OTHER MICROORGANISMS  
ON CHEDDAR CHEESE FLAVOUR,  
WITH SPECIAL REFERENCE TO PROTEOLYSIS

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## A B S T R A C T

The investigation was undertaken to determine the features of different strains of lactic streptococci (starters) associated with good or poor flavour development in Cheddar cheese. An attempt was made to differentiate the roles of the starter streptococci, other microorganisms and rennet, particularly with respect to the influence of their relative proteolytic activities on the acceptability of the cheese and the formation of bitterness.

An improved agar medium was developed for the detection of proteolytic organisms in total bacterial counts, and used to assess the bacteriological quality of the milk received for cheesemaking over two dairying seasons. In spite of wide variations in the quality of the milk, the proteinases of the raw milk flora had no significant influence on the development of flavour, or off-flavours such as bitterness, in cheeses made with specific "non-bitter" or "bitter" starters.

Starter strains which characteristically made good-flavoured cheese possessed either one or both of the following features:

- (i) low rate of cell division at the temperature of cheesemaking which resulted in relatively low numbers of cells being produced;
- (ii) low proteolytic activity as determined in pasteurized skim milk (PSM) cultures in the presence of 4 or 5% NaCl.

Although the total quantities of free amino acids varied between cheeses having either good flavour or bitter or "burnt" flavour defects, the proportions of individual free amino acids formed by proteolysis of the casein were very similar in all the cheeses. This suggested that the specificities of the proteinases of the different starter strains used to make these cheeses were

not markedly different.

A comparison of proteolysis by starter strains and by rennet in PSM under similar conditions suggests that the starters, and particularly the more active strains, contribute significantly to overall proteolysis during cheesemaking and in the young cheese, but to a lesser extent in the later stages of cheeseripening.

A possible pathway of casein breakdown is suggested to explain the roles of rennet and starter in determining whether or not bitterness will be found in cheese. It is suggested that rennet proteolysis of the casein forms a pool of predominantly high MW non-bitter peptides. The extent to which the precursors are degraded by the starter proteinases determines the level of bitter peptides in the cheese.

Good-flavoured cheese is associated with a low level of starter proteolysis, while more extensive proteolysis by the "bitter" starters of the non-bitter precursor peptides results in the formation of bitterness. "Burnt" off-flavours in cheese associated with the use of certain starter strains probably reflect a further degree of starter proteolysis and the accumulation of relatively high levels of amino acids. The increase in the intensity of bitterness in cheese when higher levels of rennet are used presumably results from the production of greater amounts of precursor peptides available for subsequent degradation to bitter peptides by the starter proteinases.

The level of starter proteinase in the cheese appears to be the most important factor determining the development of bitterness. However, it is likely that the salt-in-moisture levels in the cheese will also exert some control on the development of

bitterness since NaCl inhibited proteolysis by both rennet and starter proteinases.

It is concluded that it should be possible to exert considerable control on cheese flavour merely by regulating the maximum population of starter streptococci, and hence the level of starter proteinase, attained during cheesemaking. Such control would be important in reducing the incidence or intensity of bitterness in Cheddar cheese.

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## C O N T E N T S

	<u>Page</u>
ABSTRACT . . . . .	ii
ACKNOWLEDGEMENTS . . . . .	v
LIST OF FIGURES . . . . .	xi
LIST OF TABLES . . . . .	xii
INTRODUCTION . . . . .	1
The influence of the microflora on Cheddar cheese flavour	1
Role of starter streptococci	2
Survival of starter streptococci	4
Role of non-starter organisms	5
Bacteriological quality of the milk	6
Component balance theory of cheese flavour	7
Role of lipolysis in Cheddar cheese flavour	8
Role of glycolysis in Cheddar cheese flavour	9
Role of proteolysis in cheese flavour	9
Amino acids	9
Compounds formed from amino acids	10
Peptides and bitterness	11
Enzymes involved in casein degradation	14
Milk protease	14
Proteinases of non-starter organisms	14
Proteinases of starter streptococci	16
Rennet	17
Effect of pH and NaCl on rennet activity	18
Phosphatases	19
Influence of salt on Cheddar cheese flavour	20
AIMS OF THE PRESENT INVESTIGATION . . . . .	22
EXPERIMENTAL . . . . .	25
I. General procedures . . . . .	25
Cultures	25
Maintenance of the cultures	27
Skim milk	27
Spectrophotometric readings	28
II. Plate counting of starter streptococci . . . . .	28
A: Media	28
B: Plate counting and chain lengths of streptococci	29
Methods	
Cultures	31
Variation of chain length during growth	32
Effect of blending on chain length	32
Effect of blending on plate count	32
Effect of blending on viability	33



	<u>Page</u>
Results	33
Variation of chain length during growth	33
Effect of blending on chain length	34
Effect of blending on viability and plate count	36
Conclusions	38
III. Measurement of proteolysis . . . . .	39
Proteolytic activity of starter strains determined by Hull and Kjeldahl methods of analysis	41
Methods	41
Cultures	41
Proteolysis	41
Results	42
Comparison of Hull and Kjeldahl methods of analysis	42
Proteolysis by starter strains	45
IV. Growth, survival and proteolytic activity of starters . . . . .	46
Methods	46
Proteolysis	47
Growth and survival of starter	47
Results	48
Populations attained	48
Effect of CaCO <sub>3</sub> and NaCl on pH	50
Starter survival in the absence of NaCl	50
Starter survival in the presence of 4% NaCl	50
Starter survival in the presence of 5% NaCl	53
Proteolysis by the starter strains	55
Effect of NaCl on starter proteolysis	55
Influence of NaCl on starter survival and proteolysis	55
V. Proteolysis by rennet . . . . .	60
A: The effect of NaCl	60
Methods	60
Results	61
Relative proteolytic activities of rennet and starter	64
B: The influence of starter on determining rennet concentration in cheese	65
VI. Phosphatase activity of starters . . . . .	69
Methods	69
Cultures	69
Assay	69
Results	70
Preliminary experiments	70
Acid phosphatase of milk cultures and the relationship to proteolytic activity	72
VII. Influence of milk quality on cheese flavour . . .	74
A: Bacteriological quality of milk	74

	<u>Page</u>
B: Influence of milk quality on cheese flavour	77
Methods	77
Cheeses	77
Milk quality	77
Results	78
Bacteriological quality of the milk	78
Cheese flavour	79
Conclusions	84
VIII. Manufacture and analyses of selected cheeses . . .	85
Methods	85
Manufacture of cheeses	85
Counts of starter during cheesemaking and maturation	86
Routine analyses at 14 days	88
Proteolysis during maturation	88
Determination of free amino acids	88
Determination of acid phosphatase activity in cheese	89
Flavour assessments	89
Results	90
Manufacturing and analytical data	90
Starter populations during cheesemaking	90
Survival of starter	95
Acid phosphatase	99
Proteolysis	100
Amino acid analyses of the cheeses	103
Flavour assessments	109
Contribution of free amino acids to the flavour of the cheeses	111
DISCUSSION . . . . .	114
Growth of starters	114
Proteolysis by starters	116
Specificity of starter proteinases	117
Survival of starter in relation to proteolysis	117
Bitterness	118
Relationship of starter populations to proteolytic activity	121
Effect of NaCl on proteolysis and bitterness	124
Relationship of the pH of the cheese to the incidence of bitterness	125
Conclusions	126
REFERENCES . . . . .	129
APPENDIX I . . . . .	138
An improved medium for the detection of proteolytic organisms in total counts	

Page

APPENDIX II . . . . . 155  
The typing of coagulase-positive staphylococci by  
proteolytic activity on caseinate-agar, with  
special reference to phage nontypable strains

APPENDIX III . . . . . 167  
The rapid screening of milk samples for proteolytic  
and total bacterial counts

## LIST OF FIGURES

	<u>Page</u>
1. Increase in chain lengths of starter streptococci during growth in pasteurized skim milk	35
2. The effect of blending on the chain lengths and plate counts of starter streptococci	37
3. Spectrophotometric measurement of tyrosine	43
4. Proteolysis by starter strains measured by Kjeldahl and Hull methods	44
5. Survival of starters in pasteurized skim milk in the absence of NaCl	51
6. Survival of starters in pasteurized skim milk in the presence of 4% NaCl	52
7. Survival of starters in pasteurized skim milk in the presence of 5% NaCl	54
8. Proteolysis by starters in pasteurized skim milk in the absence of NaCl	57
9. Proteolysis by starters in pasteurized skim milk in the presence of 4% NaCl	58
10. Proteolysis by starters in pasteurized skim milk in the presence of 5% NaCl	58
11. Rennet proteolysis in pasteurized skim milk at pH 5.2 in the presence of NaCl	62
12. Influence of NaCl on rennet proteolysis of pasteurized skim milk	63
13. Casein breakdown in Cheddar cheeses measured by gel electrophoresis	68
14. Effect of pH on phosphatase activity of strain US <sub>3</sub>	71
15. Bacteriological quality of milk used for cheese-making during the 1969-70 dairying season	80
16. Overall flavour acceptability scores of 6-month-old Cheddar cheeses made during the 1969-70 season	81
17. Bitterness scores of 6-month-old Cheddar cheeses made during the 1969-70 dairying season	82
18. Starter populations during the making and ripening of Cheddar cheeses made with strains ML <sub>1</sub> , US <sub>3</sub> , Z <sub>8</sub> and HP	97
19. Starter populations during the making and ripening of Cheddar cheeses made with strains ML <sub>1</sub> , AM <sub>2</sub> , AM <sub>1</sub> and HP	98

(list of Figures cont.)	<u>Page</u>
20. Proteolysis in cheeses made with starter strains ML <sub>1</sub> , US <sub>3</sub> , Z <sub>8</sub> and HP	102
21. Chart trace of free amino acids in a 6-month-old Cheddar cheese	104
22. Comparison between the individual free amino acids in 3-month-old Cheddar cheeses and the amino acid composition of casein	107
23. Comparison between the individual free amino acids in 6-month-old Cheddar cheeses and the amino acid composition of casein	108
24. Role of rennet and starter in the formation of bitterness according to the hypothesis of Czulak	119
25. The suggested major pathways of casein breakdown by the proteinases of rennet and starter streptococci	122

#### L I S T O F T A B L E S

I. Strains of starter streptococci used	26
II. Increase of starter populations during growth in pasteurized skim milk	49
III. Proteolysis by starter strains during growth in pasteurized skim milk	56
IV. Comparison of acid phosphatase and proteolytic activities of starter strains	73
V. Manufacturing details for cheeses	91
VI. Routine analyses of cheeses at 14 days	92
VII. Starter populations during cheesemaking	94
VIII. Proteolysis in cheese curd samples immediately prior to salting	101
IX. Free amino acids in cheeses during the course of ripening	105
X. Flavour scores of the first series of cheeses	110
XI. Flavour scores of the second series of cheeses	112

THE INFLUENCE OF THE MICROFLORA ON  
CHEDDAR CHEESE FLAVOUR

It has generally been assumed that the only micro-organisms that make any significant contribution to Cheddar cheese flavour are those which are present in large numbers in the mature cheese. The "starter" organisms, predominantly lactic streptococci (Lancefield group N), tend to die out rapidly and, hence, the classical approach in earlier studies to determine the contribution of various organisms to flavour development has been to inoculate the cheesemilk with non-starter organisms isolated from cheese of good flavour. Such studies have been reported involving lactobacilli (Sherwood, 1939), organisms which frequently predominate in the flora of mature Cheddar cheese, and other organisms such as propionibacteria (Harris & Hammer, 1940), Leuconostocs, enterococci (Tittsler, Sanders, Lochry & Sager, 1948) and micrococci (Alford & Frazier, 1950). However, many of these earlier investigations were carried out at a time when adequate control of the bacterial flora, both of the raw milk and of the starter, was not possible. The commercial mixed-strain starters which were formerly widely used generally included heterofermentative organisms such as Streptococcus diacetylactis in addition to the essentially homofermentative S. lactis and S. cremoris.

During the 1930s Whitehead established that the use of pure single-strain cultures of starter in place of unknown mixtures resulted in greater reliability of cheesemaking in

terms of both the making process and the quality of the finished cheese. Single-strain starters, which most commonly are strains of S. cremoris, are now used almost exclusively for the manufacture of Cheddar cheese in New Zealand and Australia (Robertson, 1966).

The improved raw milk quality and particularly the introduction of pasteurized milk for cheesemaking into New Zealand in 1961 have effected a considerable amount of control on the flora of the cheesemilk. The possible roles of starter and non-starter in the formation of cheese flavour can now, therefore, be more effectively evaluated.

#### Role of starter streptococci

It became apparent that certain starters were regularly associated with certain characteristic flavours. Perry (1961), for example, described an unclean or fruity flavour in cheeses that was attributable to the Streptococcus lactis single-strain starter used, different strains of S. lactis being associated with different intensities of the "lactis" flavour. It was also established that certain pure strains of starter streptococci were more frequently associated with the development of bitterness than others which regularly gave good-flavoured cheeses (Emmons, McGugan, Elliott & Morse, 1962a; Lawrence & Pearce, 1968).

The development of the aseptic vat (Mabbitt, Chapman & Sharpe, 1959; Perry & McGillivray, 1964) afforded a means of making experimental cheeses under closely controlled conditions using essentially sterile milk and known strains of bacteria. It was then possible to determine more precisely the contributions

that the various bacteria commonly found in cheese made towards the development of Cheddar flavour. Cheeses made in the aseptic vat with sterile milk and gluconic acid lactone (GAL) as the acid-forming agent instead of starter were devoid of bacterial flora, and no typical cheese flavour developed. However, "aseptic" cheeses made with single strains of starter only, formed distinct Cheddar flavour, although they lacked the fullness of flavour of mature Cheddar cheese (Perry & McGillivray, 1964; Reiter et al., 1967). The characteristic fruity flavour of S. lactis ML<sub>3</sub> and bitterness associated with S. cremoris HP were detected, so confirming earlier reports concerning the flavours associated with these strains in cheeses made under normal (non-aseptic) conditions (Perry & McGillivray, 1964).

In the past it was considered that starter influenced flavour development only indirectly (Sherwood & Whitehead, 1934) since the activity of non-starter enzymes and of rennet would be determined by the pH, which is in turn largely controlled by acid production by the starter. Results obtained not only from aseptic vat experiments but also from extensive commercial trials in New Zealand have shown conclusively, however, that starter organisms do contribute directly to the basic Cheddar flavour.

Lawrence & Pearce (1968) grouped single-strain starters into three major groups on the basis of their flavour characteristics in normal cheeses, viz. those which gave no off-flavours, those which had a tendency to produce bitterness, and those which produced varying degrees of fruitiness. It was found that the intensity of the off-flavours of the starters of the bitter and



fruity groups could be substantially reduced by pairing them with starters of the first group. Emmons et al. (1962b) had similarly reported on the ability of "non-bitter" starters to decrease the intensity of the bitter flavour formed by "bitter" starters. In large-scale commercial trials in New Zealand, Sellars (1969) found that the proportion of unsatisfactorily flavoured cheeses associated with the use of S. lactis ML<sub>8</sub> alone could be decreased substantially if the lactis strain was paired with either of two cremoris strains which on their own gave good-flavoured cheeses. The reduction of bitterness in cheese by pairing a bitter starter with a non-bitter starter was explained as solely a dilution effect by Creamer, Lawrence, Gilles & Humphries (1970) who statistically analyzed the results of flavour trials of cheeses with combinations of such starters.

#### Survival of starter streptococci

The results of Dawson & Feagan (1957) that strains of S. lactis used as starter generally attained higher populations in the cheese and survived longer than S. cremoris strains were confirmed by Perry (1961), and later by Perry & McGillivray (1964) using the aseptic vat. The latter authors suggested that enhanced survival of the starters could be associated with stronger Cheddar flavour, and Perry (1961) considered that the longer survival of S. lactis could be significant for the development of "lactis" flavour. Anders & Jago (1964) postulated that bitterness in cheese was associated with poor survival of starter which resulted in a low level of peptidase activity. The ability of S. lactis strains to persist longer than strains of S. cremoris was closely correlated with the salt tolerances of the strains (Dawson &

Feagan, 1957). However, a more recent report (Anders & Jago, 1970) considers that the early loss of viability by certain strains may be due to the accumulation in cheese of long chain fatty acids, in particular oleic acid.

#### Role of non-starter organisms

Cheese made from pasteurized milk is generally not considered to develop as mature a flavour as that made from raw milk (Mabbitt, 1961) and numerous investigations have been reported on the effect of various non-starter organisms such as lactobacilli and micrococci on flavour (e.g. Franklin & Sharpe, 1963). Two organisms known to enhance Cheddar flavour, Lactobacillus casei-plantarum 25.2 (Sherwood, 1939) and Micrococcus L1 (Robertson & Perry, 1961) were investigated by Perry & McGillivray (1964) for their effects on the flavour of cheeses made in the aseptic vat. "Aseptic" cheeses made with GAL and with either the lactobacillus or the micrococcus were devoid of cheese flavour, suggesting that these organisms were not alone responsible for flavour development. However, "aseptic" cheese made with starter and either or both of these two non-starter organisms developed a more intense flavour than the corresponding cheese made with starter only.

The mechanism by which these non-starter organisms enhance flavour is unknown. It is possible that they make a direct contribution towards flavour as suggested by Robertson & Perry (1961), or that a symbiotic relationship may exist between the non-starter and starter populations with extended survival of the starter streptococci in the presence of non-starter organisms (Perry & McGillivray, 1964). It is also well known that

lactobacilli have complex growth requirements and their ability to grow actively in the cheese may depend on the supply of essential growth factors provided as a result of the earlier metabolic activities of the starter streptococci. In the absence of starter, lactobacilli in aseptic GAL cheeses died out, but the possibility of inhibitory properties of GAL or its breakdown products could not be excluded (Perry & McGillivray, 1964).

#### Bacteriological quality of the milk

It is apparent that, although the starter streptococci are primarily responsible for the development of basic Cheddar flavour, the non-starter flora, whether derived from either the raw milk, having survived pasteurization, or from the factory environment as adventitious contaminants (Naylor & Sharpe, 1958b) can sometimes considerably modify the basic flavour. Depending on the particular strains of micro-organisms involved, the non-starter population may have either little or no effect on flavour, or enhance it, or be responsible for the development of undesirable flavour characteristics. It is well known that the enzymes of many bacteria are relatively stable at the temperatures commonly used for pasteurization (Stadhouders, deVries & Mulder, 1959; Kishonti & Sjöström, 1970). Hence, although pasteurization may destroy over 90% of the raw milk flora, such enzymes as proteinases and lipases generated by the raw milk flora prior to pasteurization can still be expected to be active.

The lower flavour scores of cheeses made from pasteurized milk of initial poor bacteriological quality probably reflect the continued enzymatic activity derived from the initial raw

milk flora (Wilson, Hall & Rogers, 1945; Hattowska, 1970). However, off-flavours have been reported in cheeses made from pasteurized low count raw milk, the underlying cause being attributed to the "persistently occurring milk flora" in particular micrococci and lactobacilli (Teagan & Dawson, 1959). Hence, in any assessment of raw milk quality it may be important to take account of not only numbers but also the types of micro-organisms present.

#### COMPONENT BALANCE THEORY OF CHEESE FLAVOUR

The earliest investigators of the chemical nature of Cheddar cheese flavour considered that a single compound or group of compounds was responsible for typical Cheddar flavour (Suzuki, Hastings & Hart, 1909/10). However, Mulder (1952) and Kosikowski & Mccquot (1958) suggested that cheese flavour was due to a mixture of compounds derived from the degradation of fats, protein and lactose. Although individually these compounds may have flavour, but not necessarily cheese flavour, it is only when they are in the appropriate balance that the resultant flavour is that of typical cheese.

The component balance theory of cheese flavour has been generally accepted (e.g. see reviews by Harper, 1959; and Mabbitt, 1961) and characteristic flavour development is considered to be related to the enzymatic processes of glycolysis, lipolysis and proteolysis which must proceed interdependently at definite rates for optimum flavour formation. Most work to date on the chemical composition of cheese flavour has concentrated on the volatile odour components, although it is likely that the non-volatile

compounds involved in the taste of the cheese are most important, at least to the consumer.

#### ROLE OF LIPOLYSIS IN CHEDDAR CHEESE FLAVOUR

Although changes in the milk fat probably do contribute to some of the flavour precursors in Cheddar cheese, a direct relationship between increased lipolytic activity and the development of typical flavour has not been proven. With the use of milk of improved bacteriological quality and the widespread use of pasteurization, less importance is now attached to the lipolytic activity of many of the strongly lipolytic contaminants such as micrococci and Gram negative organisms. The major flora of importance in lipolysis in cheese, therefore, are the starter streptococci and such adventitious organisms as the lactobacilli, and these have been shown to have definite but weak lipolytic activity (Fryer, Reiter & Lawrence, 1967; Reiter et al., 1967). However, the free fatty acid level of the cheese is dependent also on the initial level in the cheesemilk, and this varies according to the milking procedure employed and the feeding regime of the cows (Reiter, Sorokin, Pickering & Hall, 1969). It is unlikely that the activity of milk lipase, which is greater than that of the starter organisms, is significant in other than raw milk cheeses on account of its inactivation by pasteurization.

It seems unlikely that fatty acids are essential flavour components, as typical Cheddar flavour has been reported in mature cheeses with widely differing acetic acid content (Reiter et al., 1967) and in cheeses in which fatty acids with more than 4 carbon atoms could not be detected (Lawrence, 1967).

### ROLE OF GLYCOLYSIS IN CHEDDAR CHEESE FLAVOUR

The content of acetic and propionic acids in Cheddar cheese is dependent on the strain of starter used (Reiter et al., 1967) and these acids are most commonly derived, along with other compounds such as diacetyl and acetoin, as by-products of the heterofermentative utilization of carbohydrate (Vedamuthu, Sandine & Elliker, 1966). These low molecular weight compounds are likely to play a significant role in the volatile component of Cheddar flavour and this may be particularly enhanced in the presence of hydrogen sulphide (Lawrence, 1963). The production of certain fruity flavours may result from the presence of esters and alcohols in cheese made with certain strains of starter (Bills, Morgan, Libbey & Day, 1965).

### ROLE OF PROTEOLYSIS IN CHEESE FLAVOUR

The gradual degradation of casein, principally under the influence of rennet and bacterial proteinases, results in the formation of a complex mixture of peptides and amino acids. Whereas undenatured proteins appear to have little or no taste (Mulder, 1952) it is considered, however, that the products of casein breakdown provide a background to cheese flavour (Mabbitt, 1961; Reiter, Fryer, Sharpe & Lawrence, 1966).

#### Amino acids

The possibility that free amino acids derived from the proteolytic breakdown of casein contribute to the taste components of cheese flavour has been considered by a number of investigators. It is well known that different amino acids when tasted alone in

solution have characteristic flavours such as bitter, sweet or tasteless (Mulder, 1952). However, experiments involving the tasting of mixtures of amino acids, or the addition of amino acids to fresh cheese curd followed by tasting after ripening, were generally inconclusive.

Mabbitt (1955) prepared a mixture of amino acids in a 4.8% NaCl solution in concentrations equivalent to those determined in the aqueous phase of 6-month-old Cheddar cheeses. Although the flavour of this mixture could not be described as typically Cheddar, it was considered that its "brothy" taste provided a most important background flavour to Cheddar cheese. A similar flavour developed in skim milk Cheddar cheese after ripening (Mabbitt & Zielinska, 1956).

Kristoffersen & Gould (1960) considered that although a correlation could not be established between the presence of individual amino acids and characteristic Cheddar flavour, a relationship between the intensity of flavour and the total concentration of free amino acids in the cheese was apparent.

#### Compounds formed from amino acids

Decomposition of sulphur-containing amino acids such as methionine and cysteine can lead to the formation of  $H_2S$ , and this may be associated with cheese flavour (Kristoffersen & Gould, 1960). Lawrence (1963) examined Cheddar cheeses made with single-strains of S. lactis and S. cremoris and found that although there were considerable differences between the cheeses in intensity of Cheddar flavour, the patterns of  $H_2S$  production were very similar. It was, however, considered possible that  $H_2S$  could contribute indirectly to the formation of Cheddar

flavour by combining with some other product of ripening such as carbonyl and carboxyl groups.

Although present in only trace amounts, methyl disulphide and dimethyl sulphide were the only volatile compounds consistently detected in higher concentrations in aseptic and control Cheddar cheeses made with starter compared to an "aseptic" cheese made without starter (McGugan et al., 1968). The starter organisms, therefore, are implicated in the formation of these sulphur compounds which are probably derived from the breakdown of methionine.

#### Peptides and bitterness

It is generally agreed that bitterness in cheese is caused by bitter peptides formed by proteolytic action on the casein and much work has recently been directed at elucidating the chemical structure of bitter peptides. Both Harwalker & Elliott (1965) and Raadsveld (1953) reported a high proportion of proline in trichloroacetic acid-soluble bitter peptides isolated from Cheddar and Gouda cheeses, respectively. Gordon & Speck (1965a) found that bitterness in skim milk cultures of S. cremoris strain HP was associated with a peptide of molecular weight approximately 2350, possibly possessing a cyclic portion, and comprising 19 amino acid residues, five of which were proline and four valine. Recently Matoba, Hayashi & Hata (1970) have extensively characterized bitter peptides obtained from the tryptic hydrolysis of casein. The bitter peptides isolated contained 6, 12 and 8 amino acid residues and had molecular weights of 629, 1385 and 980, respectively.

Richardson & Creamer (1970) could find no detectable



differences in the patterns of casein breakdown, as determined by polyacrylamide gel electrophoresis, between a bitter and a non-bitter Cheddar cheese made from the same milk. In both types of cheese  $\alpha_{s1}$ -casein was the only component degraded to any extent, supporting similar observations by Ledford, O'Sullivan & Nath (1966). The most bitter fraction of an extract of the bitter cheese occurred in the low molecular weight range, a large number of peptides being present.

Although much of the knowledge of the chemical nature of the bitter peptides derived from casein is still fragmentary it is apparent, however, that these peptides do have certain similarities such as a molecular weight of below 3000, and the possession of bitter and hydrophobic amino acids.

Czulak (1959) postulated a mechanism for the development of bitterness which involved the combined action of rennet and bacterial proteolytic enzymes. Bitter peptides were considered to be released by rennet action and to be hydrolysed further by "non-bitter" strains of lactic streptococci. "Bitter" strains were unable to hydrolyse these peptides which thus accumulated to cause the bitter taste; in other words, "bitter" strains were considered to be relatively non-proteolytic. In general, Czulak's hypothesis has been supported by a number of other investigators (Emmons et al., 1962a; Czulak & Shimmin, 1961; Jago, 1962; Stadhouders, 1962; Sullivan & Jago, 1970a). In contrast, however, Gordon & Speck (1965b) reported that "bitter" starters formed bitter compounds from casein in the absence of rennet, whilst "non-bitter" starters did not, and concluded that "bitter" strains exhibited greater proteolysis than "non-bitter"

strains. Klimovsky, Zvyagintsev, Gudkov & Medvedeva (1970) have also indicated that "bitter" strains of lactic streptococci are more proteolytically active than "non-bitter" strains. Harwalkar & Elliott (1971) have also suggested that bitterness in cheese could be produced by a mechanism that does not require the action of rennin, and have conclusively shown that proteolysis of casein by bacterial enzymes alone can be a source of bitter flavour components.

Lawrence & Gilles (1969) found that bitterness in Cheddar cheese is dependent on two main factors, viz. the use of specific fast acid-producing strains of S. cremoris and S. lactis, and the level of salt in the moisture of the cheese. The concentration of salt was considered to control the formation of bitterness by its influence on the activity of both the rennet and starter proteolytic enzymes. Although the rate of acid development in the vat has been claimed to affect the development of bitterness (Czulak, 1959), Lawrence & Gilles (1969) considered that the acidity in the curd influences bitterness only indirectly by determining the quantity of salt retained and thus the "salt in moisture" level of the cheese.

Sullivan & Jago (1970a) have suggested that bitterness in cultured dairy products may result from the combined action of rennet and bacterial proteolytic activity on casein giving rise to a bitter peptide resistant to further proteolysis on account of the formation of a pyrrolidonyl residue caused by cyclization of an N-terminal glutamine. The enzyme pyrrolidonyl peptidase is known to occur in a number of micro-organisms (Doolittle & Armentrout, 1968; Szewczuk & Mulczyk, 1969) and they consider the possession of this enzyme by "non-bitter" strains of lactic

streptococci would allow the removal of the pyrrolidonyl residue from the bitter peptide and explain the absence of bitterness. Pyrrolidonyl peptidase activity was subsequently described in the "non-bitter" S. cremoris strain ML<sub>1</sub> (Sullivan & Jago, 1970b).

#### ENZYMES INVOLVED IN CASEIN DEGRADATION

The activities of the proteolytic enzymes involved in the degradation of casein during cheesemaking and cheese-ripening are obviously important in any consideration of cheese flavour development. There are three general sources from which these enzymes can be derived - the milk itself; added rennet; and the micro-organisms of all or either of the raw milk flora, the starter streptococci and other adventitious flora.

##### Milk protease

Milk protease is one of a variety of enzymes found in normal milk and is present in low concentration associated with the casein fraction (Shahani, 1966). Little is known of its importance in cheese-ripening and there is little agreement regarding its heat stability and the optimum conditions for activity. However, recent work by Reiter et al. (1969) has indicated that milk protease can resist the heat treatment of pasteurization and is active at the pH of ripening Cheddar cheese, liberating low amounts of amino acids.

##### Proteinases of non-starter organisms

The widespread refrigeration of milk and the subsequent longer periods of holding milk between milking and processing have focussed attention on the psychrotrophic bacteria, a large

proportion of which are recognized as actively proteolytic (Witter, 1961). The significance of the heat resistance of their enzymes has already been mentioned.

Micrococci, Gram-positive spore-forming rods and corynebacteria are the major types of thermophilic organisms which may be present in the cheesemilk (Thomas, Druce, Peters & Griffiths, 1967) and micrococci appear to predominate under New Zealand conditions (Twomey & Crawley, 1969).

In comparison with the psychrotrophs, the proportion of thermophilic organisms generally reported as proteolytic is low and the importance of their proteolytic activities will depend on a number of factors such as the particular strains involved, their numbers in the milk initially, their ability to survive in the cheese, and the relative activities of their proteinases under the conditions of temperature, pH and salt concentration encountered in the cheese.

As some micrococci had been implicated in flavour enhancement in Cheddar cheese, McDonald (1964) investigated the proteinases of three strains of coagulase-negative staphylococci which were isolated from Cheddar cheese. Although the enzymes were most active at an alkaline pH and at high temperatures, they also had considerable activity at pH 5.5-6.0 and two strains had considerable activity at 15°C, a temperature close to that of cheese-ripening. Hence, it was considered that although the proteinases of these strains were not acting at their optimum pH or temperature, they could still contribute to overall protein hydrolysis during the ripening process.

The proteolytic activities of the lactobacilli have

attracted the attention of many workers on account of their frequent predominance particularly in the later stages of cheese-ripening. Considerable quantities of amino acids are formed in chalk-milk cultures of lactobacilli in which the pH is maintained at c. 5.4. Sharpe & Franklin (1962) isolated strains that were capable of producing  $H_2S$  from S-containing amino acids under the conditions of cheese-ripening. Although the lactobacilli probably contribute little to proteolytic breakdown in young cheese, their presence in large numbers during the later stages of maturation may result in a significant contribution to casein breakdown (Tourneur, 1970).

#### Proteinases of starter streptococci

The lactic streptococci are generally regarded as exhibiting only a very low level of proteolytic activity compared to many other organisms. They are, nevertheless, capable of breaking down casein to small peptides and amino acids (Morgan & Nelson, 1951). Although it has been reported that few, if any, differences exist between strains of S. cremoris and S. lactis in regard to their ability to liberate amino acids (Miller & Kandler, 1967), it seems likely that differences in proteolytic activity or specificity do exist between the "bitter" and "non-bitter" strains of lactic streptococci.

However, most workers have reported little, if any, proteolytic activity at pH 5. Optimal pH for the activity of the intracellular proteinases (van der Zant & Nelson, 1954; Vadehra & Boyd, 1963; Sato & Ohmiya, 1966) and the extracellular proteinases (Sasaki & Nakae, 1959; Williamson, Tove & Speck, 1964) was in the range pH 6.0-8.5, although Vadehra & Boyd (1963)

reported that their strain of S. lactis possessed a second peak of activity at pH 5.5.

It is apparent that the presence of very large numbers of starter streptococci in the early stages of cheesemaking accounts for a significant level of enzyme activity over a considerable period during ripening and may be responsible for the formation of amino acids in the cheese (Yamamoto & Yoshitake, 1962; Reiter et al., 1969). There are no reports that the levels of amino acids in the cheese are dependent on the starter strain used.

Investigations into the proteolytic activity of starter and other lactic acid bacteria in cheese are complicated by the presence of rennet. Jespersen (1966) supported a number of earlier reports that the proteolysis by lactic acid bacteria was accelerated in the presence of rennet. Amundstad (1950), however, concluded that although the addition of rennin to enzyme extracts from various bacteria increased the amount of proteolysis beyond that due to the bacterial proteinases alone, the total amount of proteolysis was the sum of their individual activities.

#### Rennet

Although the important contribution made by micro-organisms to proteolysis in cheese has been stressed by some workers (Peterson, Johnson & Price, 1948; Ledford et al., 1966), others have generally considered rennet to be the most significant proteolytic enzyme involved in cheese-ripening (Sherwood & Whitehead, 1934; Reiter et al., 1966). The principal use of rennet in cheesemaking is to rapidly coagulate the milk at its

normal pH of 6.6 to form a uniform, smooth coagulum. While rennin is considered only a weakly proteolytic enzyme (Bang-Jensen, Foltmann & Rombauts, 1964), it is capable of breaking casein down extensively during prolonged maturation (Lawrence, unpublished results) and has been shown to remain active for long periods in curd samples held at 10°C (Ohmiya & Sato, 1970). However, it appears that the degradation of casein to amino acids is not likely to be due to rennet (Yamamoto & Yoshitake, 1962; Reiter *et al.*, 1969).

#### Effect of pH and NaCl on rennet activity

Stadhouders (1962) studied the effect of pH and NaCl on the proteolytic activity of rennet on paracasein. Rennet activity increased markedly to an optimum at a concentration of 3% NaCl at pH 5.2. The stimulating effect of NaCl was attributed to the higher solubility of the paracasein making the substrate more readily available to the enzyme.

In contrast to Stadhouders' work, Amundstad (1950) found only a slight enhancement of rennin proteolysis with 2% NaCl at pH 5.5, but otherwise activity decreased in proportion to an increasing NaCl concentration over the range 0.5-10.0%.

Using both standard Kjeldahl analysis to measure low molecular weight degradation products, and gel electrophoresis to detect high molecular weight degradation products, Fox (1969) found that the nature of the products formed by rennet proteolysis varied considerably depending on the temperature and pH employed. Moreover, he suggested that the bitter flavour reported prevalent in acid cheese could be due to the additional products produced by rennet at low pH's and not to the inability of the bacterial

proteinases to further degrade the polypeptides produced by rennet action as reported by Czulak (1959). More recently it was suggested that the inhibitory effect of NaCl on the proteolysis of  $\beta$ -casein by rennet might be important in controlling the development of bitterness in Cheddar cheese (Fox & Walley, 1971).

### Phosphatases

It has been postulated that the degradation of casein, a phosphoprotein, is due to the combined action of phosphatases as well as proteinases (Schormüller, 1968). High molecular weight peptides appeared to be split by bacterial proteinases into phosphorus-rich residues with three to four phosphoserine groups. El-Negoumy (1970) has suggested that rennin action on  $\alpha_s$ - and  $\beta$ -caseins may also yield phosphopeptides. According to Schormüller, phosphoserine groups exert a protective effect towards further proteolytic hydrolysis of the peptide and, hence, further degradation is dependent on the presence of phosphatases. The phosphatase activity in ripening cheese was, therefore, considered an important factor in the splitting of peptides.

There is no evidence that either of the two native milk phosphatases is active in cheese made from pasteurized milk. However, the acid phosphatase is likely to be more important in cheese-ripening on account of its greater heat stability during pasteurization and its greater activity at the lower pH's (Bingham & Zittle, 1963) in spite of being present in lower concentrations than the alkaline phosphatase. Schormüller considered that as phosphatases are widely distributed in nature, it was reasonable to assume that the phosphatase activity of the micro-organisms involved in cheese-ripening could be significant.



However, there are few reports concerning the phosphatase activity of either the lactic streptococci or lactobacilli. Hammer & Olson (1941) considered the genera important in cheese-making, Streptococcus, Lactobacillus and Propionibacterium, had no phosphatase activity, but Jacquet, Villette & Richou (1956) reported that although a strain each of S. cremoris and S. lactis had comparatively little phosphatase activity at alkaline pH's, the activity at pH 4-5 was considerable, particularly in the case of milk cultures.

#### INFLUENCE OF SALT ON CHEDDAR CHEESE FLAVOUR

The salt added to cheese serves to aid moisture expulsion, retard the growth of undesirable organisms, and also as a background to flavour. For these reasons it has been regarded as one of the most important factors controlling the characteristics of the cheese (Davies, Davis, Dearden & Mattick, 1937) and will thus have either a direct or an indirect effect on flavour development.

It has long been recognized that heavily salted cheeses do not exhibit as great a rate of protein degradation as cheeses containing less salt (van Slyke & Hart, 1903; Davies et al., 1937; Kristoffersen, 1967). Furthermore, Lawrence & Gilles (1969) found that the bitter flavour in Cheddar cheese associated with specific starter strains developed less frequently at higher levels of salt, so substantially confirming the earlier report of Tuckey & Ruehe (1940) that bitterness occurred more frequently in cheeses with lower levels of salt.

It is thus apparent that NaCl affects the activities of

the proteolytic enzymes in the cheese. Mention has already been made of its effect on rennet, but there appears to be no account of its effect on the activity of the proteinases of the starter streptococci. It may, nevertheless, be of indirect importance in increasing the availability of intracellular enzymes necessary for flavour development by affecting the survival of different starter strains (Reiter et al., 1967).

AIMS OF THE PRESENT  
INVESTIGATION

It is now well established that single strains of Streptococcus cremoris and S. lactis used as starters in Cheddar cheesemaking are responsible for the development not only of the basic Cheddar flavour, but also of defects such as bitterness. The problem of bitterness has been accentuated in recent years by the growing Japanese market for New Zealand Cheddar cheese. The Japanese differ from persons of predominantly European origin in their ability to detect low levels of bitterness and it has thus become particularly important to avoid the development of such flavour defects in Cheddar cheese.

At the present time, the most practical way to control flavour is by the use of starter strains known from previous cheesemaking experience to give the desired flavour characteristics in cheese. In assessing the flavour characteristics of new starter strains, it would be valuable as an alternative to cheesemaking trials, which are of necessity at least six months in duration, to be able to identify quickly those strains which are associated with either good or poor flavour development. However, nothing is yet known regarding the features of single-strain starters which may be associated with the development of either good or poor flavours in cheese.

As many of the compounds which play a part in the development of cheese flavour are derived from the breakdown of casein, an attempt was made to determine the importance of the various proteinases that might be active during cheese-ripening. The starter streptococci are the predominant flora in cheese up to

at least two months. They are known to be only weakly proteolytic organisms, but their presence in large numbers and the long period of maturation of the cheese would be expected to result in a significant level of enzyme activity. As some of the proteolytic enzymes may be intracellular and liberated on autolysis of the cell, the death rate of the organisms in the cheese has also been considered significant, although no evidence exists to support this hypothesis. It was decided, therefore, to examine starter strains with known flavour attributes for their proteolytic activity and survival in milk cultures under the conditions of pH and NaCl concentration which are encountered in Cheddar cheese. The phosphatase activity of these starters has also been investigated since this has been implicated in casein breakdown and it was considered possible that the phosphatase and proteolytic activities of the starter streptococci might be related.

As this investigation proceeded, differences in proteolytic activity, phosphatase activity and cell populations became apparent in milk cultures of the different starter strains. To determine whether these same differences between the starter strains could also be detected in cheese, Cheddar cheeses were made using selected single-strain starters. These cheeses were examined to determine if their differences in flavour could be related to the maximum populations attained by the different starter strains, their survival, proteolytic and phosphatase activities in the cheese, and their ability to form free amino acids during the course of ripening.

There is considerable disagreement in the literature regarding the relative contributions of calf rennet and bacterial.

proteinases to the overall proteolysis in cheese during maturation. An attempt was made to resolve this point by measuring rennet proteolysis under the same conditions of pH and NaCl concentrations that were used to determine the proteolytic activities of the starters.

The activities of non-starter organisms, derived from the milk or as adventitious contaminants, may also considerably modify the basic Cheddar flavour. To determine the incidence of proteolytic organisms in the milk used for cheesemaking, it was necessary to have a suitable medium for their isolation and identification. While many investigators have come to the conclusion that the hydrolysis of casein in an opaque "milk agar" is a suitable indicator of proteolytic organisms, it is apparent from the many attempts that have been made to improve the existing methods that a satisfactory medium has yet to be devised. An improved medium for the identification of proteolytic organisms was, therefore, developed in the present investigation. As this medium was also suitable for determining the total counts in milk, an assessment could be made of the influence of the milk quality, described in terms of both total and proteolytic organisms, on the flavour developed by the cheese.

## E X P E R I M E N T A L

I. GENERAL PROCEDURES

Cultures. The strains of Streptococcus cremoris and S. lactis used in this study were obtained from the stock culture collection of single-strain Cheddar cheese starters maintained at the New Zealand Dairy Research Institute. Strains were selected on the basis of their ability to consistently give cheese with no off-flavours, or with flavour defects such as bitterness, fruitiness or other off-flavours (Table I).

The rates at which these different starter strains form acid during cheesemaking are normally assessed by standardized cheesemaking trials at the Institute. The different strains can then be grouped on the basis of the time they take from "setting" to form 0.65% of acid when used alone at 2% inoculum. Strains AM<sub>1</sub>, AM<sub>2</sub>, E<sub>8</sub>, ML<sub>4</sub> and US<sub>3</sub> are recognized as "slow" strains, whilst the remainder are comparatively "fast" (Lawrence & Pearce, 1968; Lawrence & Gilles, 1969).

The terms "slow" and "fast" apply to the relative acid-forming activities of different strains in pasteurized cheesemilk, in the presence of rennet and at temperatures in the range of approximately 37° to 39°C. They should be differentiated from the same terms used by other authors (Dawson & Feagan, 1957; Garvie, 1959; Citti, Sandine & Elliker, 1965) in describing variants of a particular strain. All the strains used in this investigation when inoculated at the rate of 1% from 18 hr cultures grown at 21°C into either sterile or pasteurized skim milk coagulated the milk within 18 hours at 21°C, and would, therefore, be termed "fast" strains according to the definition of Citti et al. (1965).

Table I. Strains of starter streptococci used in this investigation, and the typical flavours\* of cheese associated with their use as described in the literature.

<u>Species of Streptococcus</u>	Strain designation	Lawrence & Pearce (1958)	Lawrence & Gilles (1969)	Emmons et al. (1962a)	Czulak & Shimmin (1961)
<u>cremoris</u>	AM <sub>1</sub>	NOF	NB		
"	AM <sub>2</sub>	NOF	NB		
"	C <sub>13</sub>	B	B/A	B	
"	E <sub>8</sub>	NOF	NB	NB	
"	HP	B	B	B	B
"	ML <sub>1</sub>	NOF		NB	
"	R <sub>1</sub>			NB	NE
"	US <sub>3</sub>		NB		
"	Z <sub>8</sub>	B		B	
<u>lactis</u>	H <sub>1</sub>	F			
"	ML <sub>8</sub>		B		

\* Abbreviations: A, astringent;  
 B, bitter;  
 NB, non-bitter;  
 NOF, no off-flavours;  
 F, fruity.

Maintenance of the cultures. The cultures were maintained by twice-weekly subculture (1% inoculum) in sterile skim milk, with incubation at 22°C for 18 hr and storage at 4°C between subcultures. To ensure that the activity and purity of the strains was maintained, the cultures were replaced at approximately 8-week intervals with fresh cultures from the Institute's stock culture collection. These stock cultures in turn were regularly checked for purity by plating, and for their acid-forming activity under simulated cheese conditions by the method of Pearce & Limsowtin (1969).

Skim milk. To standardize the milk source, skim milk used throughout this study was reconstituted in distilled water from low-heat, spray-dried skim milk powder (New Zealand Dairy Board, Wellington, New Zealand) to 9.5% solids, the average level of milk solids in fresh skim milk (Dolby, Creamer & Elley, 1969).

For use in the routine subculture of the stock strains, the milk was sterilized by autoclaving at 118°C for 20 min. When pasteurised milk was required, the skim milk powder was reconstituted in sterile distilled water, observing aseptic precautions as far as was practicable. After pasteurization at 63°C for 30 min the milk was stored at 4°C for not longer than 24 hr before use. In this way a low count of <400 organisms/ml, determined by plating on Standard Methods agar (Baltimore Biological Laboratories, BBL) with 72 hr incubation at 30°C, could regularly be attained. The few organisms that survived would, therefore, reflect the count of thermoduric organisms in the milk powder. These organisms are comparatively slow-growing and inactive in milk (Thomas, Druce, Peters & Griffiths, 1967).



and their influence on the results of experiments in which comparatively large populations of fast-growing lactic streptococci are used would be negligible.

Spectrophotometric readings. Readings of colourimetric assays and determinations of cell densities of broth cultures were carried out at the appropriate wavelengths on a Unicam SP-500 spectrophotometer (Unicam Instruments Ltd, Cambridge, England) using 1 cm glass cells.

## II. PLATE COUNTING OF STARTER STREPTOCOCCI

### A: MEDIA

It is well known that lactic streptococci, and particularly strains of S. cremoris, have complex growth requirements and that comparatively rich media are required for their successful cultivation (Sharpe & Fryer, 1965). The media commonly used are seldom entirely satisfactory for all strains and this has led a number of workers to devise more suitable media or to evaluate existing media (Eliker, Anderson & Hannesson, 1956; Robertson, 1964; Baughman, 1964).

As a number of strains were to be used in this investigation, preliminary experiments were undertaken to select the most suitable agar media for use in pour plate counting with incubation at 30°C. The media examined included the following: lactose yeast phosphate agar (Robertson, 1960a); yeast dextrose agar (YDA) and tomato dextrose agar (Naylor & Sharpe, 1958a); the broth of deMan, Rogosa & Sharpe (1960) solidified with 1% Davis (New Zealand) agar (MRSa); and Trypticase Soy Broth (BBL)

supplemented with 0.5% yeast extract (BBL) and solidified with 1% Davis agar (TSBYA). TSBYA has been used by King & Koburger (1969) for plate-counting *S. lactis*.

Strains AM<sub>1</sub> and C<sub>13</sub> in particular grew poorly or erratically in many of the media tested. However, satisfactory and reproducible growth of these strains was obtained in YDA and MRSA, respectively, with 72 hr incubation. Many of the other strains grew equally well in more than one but not in all the media. While Trypticase Soy Broth solidified with 1% agar supported reasonable growth, the addition of 0.5% yeast extract caused a marked improvement and excellent growth was obtained with 48 hr incubation. TSBYA was, therefore, used for plating all strains other than AM<sub>1</sub> and C<sub>13</sub>, for which YDA and MRSA, respectively, were used.

The medium of Lowrie & Pearce (1971) which supports good growth of most strains of lactic streptococci, including AM<sub>1</sub> and C<sub>13</sub>, became available during the later stages of this investigation and was subsequently used in preference to YDA and MRSA. The composition of this medium (g/l) is as follows: polypeptone (BBL), 5.0; phytone (BBL), 5.0; yeast extract (BBL), 2.5; beef extract (BBL), 5.0; sodium acetate. 3H<sub>2</sub>O, 2.8; ascorbic acid, 0.5; lactose, 2.0; glucose, 2.0; agar (Davis), 10.0; pH, 6.8 to 6.9.

#### B: PLATE COUNTING AND CHAIN LENGTHS OF STREPTOCOCCI

The accurate enumeration of streptococci by plate counting has always been difficult because of the formation of chains and clumps of cells. Colonies which develop from a chain or clump cannot be regarded as developing from a single cell.

Although chains of S. lactis are generally shorter than those of S. cremoris (Breed, Murray & Smith, 1957), earlier work has indicated that the length of lactic streptococcal chains can vary considerably not only between different strains, but also according to the environment (Orla-Jensen, 1942; Morris & Edwards, 1947) and the age of the culture (Hammer, 1948). Smith (1965) also noted that there was a tendency for the chain length of S. lactis to increase during the growth of a culture.

A valid comparison of plate counts of different strains in terms of colony-forming-units (cfu) per ml or per gram may be made if it is determined that streptococcal chains are of equivalent lengths (Dawson & Feagan, 1957; Citti et al., 1965). The count of streptococci may also be expressed as cells per ml or per gram by multiplying the count of cfu obtained from pour plating by the average number of cells per chain determined microscopically (Smith, 1965). However, where large numbers of counts are to be undertaken on different strains and at different stages of growth, this procedure would obviously be very time-consuming and thus impractical.

Slade & Slamp (1956) obtained considerably increased plate counts of S. faecalis by subjecting the cultures to sonic oscillation. Microscopic examination of the cultures showed that the chains of initially from 7.0 to 12.5 cells were fragmented to smaller units of average length 1.7 to 2.7 cells after 1 min of treatment, and that an increase of from 2.0 to 3.4 times in the plate count was obtained. A longer period of treatment resulted in a marked lethal effect, although their data suggest that even 1 min of oscillation resulted in some cells being killed.

Robertson (1960b) obtained an increased plate count of lactic streptococci on subjecting the cultures to vigorous agitation in an Ato-Mix blender. Peebles, Gilliland & Speck (1969) blended the initial dilution of S. cremoris cultures in a chilled Waring blender for 5 min prior to plate counting. Chains of Leuconostoc citrovorum were found to break up readily on vigorous manual shaking to yield a majority of chains possessing four cells or fewer (Goel & Marth, 1969). Some bacteria have been reported to be sensitive to shear stresses encountered in routine pipetting of dilutions (Postgate, 1969) and Thatcher & Clark (1968) indicate that, with regard to homogenization of samples prior to plate counting, "excessive speed of the cutting blades or unduly prolonged use of the mixer may cause injury to microbial cells, either mechanically or by the heat generated". In view of this, the 5 min blending used by Peebles et al. (1969) seems to be an unnecessarily severe treatment.

It was decided, therefore, to determine firstly the chain lengths of the strains most frequently used in this investigation at different stages of their growth, and then to determine the suitability of mechanical agitation in fragmenting the chains to equivalent-sized cfu's as an aid to plate counting.

#### METHODS

Cultures. The following eight strains were inoculated (2% v/v) into 100 ml amounts of pasteurized skim milk (PSM) and incubated at 35°C: AM<sub>2</sub>, E<sub>8</sub>, HP, ML<sub>1</sub>, ML<sub>8</sub>, R<sub>1</sub>, US<sub>3</sub> and Z<sub>8</sub>. The inocula had been transferred twice in PSM with 12 hr incubation at 30°C from the stock cultures maintained in sterile skim milk (SSM).

Variation of chain length during growth. At hourly intervals during the growth of the different strains, the number of cells in 100 chains was determined by microscopic examination of Gram-stained smears made directly from the cultures. The results were averaged to give the average chain length.

Effect of blending on chain length. At hourly intervals during the growth of the different strains,  $10^{-2}$  dilutions, prepared by adding 1 ml of culture to 99 ml of 0.1% peptone diluent, were blended at full speed for 30 sec in a chilled ( $0^{\circ}$  to  $4^{\circ}\text{C}$ ) Ato-Mix blender (Measuring & Scientific Equipment Ltd, London). The chain lengths after blending were then determined and compared to the chain lengths in the unblended cultures.

Effect of blending on plate count. After 4 hr growth, when most strains exhibited their maximum chain lengths, five identical  $10^{-2}$  dilutions of each culture, prepared as described in the previous section, were blended for 0 (unblended), 15, 30, 60 and 120 sec in a chilled, sterile Ato-Mix blender at full speed. After blending, samples were taken to determine chain lengths by microscopic examination, and the blended  $10^{-2}$  dilutions were then further diluted decimally according to standard procedures (Postgate, 1969). A vortex mixer (Scientific Industries Inc., New York) was used to mix successive dilutions and 1 ml of the appropriate dilutions were pour plated in triplicate with molten TSBYA tempered to  $44^{\circ}\text{C}$ . The plates were incubated at  $30^{\circ}\text{C}$  for 48 hr and the colonies counted with the aid of an illuminated colony counter (Gallenkamp, London).

Effect of blending on viability. The viability of the chains in the blended  $10^{-2}$  dilutions of 4 hr cultures described in the previous section were determined by the slide-culture technique as described by Thomas & Batt (1968) using TSBYA as the growth medium. After blending, a loopful of the  $10^{-2}$  dilution was inoculated on to the surface of the agar contained in the annulus. Instead of covering the annuli with coverslips to prevent drying during incubation, it was found convenient to place the slides in an airtight box containing moistened blotting paper. Incubation was for 3 to 6 hr depending on the rate of growth of the strain.

## RESULTS

Variation of chain length during growth. Microscopic examination of smears made directly from the culture flasks showed that the number of cells per chain for all strains examined was almost always a multiple of 2, confirming the well-known diplococcal nature of this group of organisms. However, between strains there were characteristic differences. The main features are illustrated in the results presented for four strains -  $ML_8$ ,  $US_3$ , HP and  $ML_4$  (Fig. 1). These results represent the average of two separate determinations on each strain. Although S. cremoris strains are generally recognized as having longer chains than S. lactis strains, strains  $US_3$  (cremoris) and  $ML_8$  (lactis) closely resembled each other, both being short-chained and showing only slight increases in chain lengths to maxima of 3.7 and 4.5 cells per chain, respectively, during the exponential growth phase. However, other strains of S. cremoris were longer, attaining maximum

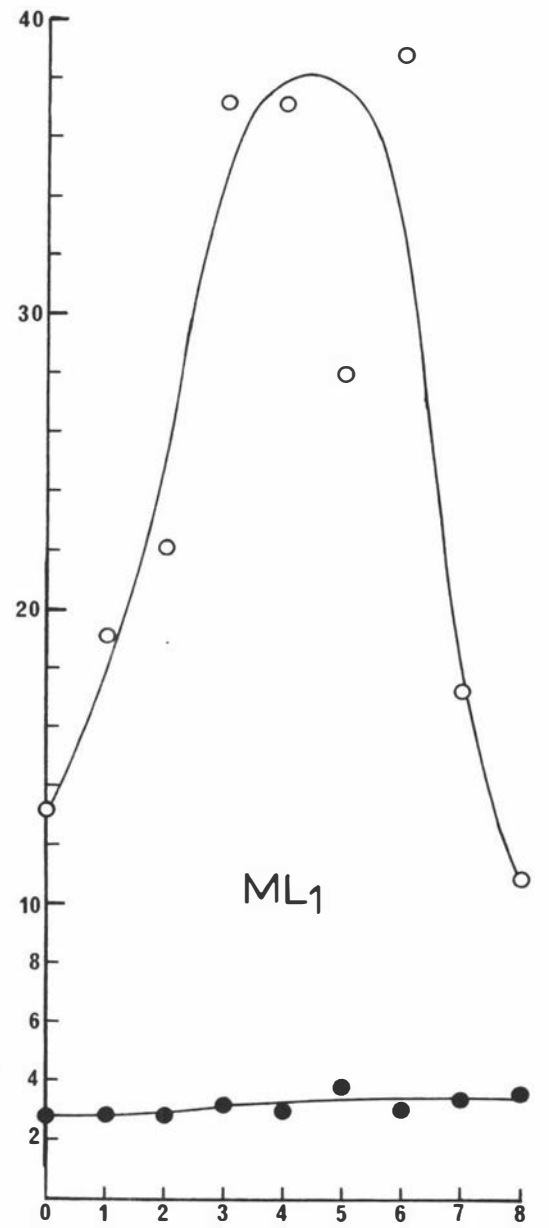
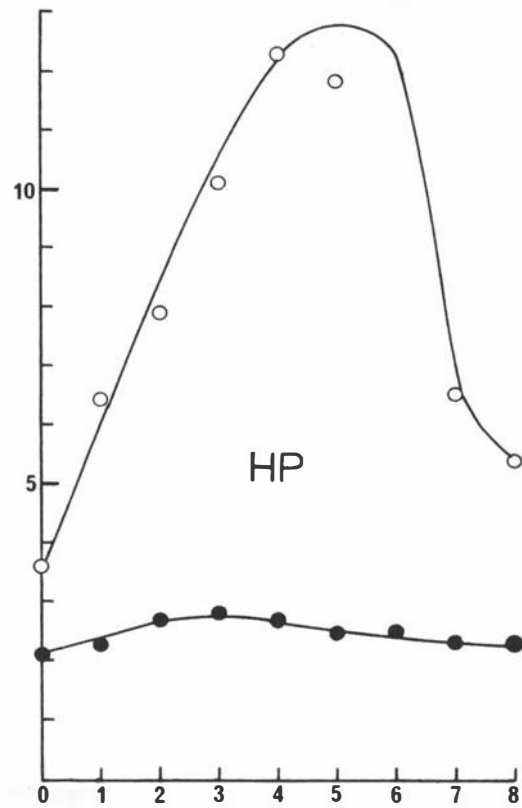
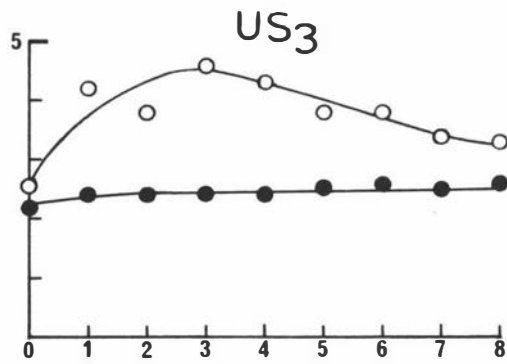
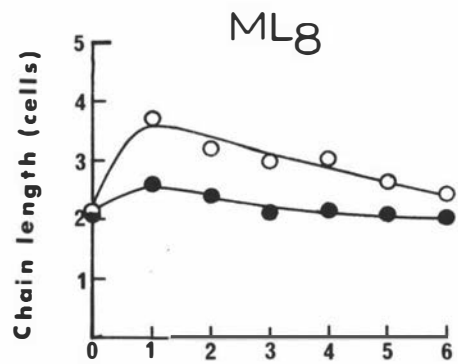
average lengths of approximately six cells per chain for strains AM<sub>2</sub> and Z<sub>8</sub>, eight for E<sub>8</sub>, 12 for R<sub>1</sub>, 13 for HP, and 38 for ML<sub>1</sub>. The relatively short-chained nature of some S. cremoris strains might be related to their growth at the higher temperatures used here compared to other investigations (Morris & Edwards, 1947; Dawson & Feagan, 1957).

There was considerable variation in the range of chain lengths observed, particularly at the times when the maximum average lengths were greatest and with the longer-chained strains. Chains of strain ML<sub>8</sub> were predominantly (89%) 2- and 4-celled, and 11% were greater than 4, with a maximum length of 10 cells. With strain US<sub>3</sub>, 73% of the chains were 2- and 4-celled and 27% were >4-celled. There were very few chains of >10 cells and the longest chain observed comprised 30 cells. By contrast, only a small proportion of the chains were short in strain HP, 2-, 4- and 6-celled chains forming 15% of the total. Twenty-six per cent of the chains were 8-celled, 48% in the range 10 to 20, and 11% had >20 cells. The longest chain comprised 42 cells. Variation in chain length of ML<sub>1</sub> was most marked, ranging from the occasional diplococcus to a few very long chains of >100 cells. Ten per cent of the chains had <10 cells, 65% were in the range 10 to 40 cells, and 25% had >40 cells.

Effect of blending on chain length. The chain lengths obtained after 30 sec blending of 10<sup>-2</sup> dilutions were compared with the corresponding values from unblended cultures of two short-chained strains (ML<sub>8</sub> and US<sub>3</sub>), one strain of intermediate chain length (HP) and a long-chained strain (ML<sub>1</sub>) at intervals during their growth in PSM at 35°C (Fig. 1). Although the

Fig. 1. Increase in chain lengths of starter streptococci during growth in PSM at 35°C, and the effect of 30 sec blending on chain lengths. The chain lengths were determined by microscopic examination of smears made from undiluted culture (○), and from 10<sup>-2</sup> dilutions blended for 30 sec (●). Note: vertical scale for strain ML<sub>1</sub> is half that for the other strains.





Age of culture (hours)

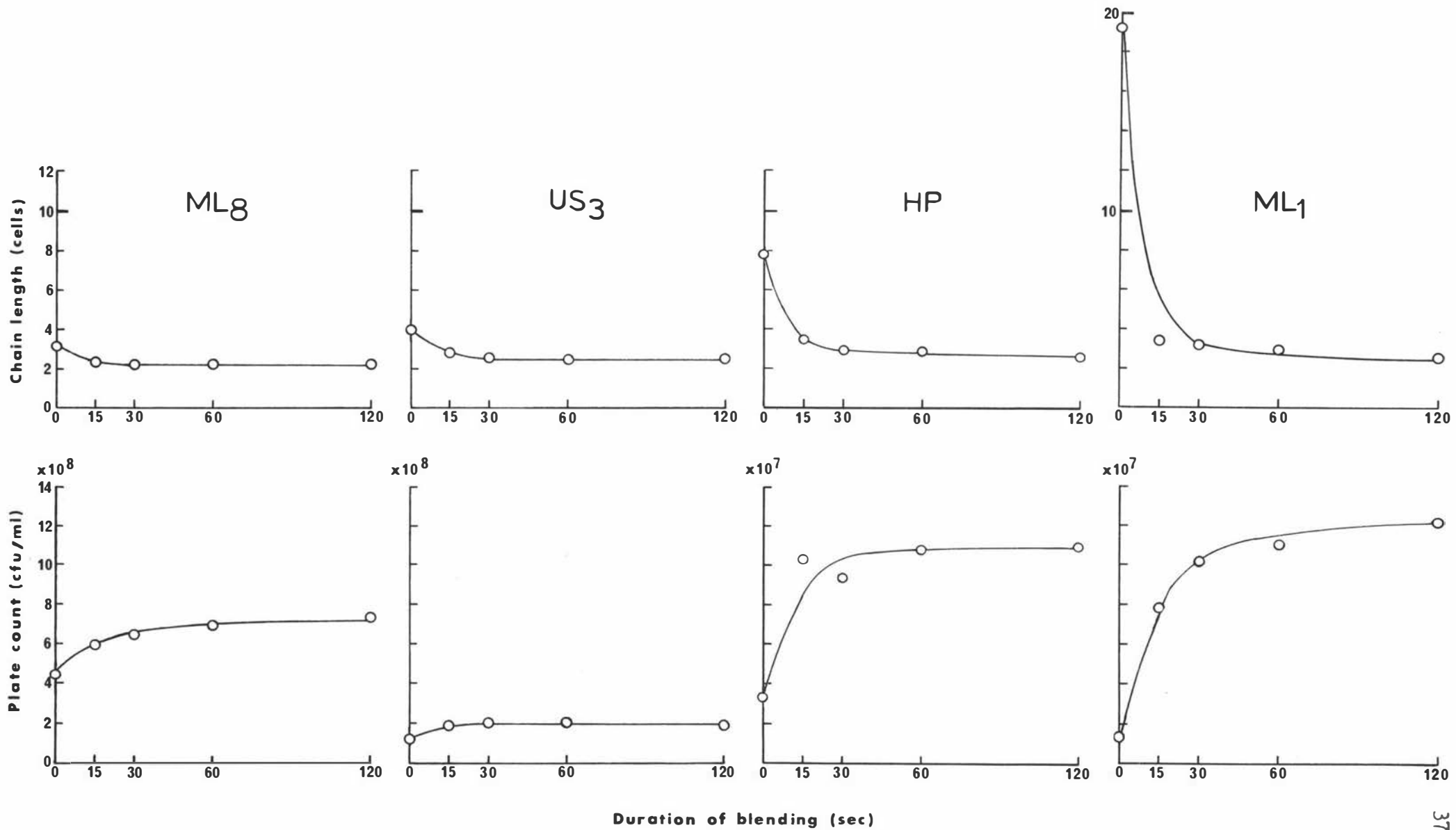
average length of the streptococcal chains in a culture depended on the strain, and also varied within the strain depending on the age of the culture, 30 sec blending was effective in reducing the average chain lengths of all the strains to an essentially constant value of between two and three cells. On average, 68% of these chains occurred as diplococci, 27% in fours, and 4% had between four and eight cells.

Effect of blending on viability and plate count. For all strains examined, viability as determined by the slide-culture technique was unaffected by blending for up to 120 sec. In these experiments the use of a chilled blender and sample ensured that excessive warming during blending did not occur, and the final temperature of the samples did not exceed 30°C. If this precaution was not taken, significant warming of the sample to >40°C occurred with 120 sec blending, and the viability of some strains, such as AM<sub>2</sub>, appeared to be adversely affected.

As the blending treatments did not affect viability, it could be expected, therefore, that a decrease in chain length obtained on blending would be proportional to an increase in plate count. The data obtained indeed showed such a relationship. The averages of duplicate determinations of chain length and plate count after blending 10<sup>-2</sup> dilutions of 4 hr 35°C PSM cultures for 0, 15, 30, 60 and 120 sec are presented for the same four strains described in the previous section (Fig. 2). Results for the other strains examined indicated essentially the same features.

With the two short-chained strains ML<sub>8</sub> and US<sub>3</sub>, blending brought about comparatively slight increases in plate count and correspondingly slight decreases in the average chain lengths.

Fig. 2. The effect of blending on the chain lengths and plate counts of starter streptococci. The cultures were grown for 4 hr in PSM at 35°C. Strains ML<sub>8</sub> and US<sub>3</sub> were typically short-chained, HP had chains of intermediate length, and ML<sub>1</sub> had long chains. Chain lengths were determined by microscopic examination of smears made from 10<sup>-2</sup> dilutions. Plate counts were determined by pour plating the appropriate dilutions.



With 30 sec blending, the plate count of  $ML_8$  increased 1.4-fold from  $4.5 \times 10^8$  (unblended) to  $6.5 \times 10^8$  cfu/ml, and the average chain length decreased 1.3-fold from 3.0 (unblended) to 2.3 cells. The corresponding figures for  $US_3$  were a 1.7-fold increase in plate count from  $1.3 \times 10^8$  (unblended) to  $2.2 \times 10^8$  cfu/ml, and a 1.6-fold decrease in the average chain length from 4.2 (unblended) to 2.6 cells.

The effect of blending on the longer-chained strains HP and  $ML_1$  was more marked. Thirty sec blending of HP increased the plate count 2.8-fold from  $3.3 \times 10^7$  (unblended) to  $9.4 \times 10^7$  cfu/ml, while the average chain length decreased 2.6-fold from 7.8 (unblended) to 3.0 cells. With strain  $ML_1$ , the values were a 7.7-fold increase in the plate count from  $1.3 \times 10^7$  (unblended) to  $1.0 \times 10^8$  cfu/ml, and a 5.9-fold decrease in the average chain length from 19.0 (unblended) to 3.2 cells.

Chain lengths in unblended  $10^{-2}$  dilutions tended to be lower than the chain lengths determined directly on undiluted cultures, particularly for the longer-chained strains. For example, the average chain length determined on an undiluted 4 hr PSM culture of  $ML_1$  was 37.0 cells (Fig. 1), whereas in the unblended  $10^{-2}$  dilution an average value of 19.0 cells per chain was obtained (Fig. 2). This probably reflects a certain amount of fragmentation of the streptococcal chains caused by agitation involved in preparing and mixing the dilution. A similar effect was noted by Goel & Marth (1969).

#### CONCLUSIONS

It is apparent that the lengths of the streptococcal chains

vary widely depending on the strain and stage of growth. Difficulties in plate counting attributable to varying cfu sizes (chain lengths) can largely be overcome by appropriate blending which reduces the chain lengths of all the strains examined at different stages of growth to the equivalent sizes. The greatest amount of fragmentation of the chains is brought about during the first 15 sec of blending. Prolonging the blending period beyond 30 sec results in only a very slight further increase in the plate count and decrease in chain length. Unnecessarily prolonged blending also carries an attendant risk of heat damage to sensitive strains unless adequately chilled equipment is used.

Consequent to these findings, a 30 sec period of blending of 100 ml amounts of  $10^{-2}$  dilutions was adopted as a routine, practical and consistent aid in the plate counting of cultures of starter streptococci.

### III. MEASUREMENT OF PROTEOLYSIS

Proteolysis can be determined as the increase in the non-protein fraction that remains after undenatured protein has been precipitated by a protein precipitant, such as trichloroacetic acid (TCA). The amount of non-protein material may be measured either in terms of nitrogen (non-protein nitrogen, NPN) by a standard Kjeldahl procedure, or by the method of Hull (1947) in which tyrosine and tryptophan residues react with the phenol reagent of Folin & Ciocalteu (1927) to form a blue colour. Kjeldahl analysis has been used to determine rennet proteolysis (e.g. Stadhouders, 1962) and proteolysis in maturing cheese

(e.g. Reiter et al., 1969). Silverman & Kosikowski (1955) considered that total tyrosine liberated in maturing cheese was a more sensitive criterion of ripening than soluble protein content. This was largely substantiated by Valkeris & Price (1959) who reported a good correlation between soluble tyrosine of cheese extracts, measured by absorption at 280 nm, and soluble nitrogen determined by Kjeldahl analysis.

Kjeldahl analysis has also been used to determine the proteolytic activities of starter streptococci, especially in relation to the development of bitterness in cheese (Czulak & Shimmin, 1961; Jago, 1962; Emmons et al., 1962a; Gordon & Speck, 1965a).

The report of van der Zant & Nelson (1953) appears to be the only one in which the proteolytic activity of milk cultures of S. lactis was determined using both Kjeldahl and Hull methods of analysis. Results obtained with the two methods differed, particularly in the first 10 to 12 hr of incubation. Marked increases in tyrosine and tryptophan determined by the Hull method were found when the production of soluble nitrogen as determined by Kjeldahl analysis was still negligible. This led these authors to consider that either two proteinase systems were involved, or that the liberation of compounds giving tyrosine and tryptophan reactions with the Folin & Ciocalteu reagent was an early phase of proteolysis. Although they calculated from their results that the amount of tyrosine and tryptophan liberated was closely related to the amount of soluble nitrogen, the relative closeness of these figures was considered fortuitous, rather than indicative that equivalent amounts of proteolysis had in fact been measured.

The Hull method offers considerable advantages over the Kjeldahl method of analysis, which is laborious and time-consuming especially when large numbers of samples are involved. It was, therefore, selected as the method for carrying out routine determinations of proteolysis. However, trials comparing the Hull and Kjeldahl methods of analysis were carried out to allow a comparison of the present work with earlier reports on starter proteolytic activity measured by Kjeldahl procedures.

PROTEOLYTIC ACTIVITY OF STARTER STRAINS DETERMINED  
BY HULL AND KJELDAHL METHODS OF ANALYSIS

METHODS

Cultures. Nine strains of S. cremoris (AM<sub>1</sub>, AM<sub>2</sub>, C<sub>13</sub>, E<sub>8</sub>, HP, ML<sub>1</sub>, R<sub>1</sub>, US<sub>3</sub> and Z<sub>8</sub>) and one strain of S. lactis (ML<sub>8</sub>) were grown in PSM from 1% inocula for 18 hr at 22° and 35°C. The lower temperature has been used in a number of investigations of proteolysis (e.g. Gordon & Speck, 1965a). The higher temperature was included as it was in the range of 31° to 39°C normally used during Cheddar cheesemaking.

Proteolysis. The amount of casein breakdown in the cultures was measured by both the method of Hull (1947) and Kjeldahl analysis. In the Hull procedure, all samples and reagents were routinely kept at 35°C, since low ambient temperatures interfere with the test (Citti, Sandine & Elliker, 1963). Protein in 1 volume of culture was precipitated with 2 volumes of 12% TCA. The blue colour that developed on the reaction of the Folin-Ciocalteu reagent (BDH) with available tyrosine and tryptophan residues in the 8% TCA



filtrates was measured colourimetrically at 650 nm against a reagent blank in which distilled water had been substituted for culture. The OD reading obtained was converted to an equivalent value of tyrosine by reference to a standard graph (Fig. 3). Separate graphs were prepared for different batches of Folin-Ciocalteu reagent.

Nitrogen in 10 ml lots of the same 8% TCA filtrates as used in the Hull procedure was determined by the semi-micro Kjeldahl method of Rowlands (1938).  $\text{CuSO}_4$  was used as the catalyst and methyl red as indicator.

## RESULTS

Comparison of Hull and Kjeldahl methods of analysis. In general, the trend of results obtained by Kjeldahl analysis was similar to that obtained by the Hull method (Fig. 4). The results are averages of duplicate experiments with duplicate determinations on all samples. With both methods of analysis, the average error in individual determinations was  $\pm 2\%$ . With the Kjeldahl method, however, this error was large relative to the small increases of NPN being measured, particularly with the less proteolytic strains. NPN determined by Kjeldahl analysis increased, from an average value of 0.042 g nitrogen/100 ml culture at the time of inoculation, by 25% for strains HP, ML<sub>1</sub> and R<sub>1</sub>, with an average increase for all strains of 17%, after 18 hr incubation at 35°C. Strain US<sub>3</sub>, however, showed no increase. By contrast, the levels of "tyrosine" determined by the Hull method increased from 35% for the least proteolytic strains (AM<sub>1</sub>, E<sub>8</sub>, and US<sub>3</sub>) to 200% for the most proteolytic strains (ML<sub>1</sub> and C<sub>13</sub>) from an initial

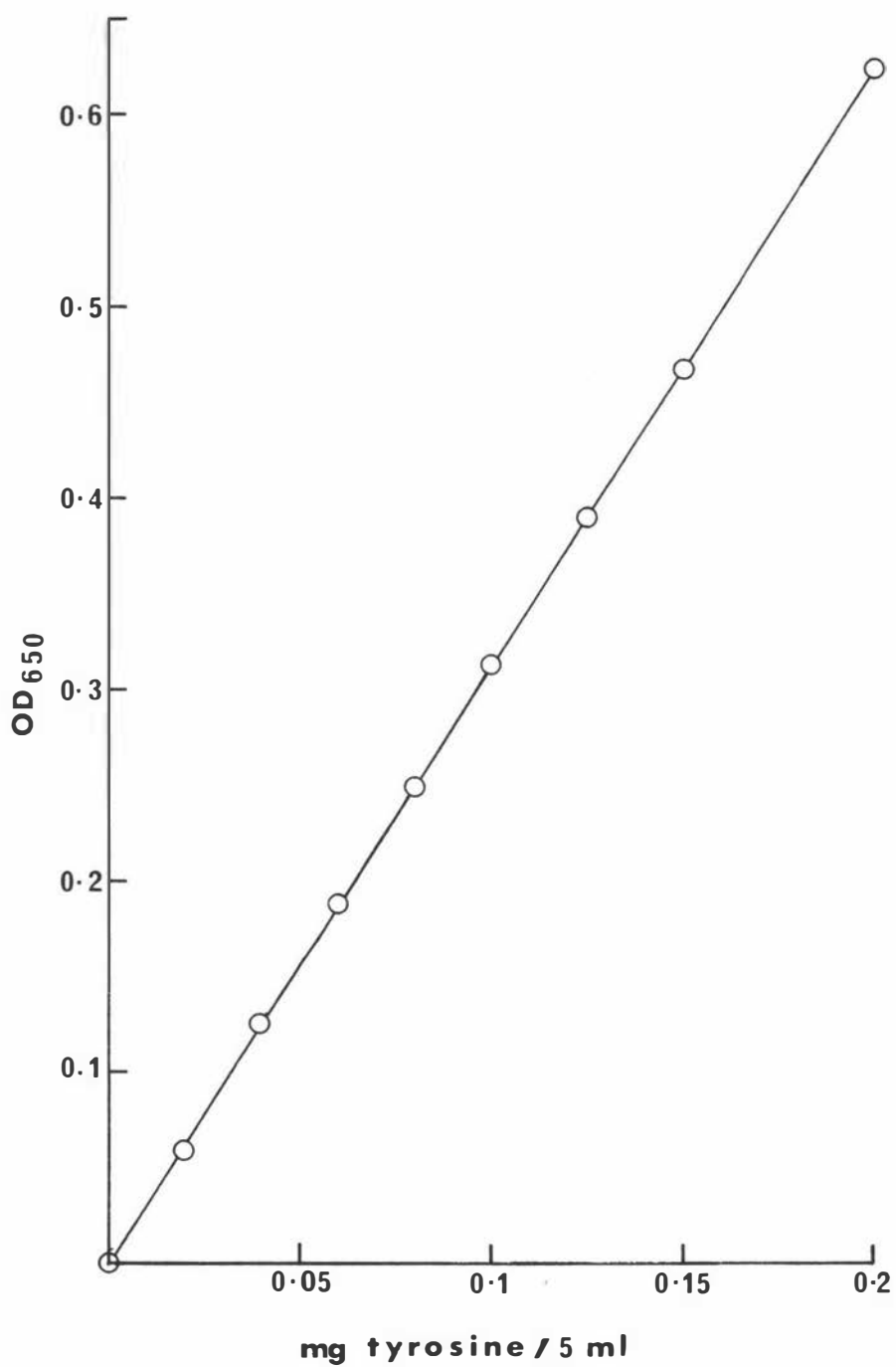
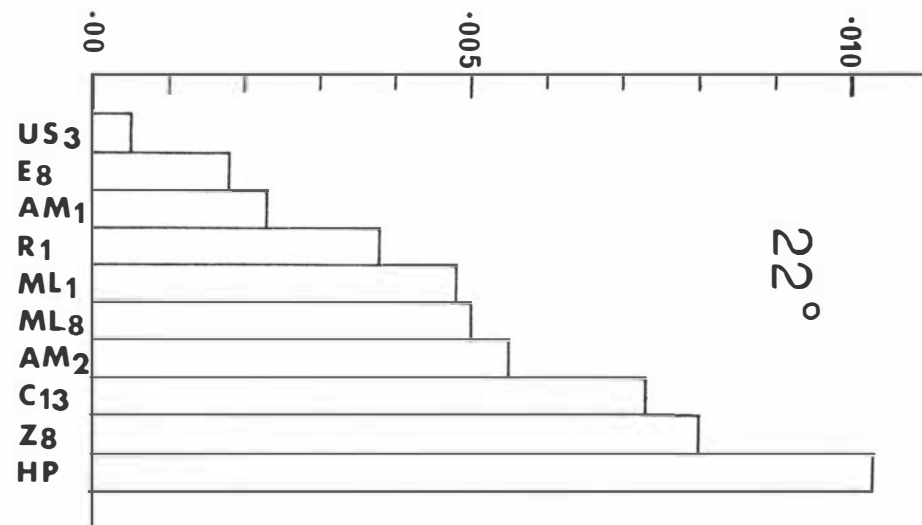
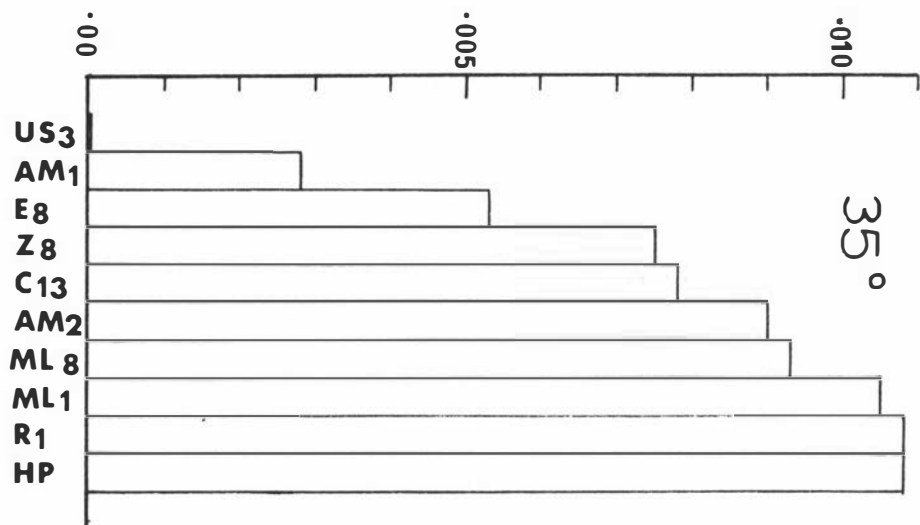


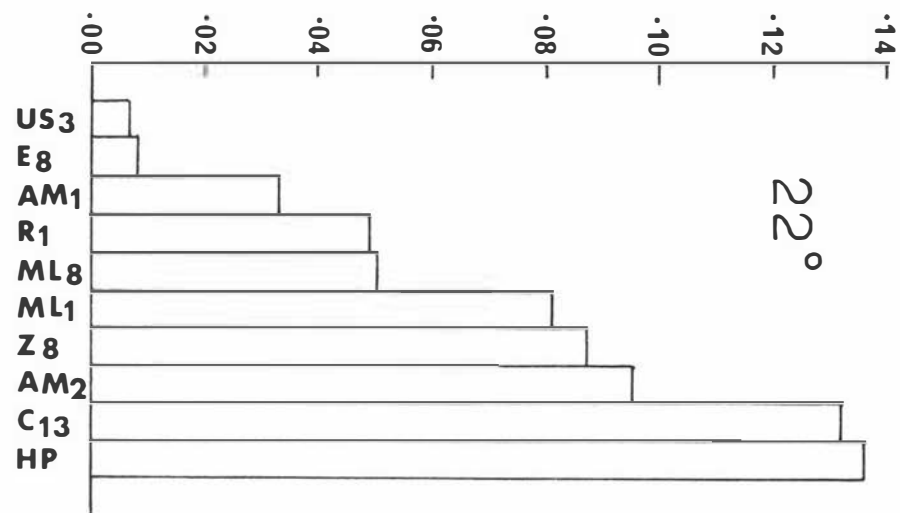
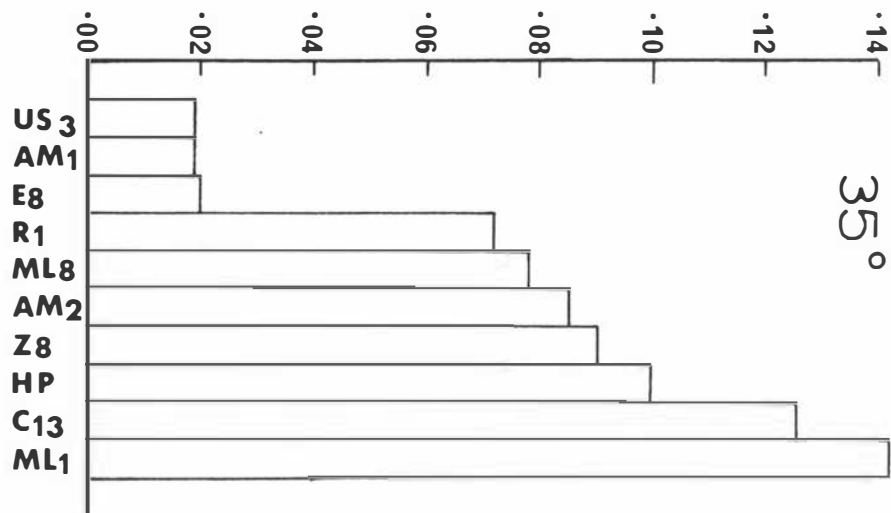
Fig. 3. Spectrophotometric measurement of the concentration of tyrosine using Folin-Ciocalteu reagent.

Fig. 4. Proteolysis by different starter strains grown in PSM for 18 hr at 22°C and 35°C from 1% inocula. Proteolysis was measured both by Kjeldahl analysis (g NPN/100 ml culture) and by the method of Hull (1947) (mg tyrosine/5 ml TCA filtrate).

Proteolysis (increase in g non-protein nitrogen/100ml culture)



Proteolysis (increase in mg tyrosine/5ml TCA filtrate)



level equivalent to 0.054 mg tyrosine/5 ml TCA filtrate. The average increase for all strains was 130%. The results at 22°C were similar but with a tendency for slightly less proteolysis than at 35°C.

Both methods of analysis gave substantially equivalent results and graded the relative proteolytic activities of the starters in almost the same order (Fig. 4). The Hull method, however, has the advantage of speed over Kjeldahl analysis, and a much greater sensitivity which is especially valuable in measuring the comparatively low levels of proteolytic activity of some starter strains.

Proteolysis by starter strains. At both 22° and 35°C the "non-bitter" strains AM<sub>1</sub>, E<sub>8</sub> and US<sub>3</sub> were less proteolytic than the "bitter" strains, such as HP and Z<sub>8</sub> (Fig. 4). However, other "non-bitter" strains, such as AM<sub>2</sub> and ML<sub>1</sub>, were as much or more proteolytic than some of the "bitter" strains. Thus the finding of Gordon & Speck (1965a) that a "bitter" strain was more proteolytic than a "non-bitter" strain in milk culture grown at either 22° or 32°C was confirmed in the present investigation for strains HP and E<sub>8</sub>, but could not be substantiated as a general feature of all "bitter" and "non-bitter" strains.

#### IV. GROWTH, SURVIVAL AND PROTEOLYTIC ACTIVITY OF STARTERS

The death rate of starter bacteria in cheese has been considered by some authors to influence cheeseripening and flavour development as a result of the liberation of intracellular enzymes (Franklin & Sharpe, 1963; Reiter et al., 1967) or the limitation of the levels of enzyme formed (Anders & Jago, 1964). The extended survival of certain strains in the cheese and their continued metabolic activities have also been implicated in the development of flavour defects (Perry, 1961; Vedamuthu et al., 1966).

An investigation was, therefore, undertaken of the growth, survival and proteolytic activity of starter strains in conditions which were designed to simulate some of those encountered in cheesemaking and maturation.

#### METHODS

The following starters were used: S. cremoris strains AM<sub>1</sub>, AM<sub>2</sub>, C<sub>13</sub>, E<sub>8</sub>, HP, ML<sub>1</sub>, R<sub>1</sub>, US<sub>3</sub> and Z<sub>8</sub>; and S. lactis strains H<sub>1</sub> and ML<sub>3</sub>. The flavour characteristics of cheese made with these strains have been detailed in Table I.

Stock sterile skim milk cultures were transferred twice through pasteurized skim milk (PSM) with 12 hr incubation at 30°C before being used as inocula. Prewarmed PSM (1,000 ml) was inoculated with 2% (v/v) of these starter cultures and incubated at 35°C until the pH reached 5.2, the pH at which Cheddar cheese curd is normally salted. At this point, 250 ml amounts of the culture were aseptically transferred to 3 sterile flasks. The

first flask contained 2.5 g  $\text{CaCO}_3$  only, the second flask 2.5 g  $\text{CaCO}_3$  and 10 g NaCl, and the third flask 2.5 g  $\text{CaCO}_3$  and 12.5 g NaCl. Thus all the cultures transferred to these flasks contained 1% (w/v)  $\text{CaCO}_3$ , while cultures in the second and third flasks contained in addition 4 and 5% (w/v) NaCl respectively. The  $\text{CaCO}_3$  held the pH constant at close to the approximate pH of cheese, and the levels of NaCl employed were of the order of the "salt-in-moisture" levels commonly found in commercial Cheddar cheeses (Lawrence & Gilles, 1969).

The cultures were then cooled to 13°C in a water bath and held for 14 days at this temperature. In commercial practice in New Zealand, Cheddar cheese is initially kept at 13°C for 14 days, followed by subsequent maturing at 7°C.

Proteolysis. The course of proteolysis of the cultures was followed by the method of Hull (1947) as described earlier (p.41). Samples, approximately 10 ml, were taken at the time of inoculation, again at the stage when the cultures reached pH 5.2 (i.e. immediately before the addition of NaCl and  $\text{CaCO}_3$ ), and at intervals during the subsequent period at 13°C. Undissolved  $\text{CaCO}_3$  in the samples was removed by centrifugation, and protein in a 5 ml sample of the supernatant culture was precipitated by the addition of 10 ml of 12% TCA.

Growth and survival of starter. The increase in cell numbers was determined by blending and pour plating as described earlier (see sections on Media and Plate counting, p. 28 and 29) at the time of inoculation and again at the stage when the cultures had reached pH 5.2. The survival of these strains in

the presence of NaCl and CaCO<sub>3</sub> during their subsequent incubation at 13°C was similarly determined. Although NaCl alone effected some fragmentation of the long chains of streptococci, as shown by microscopic examination, consistency in sizes of the colony-forming-units was ensured by the 30 sec period of blending. This allowed valid comparisons to be made both between the control cultures without NaCl and the cultures containing 4 and 5% NaCl, and between the different strains. Blending did not adversely affect the recovery of the NaCl-treated cultures.

## RESULTS

Populations attained. Plate counts on the cultures made at the time of inoculation and at the stage when the pH had reached 5.2 are presented in Table II. The results represent the averages of three separate determinations on each strain.

Except for ML<sub>1</sub>, the initial counts of the strains were very similar. The S. lactis strains (H<sub>1</sub> and ML<sub>8</sub>) had slightly higher counts ( $1.8 \times 10^7$  cfu/ml) than the other (S. cremoris) strains (average  $1.4 \times 10^7$  cfu/ml). The highest populations were attained by the S. lactis strains (average  $8.9 \times 10^8$  cfu/ml) after undergoing  $3.7 \times 10^8$  cell divisions (range  $3.4$  to  $9.0 \times 10^8$ ) corresponding to 5.55 (range 5.5 to 5.6) doublings of the populations during growth to pH 5.2. The populations of the S. cremoris strains at pH 5.2 varied considerably between strains from  $1.9 \times 10^8$  cfu/ml for ML<sub>1</sub> to  $6.2 \times 10^8$  cfu/ml for Z<sub>8</sub>, with the average for all S. cremoris strains  $4.0 \times 10^8$  cfu/ml. This represented an average of  $3.8 \times 10^8$  cell divisions (range  $1.8$  to  $6.1 \times 10^8$ ) during growth, corresponding to an average of 4.8 population doublings (range 4.2



Table II. Increase of starter populations during growth in PSM at 35°C from 2% inocula\* to pH 5.2

Strains	Populations of cultures (cfu X 10 <sup>-8</sup> /ml)**		No. of cell divisions (X 10 <sup>-8</sup> )	No. of population doublings
	initially	on attain- ing pH 5.2		
AM <sub>1</sub>	0.17	4.3	4.1	4.6
AM <sub>2</sub>	0.15	3.0	2.9	4.2
C <sub>13</sub>	0.16	4.3	4.1	4.7
E <sub>8</sub>	0.13	4.2	4.1	5.0
H <sub>1</sub>	0.18	8.6	8.4	5.5
HP	0.14	3.9	3.8	4.8
ML <sub>1</sub>	0.066	1.9	1.8	4.8
ML <sub>8</sub>	0.18	9.2	9.0	5.6
R <sub>1</sub>	0.17	4.4	4.2	4.6
US <sub>3</sub>	0.15	3.8	3.7	4.6
Z <sub>8</sub>	0.14	6.2	6.1	5.4

\* Inocula were 12 hr 30°C PSM cultures.

\*\* Determined by plate counting as described in text.

to 5.4). Strains  $ML_1$ ,  $AM_2$  and  $US_3$ , which are all regarded as "slow" starters in cheesemaking (Lawrence & Pearce, 1968), underwent fewer cell divisions during growth to pH 5.2, and it is thus readily apparent that the production of acid per cell division with these strains is high relative to the other "fast" strains such as  $ML_8$  and  $Z_8$ .

Effect of  $CaCO_3$  and NaCl on pH. After the addition of  $CaCO_3$  alone, or both  $CaCO_3$  and NaCl to the cultures at pH 5.2, the pH continued to fall to between 5.0 and 4.8 by 24 hr. Thereafter it remained fairly constant or increased slowly by 0.1 to 0.2 unit during the next 13 days.

Starter survival in the absence of NaCl. The starter populations continued to rise slightly during incubation at  $13^\circ C$  after the addition of  $CaCO_3$ . The maximum populations were reached between the first and third days, and thereafter the populations of most strains declined only slightly ( $< 2$ -fold) (Fig. 5). The exceptions were the "non-bitter" strains  $AM_1$ ,  $AM_2$  and  $US_3$  which declined 2.5-, 5- and 8-fold respectively from their maxima.

Starter survival in the presence of 4% NaCl. The population of *S. lactis* strain  $H_1$  continued to rise slightly even in the presence of 4% NaCl and the maximum population of  $1.2 \times 10^9$  cfu/ml was attained at 2 days (Fig. 6). This was almost identical to its behaviour in the absence of NaCl. Thereafter the population declined slowly c. 3-fold to  $5 \times 10^8$  cfu/ml by 14 days. The addition of NaCl checked further increase in cell numbers of the other strains. The population of *S. lactis*  $ML_8$  also declined slowly from its initial level of  $9 \times 10^8$  cfu/ml to  $2 \times 10^8$  cfu/ml at 14 days.

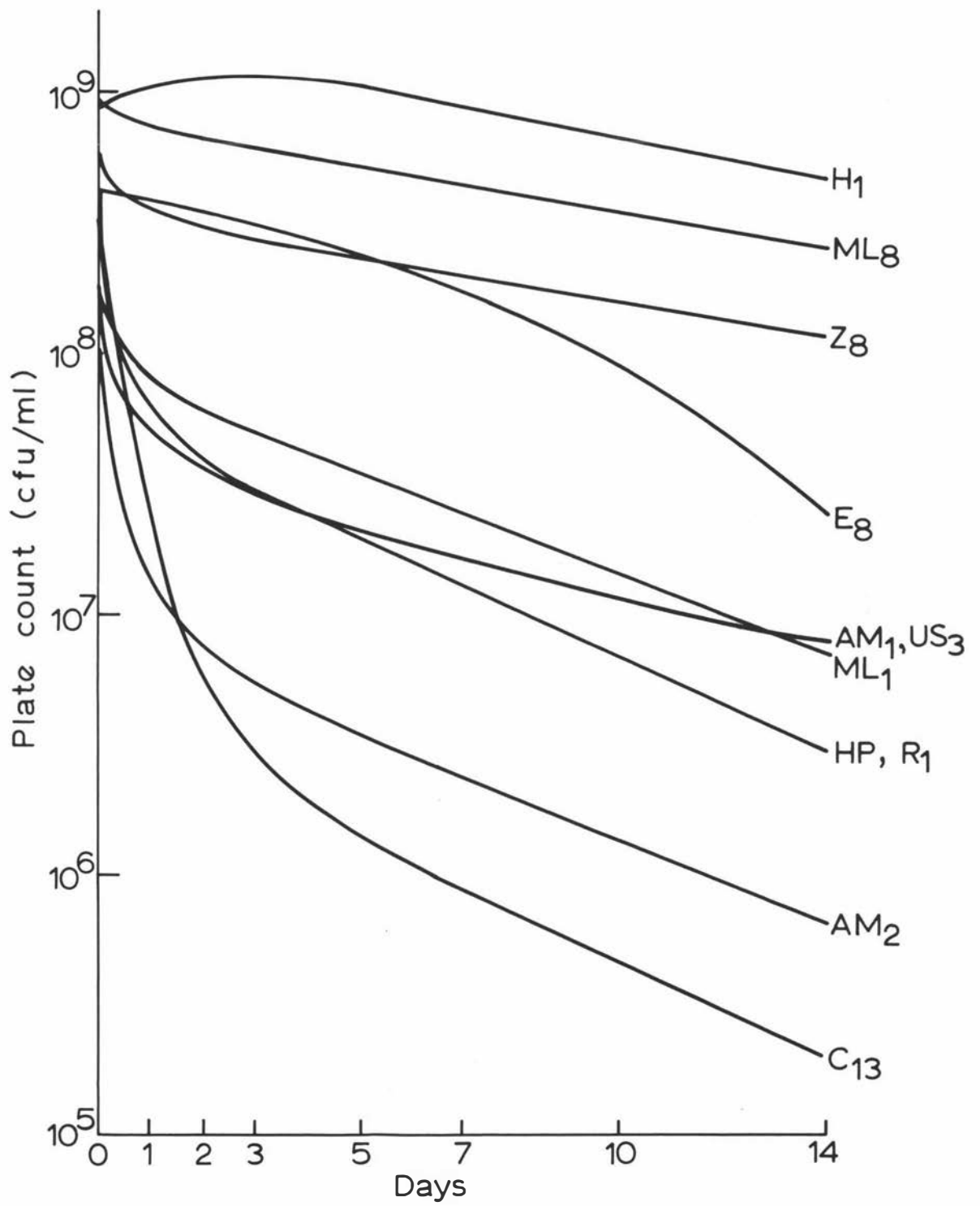
Fig. 5. Survival of starters at 13°C in PSM in the presence of 1% CaCO<sub>3</sub> and 0% NaCl. Counts at day "0" correspond to the counts of the cultures at pH 5.2 immediately before the addition of CaCO<sub>3</sub> (see Table II). For the sake of clarity the individual points have been omitted from the graphs and tabulated hereunder.

Strain	Plate count (cfu X 10 <sup>-8</sup> /ml) at							
	2 hr	1d	2d	3d	5d	7d	10d	14d
AM <sub>1</sub>	4.4	4.0	5.4	4.3	2.8	3.2	2.4	0.77
AM <sub>2</sub>	3.9	4.5	5.4	4.0	3.9	2.8	3.1	2.1
C <sub>13</sub>	4.6	5.1	4.6	5.6	5.9	5.9	5.5	4.8
E <sub>13</sub>	4.4	5.9	6.9	6.3	8.5	6.9	6.1	4.9
H <sub>1</sub>	9.3	10.8	13.9	14.0	15.6	11.5	14.0	17.2
HP <sub>1</sub>	7.3	6.8	8.6	8.6	6.4	7.0	7.2	5.1
ML <sub>1</sub>	2.7	2.6	2.4	2.5	2.9	2.3	2.4	2.2
ML <sub>10</sub>	9.6	9.8	10.9	9.1	9.5	7.3	6.5	5.7
US <sub>10</sub>	4.8	9.5	9.1	7.9	6.9	6.3	7.1	8.2
Z <sub>3</sub>	6.0	4.6	4.7	4.6	2.3	1.5	1.0	0.63
Z <sub>8</sub>	8.5	7.5	8.2	8.9	7.6	7.8	8.0	7.3



Fig. 6. Survival of starters at 13°C in PSM in the presence of 1% CaCO<sub>3</sub> and 4% NaCl. Counts at day "0" correspond to the counts of the cultures at pH 5.2 immediately before the addition of CaCO<sub>3</sub> and NaCl (Table II). For the sake of clarity, the individual points have been omitted from the graphs and tabulated hereunder:

Strain	Plate count (cfu X 10 <sup>-7</sup> /ml) at							
	2 hr	1d	2d	3d	5d	7d	10d	14d
AM <sub>1</sub>	8.3	5.6	3.0	3.4	2.1	1.1	1.3	1.3
AM <sub>2</sub>	19.0	1.4	0.56	0.48	0.44	0.25	0.094	0.093
C <sub>13</sub>	30.0	6.2	0.76	0.19	0.11	0.19	0.038	0.018
E <sub>13</sub>	42.0	44.0	35.0	29.0	23.0	17.0	10.0	1.7
H <sub>13</sub>	93.0	96.0	123.0	109.0	113.0	88.0	69.0	47.0
HP <sub>1</sub>	13.0	4.1	4.5	3.2	2.2	1.5	0.76	0.2
ML <sub>1</sub>	12.0	6.3	6.2	5.1	4.3	3.1	1.5	0.48
ML <sub>8</sub>	82.0	68.0	47.0	56.0	61.0	46.0	40.0	21.0
R <sub>1</sub>	12.0	5.8	3.2	3.5	2.2	1.3	0.58	0.14
US <sub>1</sub>	24.0	8.4	5.5	4.1	2.8	1.4	1.1	1.1
Z <sub>0</sub>	45.0	35.0	28.0	26.0	22.0	19.0	15.0	9.7



S. cremoris strains apart from  $E_0$  and  $Z_0$  exhibited very rapid rates of decline initially, particularly over the first 24 hr, with the populations generally decreasing 5-fold. Thereafter the strains did not decline so rapidly. Strain  $C_{13}$  exhibited by far the greatest decline in population, decreasing to  $10^5$  cfu/ml in 14 days, and was followed in order by strains  $AM_2$ , HP,  $R_1$ ,  $ML_1$ ,  $AM_1$  and  $US_3$ , which reached levels between  $10^6$  and  $10^7$  cfu/ml.

Strains  $Z_0$  and  $E_0$  did not decline as rapidly as the other strains, either initially or subsequently. After 14 days the  $Z_0$  population had declined about 6-fold to  $10^8$  cfu/ml and the  $E_0$  population 14-fold to  $3 \times 10^7$  cfu/ml.

Starter survival in the presence of 5% NaCl. Many of the features of survival in the presence of 4% NaCl were also obtained in the presence of 5% NaCl (Fig. 7). The rates of population decline were of the same order for most strains, but there were notable exceptions. Strains HP and  $R_1$  exhibited markedly better survival in the presence of 5% NaCl, declining to populations of c.  $4 \times 10^7$  cfu/ml at 14 days, compared to c.  $3 \times 10^6$  in the presence of 4% NaCl. Strain  $AM_2$  also survived better in the presence of 5% NaCl, declining to a population of  $4 \times 10^6$  at 14 days, than in the presence of 4% NaCl ( $9 \times 10^5$  cfu/ml). The only strains to decrease to significantly lower levels on the presence of 5% compared to 4% NaCl was  $AM_1$ , the counts at 14 days being  $8 \times 10^5$  and  $10^7$  cfu/ml respectively.

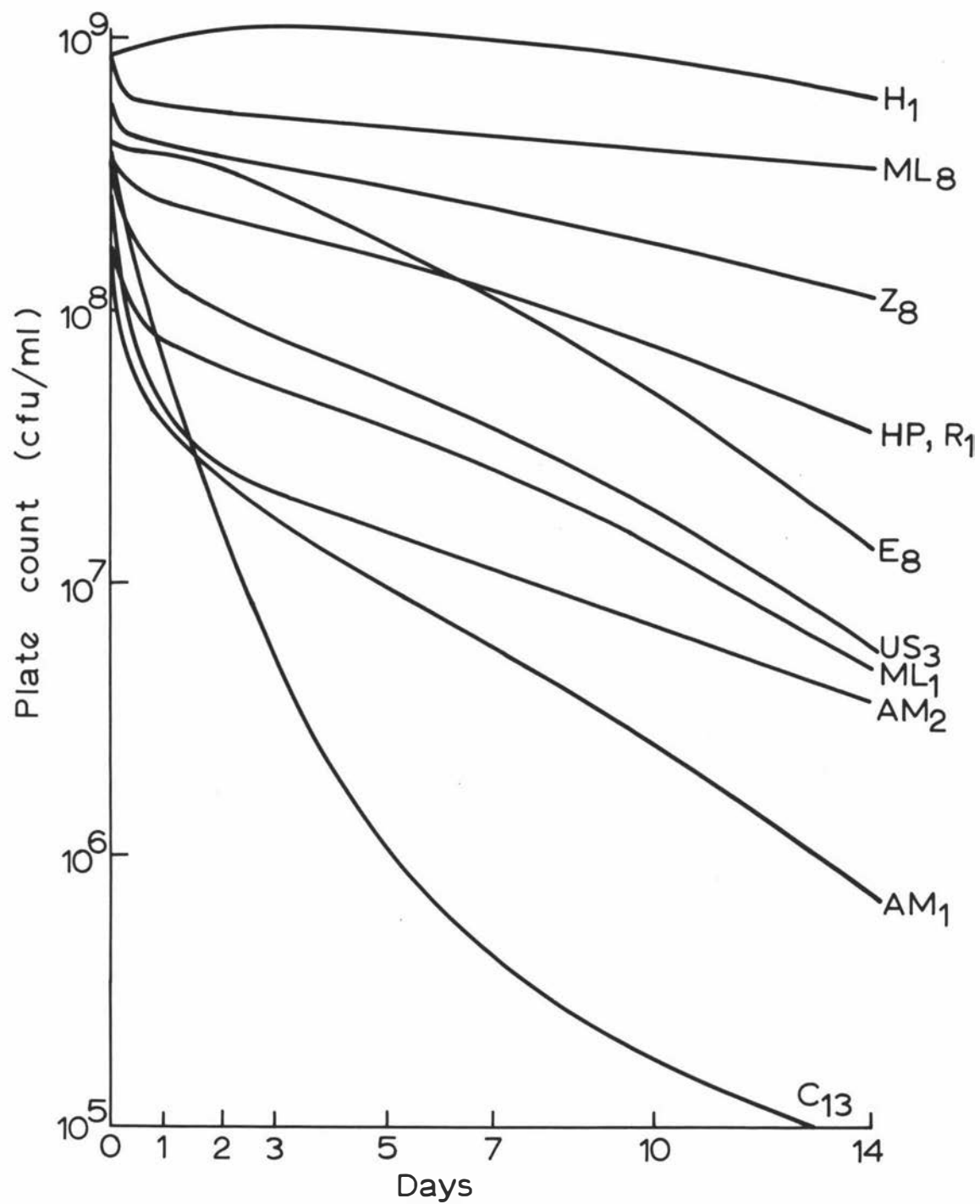




Fig. 7. Survival of starters at 13°C in PSM in the presence of 1% CaCO<sub>3</sub> and 5% NaCl. Counts at day "0" correspond to the counts of the cultures at pH 5.2 immediately before the addition of CaCO<sub>3</sub> and NaCl (Table II). For the sake of clarity, the individual points have been omitted from the graphs and tabulated hereunder:

Plate count (cfu X 10<sup>-7</sup>/ml) at

Strain	2hr	1d	2d	3d	5d	7d	10d	14d
AM <sub>1</sub>	8.0	3.4	2.8	2.6	1.5	1.0	0.13	0.032
AM <sub>2</sub>	17.0	4.5	2.6	2.3	1.7	1.3	0.65	0.39
OC <sub>13</sub>	32.0	12.0	2.0	0.5	0.11	0.05	0.019	0.009
LI <sub>13</sub>	37.0	42.0	32.0	23.0	21.0	12.0	3.1	1.4
HO <sub>1</sub>	85.0	105.0	111.0	102.0	88.0	105.0	83.0	64.0
HP <sub>1</sub>	31.0	24.0	23.0	16.0	17.0	12.0	8.2	3.6
ML <sub>1</sub>	13.0	6.7	5.5	5.9	4.4	2.7	1.3	0.5
ML <sub>2</sub>	55.0	58.0	50.0	53.0	53.0	38.0	39.0	37.0
R <sub>1</sub>	28.0	27.0	24.0	23.0	15.0	15.0	7.0	3.1
US <sub>3</sub>	26.0	8.6	10.0	10.0	3.9	6.6	2.1	0.48
Z <sub>8</sub>	41.0	40.0	37.0	37.0	32.0	25.0	15.0	10.0

Proteolysis by the starter strains. Strains R<sub>1</sub>, ML<sub>1</sub>, C<sub>13</sub> and HP effected about 10 times more proteolysis than strains US<sub>3</sub> and AM<sub>1</sub> during their growth in PSM at 35°C to pH 5.2 (Table III). The order obtained by grouping the strains from least to greatest proteolytic activity was similar to that obtained earlier with cultures grown for 18 hr at 35°C (see Fig. 4).

Effect of NaCl on starter proteolysis. The effect on further proteolysis by starters at 13°C when CaCO<sub>3</sub> and NaCl were added is detailed in Figs 8, 9 and 10. In the control cultures without NaCl (Fig. 8), strain ML<sub>1</sub> exhibited the greatest amount of proteolysis followed by strains C<sub>13</sub>, HP and R<sub>1</sub>. Least proteolysis was exhibited by strains US<sub>3</sub>, AM<sub>1</sub> and E<sub>g</sub>.

In the presence of 4% NaCl (Fig. 9), proteolysis, particularly by the more active strains was restricted to about half the values obtained in the control cultures. The level of "tyrosine" for ML<sub>1</sub> at 14 days in the absence of NaCl was 0.37 mg compared to 0.2 mg in the presence of 4% NaCl. The comparable values for strains Z<sub>g</sub> and ML<sub>g</sub> were reduced from c. 0.24 to 0.11. Proteolysis by the less active strains, US<sub>3</sub>, AM<sub>1</sub> and E<sub>g</sub>, was practically unaffected by 4% NaCl, and the values at 14 days were in the range of 0.04 to 0.07 mg "tyrosine".

The effect of 5% NaCl was to inhibit starter proteolysis slightly more than did 4% NaCl (Fig. 10). The more proteolytic strains were again greatly affected, whereas the activity of the less proteolytic strains was only slightly inhibited.

Influence of NaCl on starter survival and proteolysis. It is apparent that NaCl markedly influences the survival and

Table III. Proteolysis by starter strains during growth in PSM at 35°C to pH 5.2.

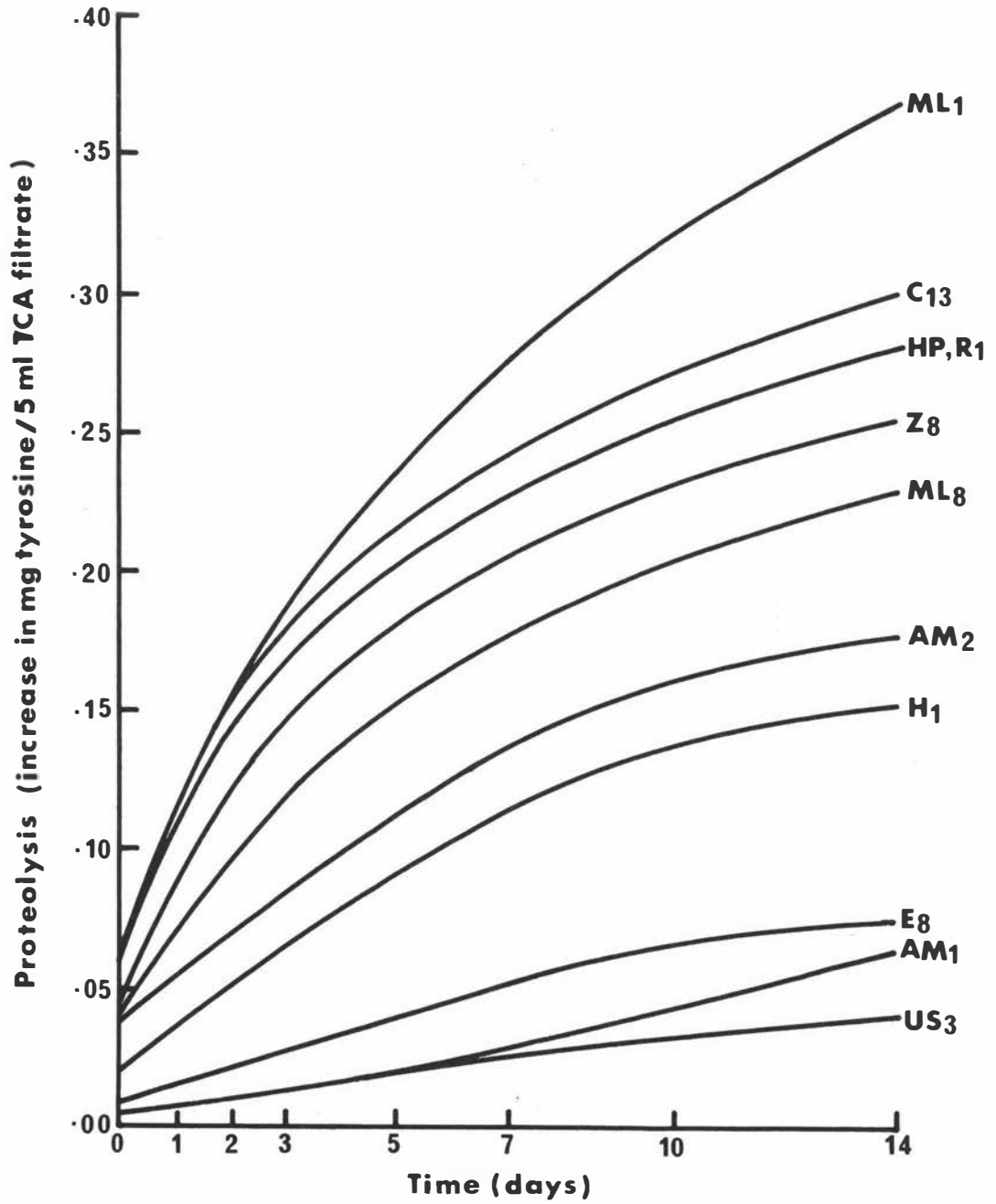
Strain	Proteolysis <sup>*</sup>
US <sub>3</sub>	0.005
AM <sub>1</sub>	0.006
E <sub>8</sub>	0.011
H <sub>1</sub>	0.017
Z <sub>8</sub>	0.039
AM <sub>2</sub>	0.040
ML <sub>8</sub>	0.040
C <sub>13</sub>	0.055
HP	0.055
ML <sub>1</sub>	0.062
R <sub>1</sub>	0.074

\* Proteolysis was measured by the method of Hull (1947) as the increase in mg tyrosine/5 ml TCA filtrate from the time of inoculation until the culture attained pH 5.2.

Fig. 8. Proteolysis by starters in PSM at 13°C in the presence of 1% CaCO<sub>3</sub> and 0% NaCl. Proteolysis was determined by the method of Hull (1947). Values at day "0" correspond to the amounts of proteolysis effected by the cultures during their growth in PSM at 35°C to pH 5.2 (Table III). For the sake of clarity, the individual points have been omitted from the graphs and tabulated hereunder:

Strain	Proteolysis* at day						
	1	2	3	5	7	10	14
AM	0.007	0.006	0.010	0.022	0.040	0.045	0.068
AM <sub>1</sub>	0.049	0.077	0.071	0.123	0.150	0.170	0.160
C <sub>22</sub>	0.135	0.166	0.181	0.211	0.238	0.257	0.306
F <sub>13</sub>	0.008	0.027	0.034	0.031	0.056	0.071	0.072
F <sub>13</sub> <sub>1</sub>	0.031	0.064	0.079	0.100	0.121	0.124	0.157
HP	0.098	0.135	0.182	0.194	0.208	0.257	0.283
ML	0.114	0.178	0.198	0.251	0.246	0.319	0.374
ML <sub>1</sub>	0.069	0.097	0.126	0.150	0.174	0.201	0.228
P <sub>10</sub>	0.130	0.160	0.161	0.200	0.208	0.250	0.280
US <sub>1</sub>	0.008	0.014	0.013	0.023	0.025	0.027	0.045
Z <sub>8</sub>	0.078	0.119	0.158	0.181	0.204	0.199	0.254

\* Increase of mg tyrosine/5 ml TCA filtrate from the time of inoculation.



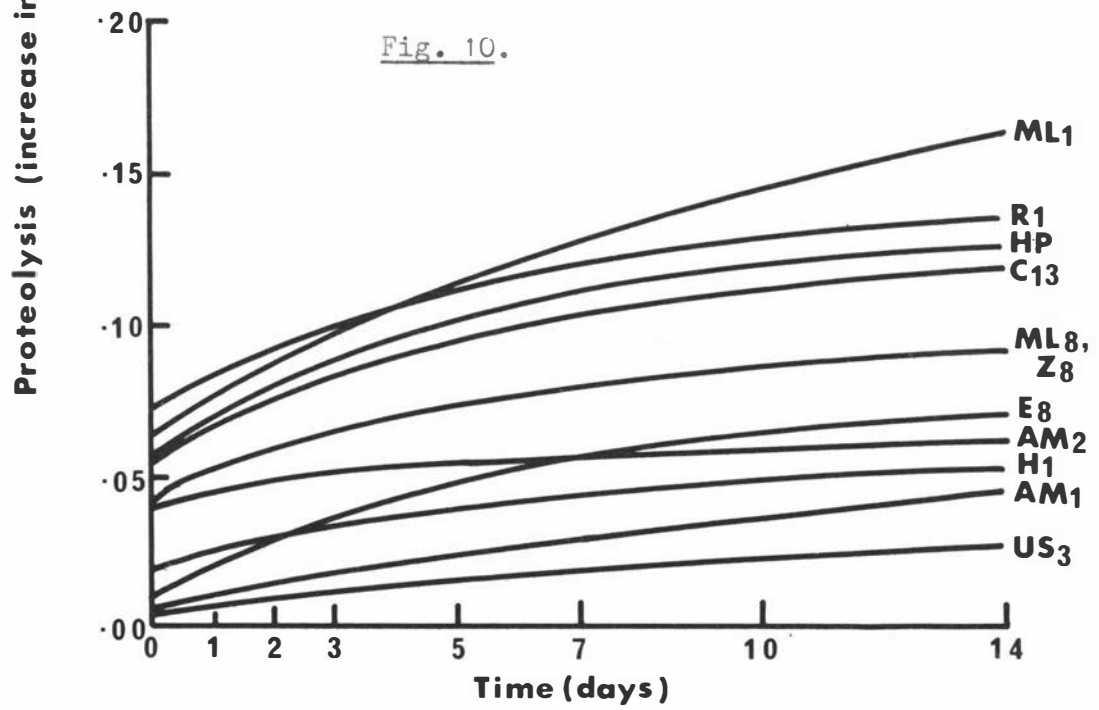
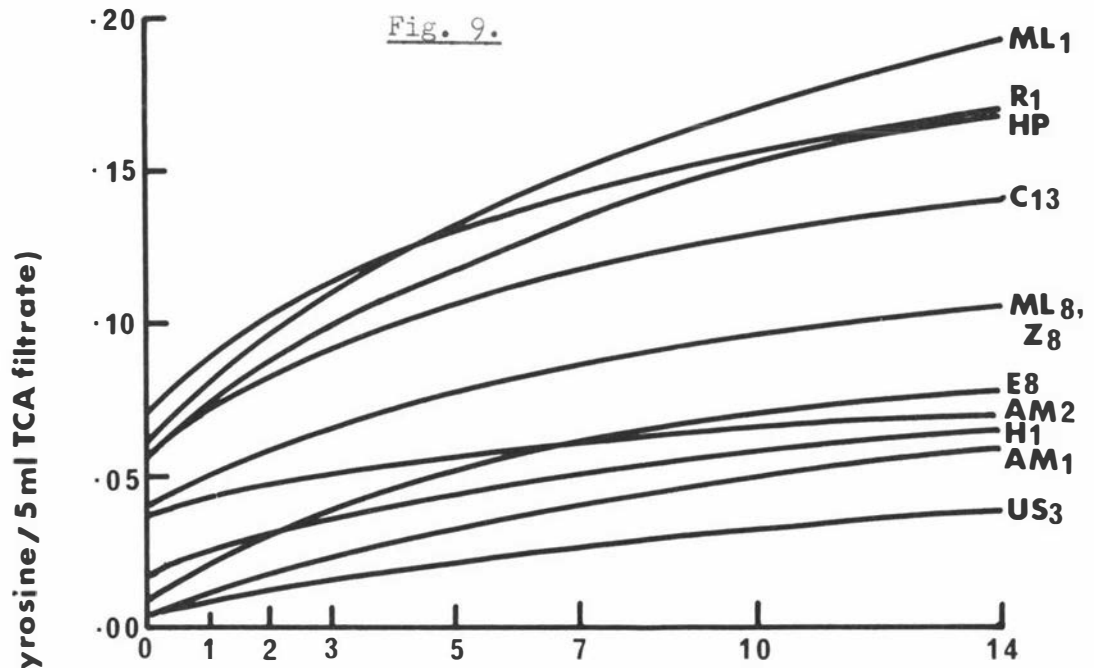
Figs 9 and 10. Proteolysis by starters in PSM at 13°C in the presence of 1% CaCO<sub>3</sub> and either 4% NaCl (Fig. 9) or 5% NaCl (Fig. 10). Proteolysis was measured by the method of Hull (1947). Values at day "0" correspond to the amounts of proteolysis effected by the cultures during their growth in PSM at 35°C to pH 5.2 (Table III). For the sake of clarity, the individual points have been omitted from the graphs and tabulated hereunder:

Fig. 9:

		Proteolysis at day						
Strain		1	2	3	5	7	10	14
AM	1	0.015	0.017	0.026	0.028	0.032	0.047	0.065
AM	2	0.038	0.049	0.053	0.057	0.056	0.065	0.067
C	2	0.076	0.084	0.094	0.093	0.119	0.115	0.149
E	13	0.022	0.032	0.033	0.060	0.059	0.064	0.077
H	8	0.037	0.030	0.042	0.040	0.043	0.055	0.064
HP	1	0.076	0.082	0.094	0.129	0.121	0.149	0.179
ML	1	0.081	0.106	0.106	0.134	0.139	0.170	0.199
ML	8	0.060	0.057	0.061	0.081	0.077	0.091	0.113
R	8	0.096	0.114	0.111	0.142	0.141	0.156	0.168
US	1	0.005	0.012	0.011	0.021	0.025	0.025	0.042
Z	3	0.044	0.054	0.083	0.077	0.082	0.092	0.110

Fig. 10

		Proteolysis at day						
Strain		1	2	3	5	7	10	14
AM	1	0.009	0.017	0.020	0.020	0.029	0.038	0.051
AM	2	0.032	0.060	0.052	0.055	0.054	0.058	0.059
C	2	0.066	0.081	0.080	0.094	0.094	0.108	0.122
E	13	0.016	0.031	0.034	0.052	0.058	0.062	0.066
H	8	0.032	0.029	0.032	0.037	0.044	0.041	0.057
HP	1	0.074	0.068	0.086	0.111	0.100	0.109	0.126
ML	1	0.077	0.094	0.093	0.115	0.114	0.138	0.170
ML	8	0.058	0.059	0.060	0.079	0.066	0.082	0.092
R	8	0.090	0.103	0.099	0.117	0.113	0.126	0.137
US	1	0.005	0.010	0.008	0.016	0.023	0.018	0.028
Z	3	0.045	0.050	0.072	0.075	0.084	0.085	0.093



proteolytic activity of the various starter strains to different extents. As was to be expected in view of previous reports (Perry & McGillivray, 1964), the strains of S. lactis, H<sub>1</sub> and ML<sub>8</sub>, survived much better in the presence of either 4 or 5% NaCl than the S. cremoris strains. Amongst these latter strains, the survival in the presence of NaCl varied considerably between strains, and even within one strain at the two levels of NaCl. The enhanced survival of some strains such as HP at the higher level of NaCl is difficult to explain, but it could possibly be attributed to slight variations in pH close to 5.0 which are known to markedly alter the solubility of the casein (Lawrence & Gilles, 1969).

Compared to the values obtained in the absence of NaCl when starter survival was good, proteolysis by the strains decreased by about half in the presence of 4% and by slightly more in the presence of 5% NaCl. This occurred whether the strains survived well (e.g. ML<sub>8</sub>) or died out very rapidly (e.g. C<sub>13</sub>). It would appear, therefore, that the survival of the starter is not necessary for continued proteolytic activity, and that the death of the cells does not enhance proteolysis as the result of the liberation of intracellular enzymes.

However, good survival of the starters may nevertheless be important for the continuation of other metabolic activities which may be responsible for the production of the "fruity" flavour defect in cheese typically associated with strains of S. lactis such as H<sub>1</sub> and ML<sub>8</sub> (Vedamuthu et al., 1966; Lawrence & Pearce, 1968).



## V. PROTEOLYSIS BY RENNET

### A: THE EFFECT OF NaCl

It is difficult to assess the relative contributions of the starter proteinases and rennet to the overall proteolysis in cheese. The starter streptococci exhibit detectable proteolytic activity after a few hours' growth in PSM at 35°C, corresponding to the period of cheesemaking, and subsequent proteolysis is affected by NaCl. It was desirable, therefore, to gain some knowledge of the proteolytic activity of rennet alone under similar conditions.

### METHODS

Rennet proteolysis of PSM was followed at pH 5.2 in the presence of 0, 1, 2, 3, 4 and 5% (w/v) NaCl and at temperatures of 35°C and 13°C. The levels of NaCl were chosen to include those levels used in the study of the effect of NaCl on proteolytic activity and survival of the starter streptococci, as well as the levels which Stadhouders (1962) reported as being optimal for rennet proteolysis.

PSM was prepared in 1000 ml lots, adjusted to pH 5.2 with 10% lactic acid (approximately 30 ml), and solid NaCl added to the levels required. Merthiolate (Elanco Products Co., Indianapolis, U.S.A.) was added (25 mg/l) to prevent the growth of the few thermoduric bacteria in the PSM. Rennet was then added at the rate of 10 ml of a 1/50th dilution in distilled water of commercial rennet extract to 1000 ml of PSM. This is equivalent to 22 ml rennet/100 l milk (3½ fluid oz./100 gal), a level commonly used in commercial cheesemaking practice in this

country. Immediately after adding the rennet, the 1000 ml lots of PSM were distributed in 100 ml lots and incubated at the required temperatures.

The course of proteolysis was followed by the Hull method (1947), observing the precautions recommended by Citti et al. (1963), by sampling at 0 hr (immediately after adding the rennet) and at 1, 2, 3, 5, 7, 10 and 14 days. The rennet coagulated the PSM containing up to 2% NaCl, and when sampling these milks to determine the amount of proteolysis, whole 100 ml samples of PSM were first homogenized by 2 min blending in an Ato-Mix blender. Protein in 5 g of sample was then precipitated by 10 ml of 12% TCA.

## RESULTS

The proteolysis of PSM by rennet proceeded linearly with time up to 14 days at all concentrations of NaCl between 0 and 5%, when incubated at either 35°C or 13°C (Fig. 11). As expected, proteolysis proceeded more slowly at the lower temperature. Increasing levels of NaCl decreased the rate of proteolysis, and, moreover, the relationship between the amount of proteolytic breakdown that had occurred at any given stage of incubation up to 14 days and the level of NaCl was linear (Fig. 12). In these experiments, therefore, the decreased proteolysis of casein by rennet was in direct proportion to an increased level of NaCl.

These results generally substantiate the findings of Amunstad (1950) that the rate of casein degradation decreases as the concentration of NaCl is increased. However, they are in contrast to the reports by Stadhouders (1962) and Fox & Walley (1971) that

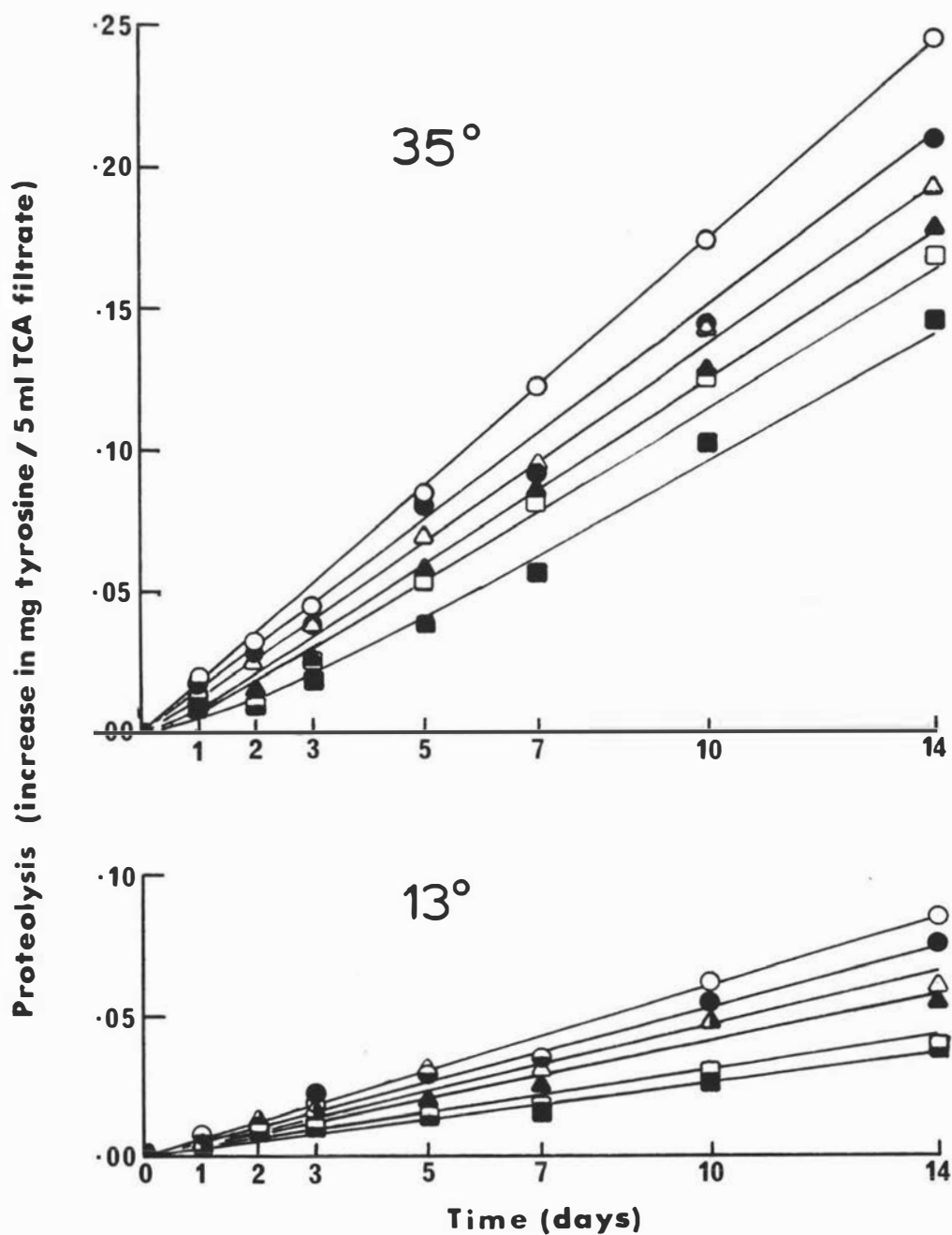


Fig. 11. Rennet proteolysis in PSM at pH 5.2 in the presence of NaCl at 35°C and 13°C. The level of rennet used was 0.2 ml/1 of PSM. Proteolysis was measured by the method of Hull (1947).

NaCl concentrations: 0%, ○ ; 1%, ● ; 2%, △ ; 3%, ▲ ; 4%, □ ; 5%, ■ .

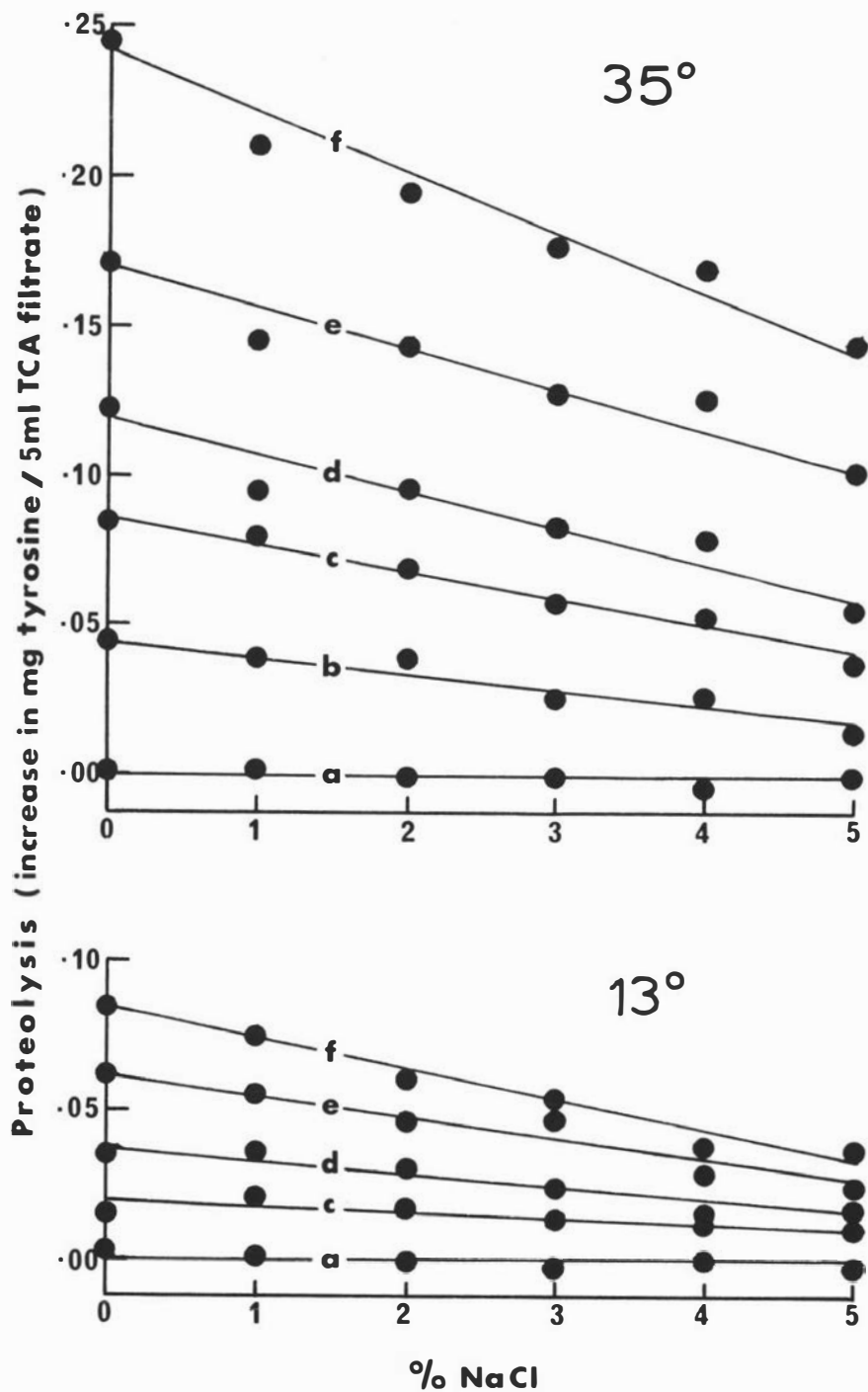


Fig. 12. Influence of NaCl on rennet proteolysis of PSM at pH 5.2 at various stages during incubation for up to 14 days at either 35°C or 13°C. The level of rennet used was 0.2 ml/1 of PSM. Proteolysis was measured by the method of Hull (1947). Stage of incubation (days): a, 0 ; b, 3 ; c, 5 ; d, 7 ; e, 10; f, 14.

rennet proteolysis was enhanced in the presence of NaCl due to the greater solubility of the substrate. The differences between the results might be attributable to the fact that the level of rennet used in the present investigation was lower than the levels used by the latter authors by 50- and 5-fold respectively. At this lower level of rennet the advantage of a more readily available substrate due to the solubilization of casein by NaCl might be negligible and, hence, only the inhibitory effect of NaCl on rennet proteolysis would be apparent.

Relative proteolytic activities of rennet and starter.

From Fig. 11 it can be estimated that 6 hr of rennet proteolysis of PSM at pH 5.2 and 35°C (in the absence of NaCl) brought about an increase of approximately 0.003 mg tyrosine/5 ml TCA filtrate. It is well recognized that general proteolysis by rennet proceeds more rapidly at a pH considerably lower than the initial pH of milk of c. 6.7 (Fox, 1969). Since the rennet would be acting at a suboptimal pH during the first 5 to 6 hr of cheesemaking as the pH shifts downwards from 6.7 to 5.2, and as some loss of rennet in the whey might also be expected, the actual amount of proteolysis brought about by rennet during this period is probably much less than the value given above.

Proteolysis by cultures of the various starters grown (in about 6 hr) in PSM to pH 5.2 brought about increases of between 0.006 and 0.07 mg tyrosine/5 ml TCA filtrate (Table III). Comparison of these results with those for rennet suggests that in the first 5 to 6 hr of cheesemaking the rate of breakdown of casein to TCA-soluble fragments is substantially due to the starter. Subsequent proteolysis in the cheese would depend on

the relative influence on the proteinases of both starter and rennet of temperature, pH and NaCl found during cheeseripening.

After 14 days in the presence of 4 or 5% NaCl and at 13°C, rennet was as proteolytic as cultures of the least proteolytic ("non-bitter") starter strains maintained under similar conditions (compare Figs 9, 10 and 11). However, proteolysis by the other "bitter" strains in this time was up to 4 times greater than by the rennet. On the other hand, in contrast to rennet, starter proteolytic activity gradually decreased with time (Figs 8, 9 and 10).

These results suggest that starters, and particularly the more active strains, will contribute relatively more than the rennet to overall proteolysis during cheesemaking and in the young cheese, but not in the later stages of maturation.

### B: THE INFLUENCE OF STARTER ON DETERMINING RENNET CONCENTRATION IN CHEESE

Stadhouders (1962) considered that the rate at which a starter formed acid during cheesemaking influenced the amount of rennet retained in the curd of the cheese and thus was a factor in determining the level of bitter peptides formed by rennet action. A fast rate of acid production was considered to be associated with a higher concentration of rennet in the cheese.

The typically "non-bitter" strains, such as AM<sub>1</sub> and AM<sub>2</sub>, are recognized as forming acid more slowly than the "bitter" strains such as HP, ML<sub>8</sub> and Z<sub>8</sub> (Lawrence & Pearce, 1968). The possibility of these strains influencing the amount of rennet retained in the curd during cheesemaking was examined in two ways.

Cultures grown in PSM containing rennet were assayed for rennet lost in the whey by the time the cultures had reached pH 5.2. Secondly, cheeses made with the same rennet levels but with different starters were examined for evidence of different levels of rennet proteolysis by gel electrophoresis.

To 100 ml PSM contained in a beaker and prewarmed to 35°C was added 1 ml of a 1/50th dilution of rennet. This represents a  $2 \times 10^{-4}$  dilution of rennet and corresponds to the level used in cheesemaking. The milk was then inoculated with 2% starter as described earlier (p. 46) and incubated at 35°C. The starter strains used were two "non-bitter" strains ( $AM_1$  and  $AM_2$ ) and three "bitter" strains (HP,  $ML_8$  and  $Z_8$ ). After 30 min the soft coagulum that had formed due to rennet action was cut into four pieces with a sterile wire to allow whey to be expelled more readily, and the incubation continued until the pH reached 5.2.

The rennet concentration in the whey was then assayed by the sensitive method of Lawrence & Sanderson (1969). In this method 0.003 ml of enzyme is placed in a 2 mm diameter well cut in a thin layer of caseinate-agar on a microscope slide. The diameter of the white precipitation zone formed around the well as the result of proteolysis during incubation is related to the concentration of the enzyme. In the present investigation it was established that the minimum concentration of rennet detectable by this method after 18 hr incubation at 37°C corresponded to a  $5 \times 10^{-5}$  dilution. Assays on the wheys from the different starter cultures at pH 5.2 indicated rennet levels corresponding to dilutions of 1 to  $2 \times 10^{-4}$  for all starter strains. There was no tendency for the "bitter" strains HP,  $ML_8$  and  $Z_8$  to lose less.

rennet in the whey, and thus to retain more in the curd, than the "non-bitter" strains  $AM_1$  and  $AM_2$  grown to the same pH.

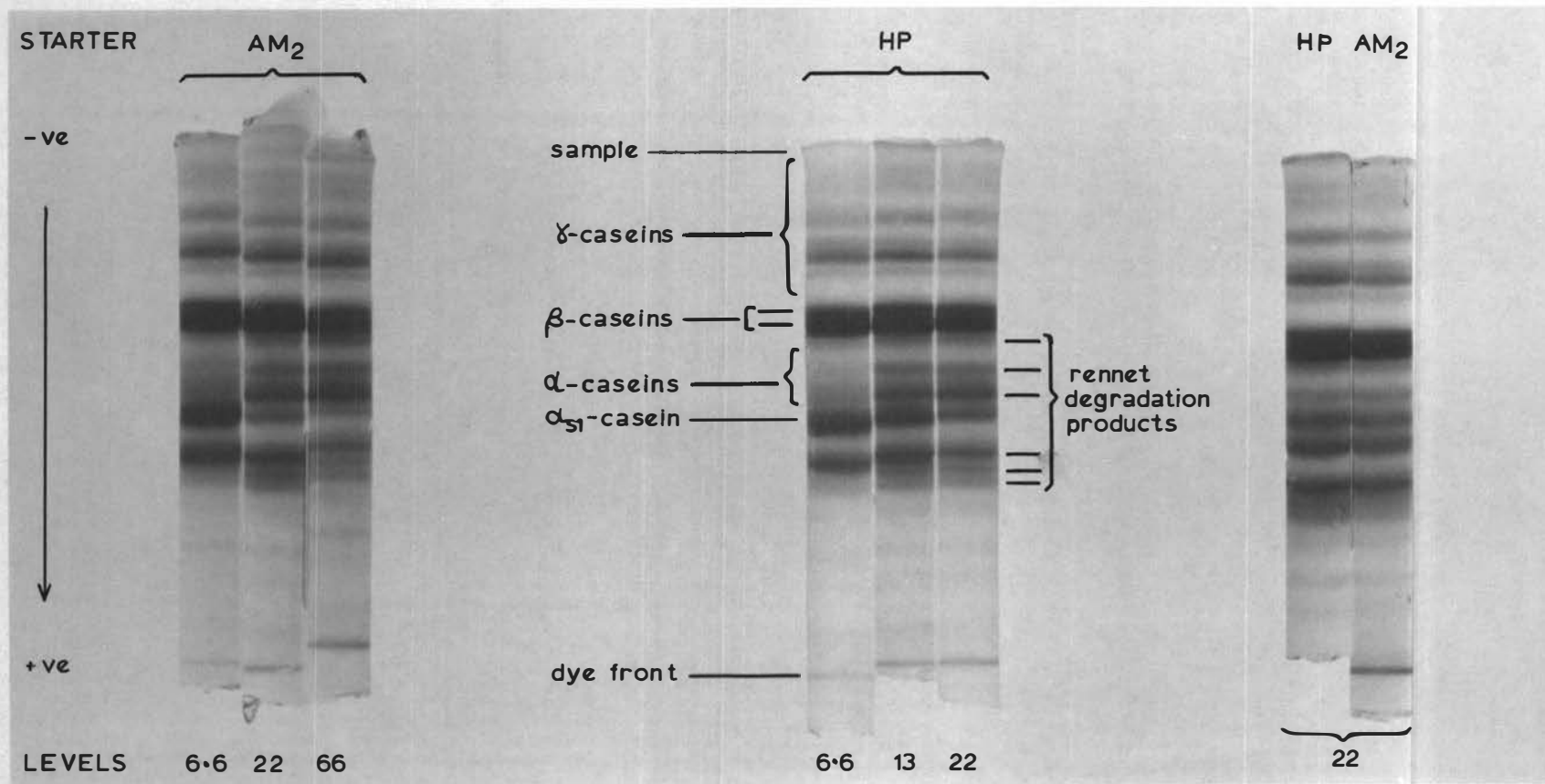
Protein breakdown in three non-bitter 19-week-old cheeses made with  $AM_2$  starter and with rennet levels of 6.6, 22 (normal), and 66 ml/100 l milk were examined by the disc gel electrophoretic method of Creamer (1970). A similar series of "bitter" HP cheeses made with rennet levels of 6.6, 13.2 and 22 ml rennet/100 l milk was also examined. In both series of cheeses differences could readily be detected in the patterns of casein breakdown between rennet levels differing by as little as 2-fold (Fig. 13).

However, an examination of a bitter HP and a non-bitter  $AM_2$  19-week-old cheese made using the same milk and the same rennet levels (22 ml/100 l milk) showed that the patterns of protein breakdown were indistinguishable (Fig. 13). This suggests that the rennet levels in the two cheeses, if not identical, must differ less than 2-fold. The similarity between the gel electrophoresis patterns of the cheeses made with HP and  $AM_2$  also suggests that the proteinases of both strains of starter have similar specificities towards the various casein fractions.

The average make times were 5 hr 15 min for the HP cheeses, and 6 hr 00 min for the  $AM_2$  cheeses, indicating that the relative rates at which the slow or fast starters form acid during cheesemaking are not likely to influence the concentration of rennet retained in the curd. This confirms the report of Lawrence & Gilles (1969) that the rate at which acid is formed during cheesemaking is not important in determining the development of bitterness.



Fig. 13. Casein breakdown measured by disc gel electrophoresis in 19-week-old Cheddar cheeses made with "non-bitter" (AM<sub>2</sub>) and "bitter" (HP) starters and different levels of rennet (expressed as ml<sup>100</sup>/<sub>l</sub> of milk).



## VI. PHOSPHATASE ACTIVITY OF STARTERS

In view of the importance attached to phosphatases in the degradation of casein during cheeseripening (Schorndtiller, 1968), strains of starter streptococci with comparatively low and high levels of proteolytic activity (Table III), and including both "bitter" and "non-bitter" strains (Table I), were examined for their ability to produce phosphatase in conditions similar to those encountered in cheesemaking.

### METHODS

Cultures. The following strains were grown in PSM at 35°C to pH 5.2 as described earlier (p. 46): AM<sub>1</sub>, AM<sub>2</sub>, C<sub>13</sub>, E<sub>8</sub>, HP, ML<sub>1</sub>, ML<sub>2</sub>, R<sub>1</sub>, US<sub>3</sub> and Z<sub>8</sub>. After removing a sample for plate counting, the growth of the cells in 100 ml of culture was stopped by the addition of 1 ml of a 3% solution of merthiolate and the culture assayed for phosphatase activity.

Assay. Preliminary experiments to determine the optimum pH for the assay for phosphatase activity were carried out on the strains grown in broth for 12 hr at 30°C, and adjusted to equivalent cell densities. The phosphate-free broth of Lowrie & Pearce (1971) was selected to minimize any inhibitory effect of phosphates on the production and activity of phosphatases, since this is known to occur with some other bacterial phosphatases (Torriani, 1960). The cultures were assayed for phosphatase activity between pH 4 and 9.5, following the assay procedure of Malveaux & San Clemente (1969) except that citrate buffer (0.1 M) was used from pH 4 to 7.5, and Tris buffer (0.1 M) from pH 7.5 to

9.5. The substitution of the acetate buffer (0.1 M, pH 5.2) used by these authors by citrate buffer at the same pH caused no loss of activity.

A similar method was then used to assay the PSM cultures for phosphatase activity. The reaction mixture, contained in a total volume of 8.0 ml, consisted of 2.8 ml of 0.1 M acetate buffer (pH 5.2), 2.0 ml of the PSM culture at pH 5.2, and 1.2 ml of 0.4% (w/v) p-nitrophenylphosphate (PNPP) (BDH) as substrate. As a control, PSM with merthiolate was substituted for culture.

After incubation for 60 min at 37°C, the reaction was stopped by precipitating protein with 1 ml of 12% TCA. After standing for 15 min at room temperature, the solution was filtered through Whatman No. 1 filter paper. The filtrate was made alkaline with 2-N NaOH to develop the yellow colour resulting from the liberation of p-nitrophenol (PNP) from the substrate. As a slight flocculent precipitate formed on adding the NaOH, the samples were centrifuged for 5 min at 3000 g before being read at 400 nm against the control set at zero OD. The OD readings of the samples were then converted to the equivalent concentration of PNP by reference to a standard graph.

## RESULTS

Preliminary experiments. The phosphatase activity of all the strains was optimal at c. pH 5.2 and very low at pHs > 8. A typical pH-activity curve is shown for strain US<sub>3</sub> (Fig. 14). Although the optimum pH for phosphatase activity of all strains was practically identical, the total amounts of activity at any one pH varied greatly with the various strains.

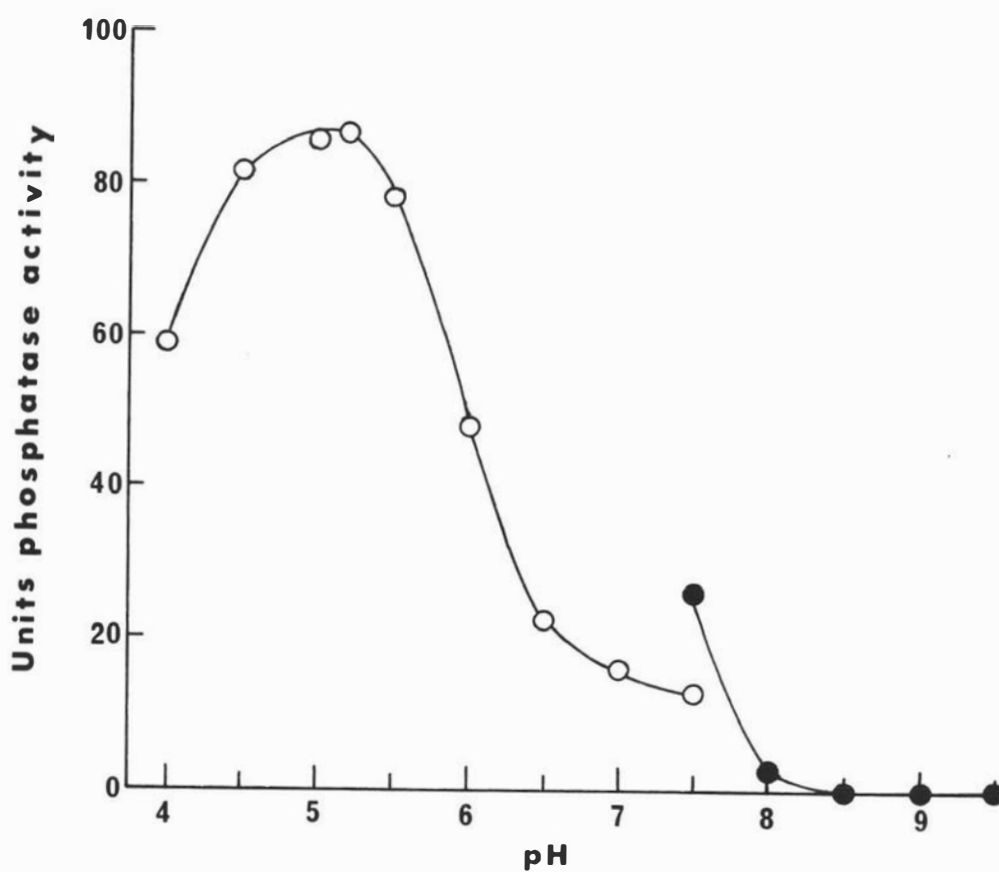


Fig. 14. Effect of pH on phosphatase activity of strain US<sub>3</sub>. The culture was grown in broth for 12 hr at 30°C and adjusted to OD<sub>580</sub> 1.0. The assay procedure of Malveaux & San Clemente (1969) was followed using either 0.1 M citrate buffer (○) or 0.1 M Tris buffer (●). 1 unit of activity liberated 0.001 micromole p-nitrophenol/ml of culture per hr under the conditions of assay.

Acid phosphatase of PSM cultures and the relationship to proteolytic activity. The acid phosphatase activity of the strains varied widely from 165 units/ml for HP to < 1.0 unit/ml for strains  $C_{13}$  and  $Z_8$  (Table IV). The populations of the strains when the cultures reached pH 5.2 were practically identical to the values obtained earlier in the investigation of proteolytic activity of the strains (Table II). Thus a valid comparison can be made between proteolytic and phosphatase activities of the strains.

When the strains are arranged in order of greatest to least phosphatase activity and compared with their proteolytic activities, it is apparent that phosphatase activity is not related to proteolytic activity (Table IV). For example, strains HP,  $AM_1$ ,  $US_3$  and  $AM_2$  all had high levels of phosphatase activity but differed widely in proteolytic activity; similarly, strains  $R_1$ ,  $ML_1$ , HP and  $C_{13}$  all exhibited relatively high levels of proteolytic activity but varied widely in phosphatase activity.

On the basis of these results, therefore, there seems little reason to expect that the starter phosphatases play a significant role in proteolysis.

There was no apparent tendency for either low or high levels of phosphatase activity to be associated with the "bitter" starters (such as HP,  $ML_8$ ,  $R_1$  and  $Z_8$ ). However, although the "non-bitter" starters ( $AM_1$ ,  $AM_2$ ,  $E_8$  and  $US_3$ ) had relatively high phosphatase activity, this relationship may be only coincidental in view of the small number of strains examined.

Table IV. Comparison of acid phosphatase and proteolytic activities of starter strains grown at 35°C in FSM to pH 5.2.

Strain	Plate count (cfu X 10 <sup>-8</sup> /ml)	Units* phosphatase activity/ml of culture	Proteolytic activity** (mg tyrosine/ 5 ml TCA filtrate)
EP	5.0	165	0.055
AM <sub>1</sub>	2.6	82.0	0.006
US <sub>3</sub>	3.5	57.2	0.007
AM <sub>2</sub>	2.7	64.0	0.040
E <sub>8</sub>	4.1	43.7	0.011
ML <sub>8</sub>	9.7	41.4	0.040
ML <sub>1</sub>	1.4	22.4	0.062
H <sub>1</sub>	8.4	17.3	0.017
R <sub>1</sub>	4.4	6.5	0.074
Z <sub>8</sub>	6.2	0.43	0.039
C <sub>13</sub>	4.5	0.01	0.055

\* 1 unit of activity liberates 0.001 micromoles of p-nitrophenol/hr under the conditions of assay (see text).

\*\* Data from Table III.

## VII. INFLUENCE OF MILK QUALITY ON CHEESE FLAVOUR

### A: BACTERIOLOGICAL QUALITY OF MILK

In assessing the bacteriological quality of milk, it is necessary to take into account not only the total numbers but also the types of organisms present. Proteinases and some other enzymes produced by the bacteria in the raw milk may survive the heat treatment of pasteurization (Stadhouders et al., 1959; Kishonti & Sjöström, 1970) and give rise to flavour defects in products such as cheese on storage.

Taylor (1967) proposed a method of assessing milk quality by counting both total numbers and types of bacteria growing in a thin film of undiluted milk sample adhering to a plain water-agar base. In preliminary trials with this method, considerable difficulty was encountered in distinguishing the three types of zones formed by proteolytic, acidoproteolytic and acid-forming organisms, because of the crowding of the colonies on the plates which occurred even in the case of comparatively good-quality milk samples.

Most investigators have concluded that the hydrolysis of casein in an opaque "milk agar" affords the best method of detecting proteolytic organisms (Druce & Thomas, 1959; Willis & Hobbs, 1959; Brown, Sandvik, Scherer & Rose, 1967; Zaadhof & Terplan, 1967). However, it is apparent from the many attempts that have been made to improve the existing methods that a satisfactory medium has yet to be devised.

The American Public Health Association (1967) recommends the addition of 10% (v/v) of sterile skim milk to Standard Methods agar, yet it has long been known that acid-forming



bacteria can produce zones of clearing on such media. Two surveys (Frazier & Rupp, 1928; Lightbody, 1961) showed that 37 to 45% of the zones of clearing on milk agar were not due to proteolysis. The most serious disadvantage, therefore, associated with the milk agar method is the need to flood the medium with a protein precipitant to confirm that zones of clearing are due to proteolysis and not caused by acid formed by the organisms as the result of sugar fermentation. In an attempt to overcome some of these difficulties, Frazier & Rupp (1928) devised an opaque carbohydrate-free casein agar medium to eliminate clearing due to acid production. The limitation of this medium for organisms requiring carbohydrate has prevented its general adoption.

Lawrence & Sanderson (1969) described a caseinate agar slide micro-technique for the quantitative estimation of proteinases. A small well cut in the caseinate agar was filled with the enzyme solution which then diffused outwards, breaking down the caseinate to form a white precipitate which consists initially mainly of para-K-casein, together with small amounts of high MW degradation products of  $\alpha_{s1}$ - and  $\beta$ -caseins. With highly active proteinases, subsequent breakdown of the white precipitate occurred to yield soluble fractions, with the formation of a white ring of precipitation and an inner clear area surrounding the well.

A more sensitive agar medium for the detection of proteolytic organisms using this principle was, therefore, developed in this investigation by adding caseinate (1% w/v), citrate (0.015 M) and  $\text{Ca}^{2+}$  (0.02 M) to Standard Methods agar (BBL). Details of this medium (Standard Methods caseinate agar (SMCA)) are given in Appendix I. Its greater sensitivity compared with existing milk agar media is related to its ability to detect the first stage of

casein breakdown as shown by the formation of a white zone of precipitation which is readily detected in the transparent medium. The extent of proteolysis exhibited by an organism is reflected both by the size and type of precipitation zone forming round the colony. No significant difference was found between total counts of raw milk samples measured on the new medium and Standard Methods agar. SMCA could thus be used for the simultaneous determination of both total and proteolytic counts in milk used for cheesemaking.

The technique of inoculating SMCA plates by flooding with a diluted milk sample and decanting the excess as described by Taylor (1967) proved very satisfactory when applied to SMCA. A quantitative determination of milk quality in terms of both total count and the proportion of proteolytic organisms could rapidly be obtained and was of value in screening the milks from individual suppliers for bacteriological quality. Further details of this procedure are given in Appendix III.

Staphylococci and micrococci (Brown et al., 1967; Forbes, 1968) are a common cause of mastitis in dairy cattle, which remains a serious cause of loss of milk production both in New Zealand and other countries. The principal method used in differentiating strains of pathogenic staphylococci in epidemiological studies of their incidence in milk has been phage typing, although up to 30% of isolates may be phage nontypable (Wallmark & Finland, 1961; Cohen & Smith, 1964; Marandon & Oeding, 1966). It is not surprising, therefore, that attempts have been made to develop more comprehensive methods of differentiating strains of staphylococci and micrococci by such means as the serological typing of their proteolytic enzymes (Sandvik & Fossum, 1965; Brown et al., 1967).

The finding that the pattern of white precipitation zone(s) formed on SMCA due to proteolysis appeared to be specific for a particular strain of organism, prompted an investigation into typing staphylococci, including the phage nontypable strains, by their proteolytic activity on SMCA. This is reported in detail in Appendix II~~4~~.

#### B: INFLUENCE OF MILK QUALITY ON CHEESE FLAVOUR

The influence on the flavour of cheeses of variations in the bacteriological quality of the cheesemilk, with particular reference to the incidence of proteolytic organisms, was investigated at frequent intervals over two dairying seasons.

#### METHODS

Cheeses. Cheeses were made at the Institute's Processing Hall using single-strain starters AM<sub>2</sub> and HP, and AM<sub>1</sub> and C<sub>13</sub>, on alternate weeks throughout the spring to autumn (September to April) period. The cheeses were made from pasteurized milk (71°C for 15 sec) using normal Cheddar cheesemaking procedures, which were not altered throughout the course of the season. The temperature of setting and cooking were 32 and 38°C respectively, and 22 ml rennet/100 l milk was used.

The flavour of the cheeses was assessed after six months by a panel of 6 to 10 experienced tasters.

Milk quality. The milk used for cheesemaking was obtained from a commercial dairy company and came from cows of predominantly Jersey breed. The individual suppliers' milks were

generally water-cooled (not refrigerated) and collected on a daily basis.

The bacteriological quality of the milk was determined both before and after pasteurization by surface plate counting on SMCA as described in Appendix I, in terms of the total and proteolytic counts. Counts were made at approximately fortnightly intervals throughout the season.

## RESULTS

As the results for both the 1968-69 and 1969-70 seasons were very similar, a description is given only of the latter season.

Bacteriological quality of the milk. The results of the total counts on the raw and pasteurized milks, and the counts of proteolytic organisms in the raw milks are detailed in Fig. 15. The total counts of the raw milks varied considerably from week to week during the season from  $2.5 \times 10^4$  to  $10^7$  organisms per ml. This erratic variation of counts probably reflects, in part at least, the lack of refrigeration on the farm and the effect of variations in ambient temperatures. The total counts of the pasteurized milks (thermoduric organisms) did not vary so greatly from week to week, but increased steadily as the season progressed from an initial level of  $10^4$  to  $2 \times 10^5$  organisms per ml. A similar increase of thermoduric organisms was found in the preceding season. This increase probably reflected the progressive build-up of milk-stone in the milking equipment during the season. At times, especially towards the end of the season, the thermoduric organisms comprised a high proportion, frequently  $>50\%$ , of the total count.

The proportion of the total raw milk flora determined on SMCA as proteolytic varied from 7 to 94% (average 41%). The trend of counts of proteolytic organisms per ml tended to follow the total counts of raw milk (Fig. 15). The proportion of proteolytic organisms of the thermoduric count was generally much lower, ranging from 1 to 24% (average 9%). Thus, the actual numbers of proteolytic bacteria in raw milk were relatively high compared to the numbers in pasteurized milk. It could be estimated that pasteurization frequently destroyed >90% of the actively proteolytic bacteria in the raw milk. The influence of these organisms on cheese flavour would depend, therefore, on the opportunity they have for growth in milk prior to pasteurization, and the heat stability of the enzymes which had been formed.

Cheese flavour. Cheeses were scored for the overall acceptability of flavour, a measure of consumer preference, on a scale of 1 to 9 (Fig. 16). On this scale a cheese with very serious flavour defects scored 1, an average cheese 5, and a cheese of exceptionally good flavour 9. The intensity of bitterness in the cheeses was scored on a 1 to 5 scale (Fig. 17) as follows: 1, flavour absent; 2, possibly present; 3, definitely present; 4, predominant flavour; 5, very intensely flavoured. The results from individual tasters were averaged for each cheese.

The overall flavour score of the cheeses made with  $AM_1$  averaged 6.83 for the whole season (range 7.4 to 5.8) and they were always judged to be non-bitter (average score 1.02, range 1.0 to 1.1). Cheeses made with  $AM_2$  were very similar to  $AM_1$ , scoring an average of 6.44 (range 7.3 to 5.2) for overall flavour, and 1.1 (range 1.0 to 1.4) for bitterness over the whole season.

Fig. 15. Bacteriological quality of milk used for cheese-making during the 1969-70 dairying season. Total and proteolytic counts were determined using Standard Methods Caseinate Agar (total count of raw milk, ○ ; total count of pasteurized milk, ● ; proteolytic count of raw milk, Δ ).

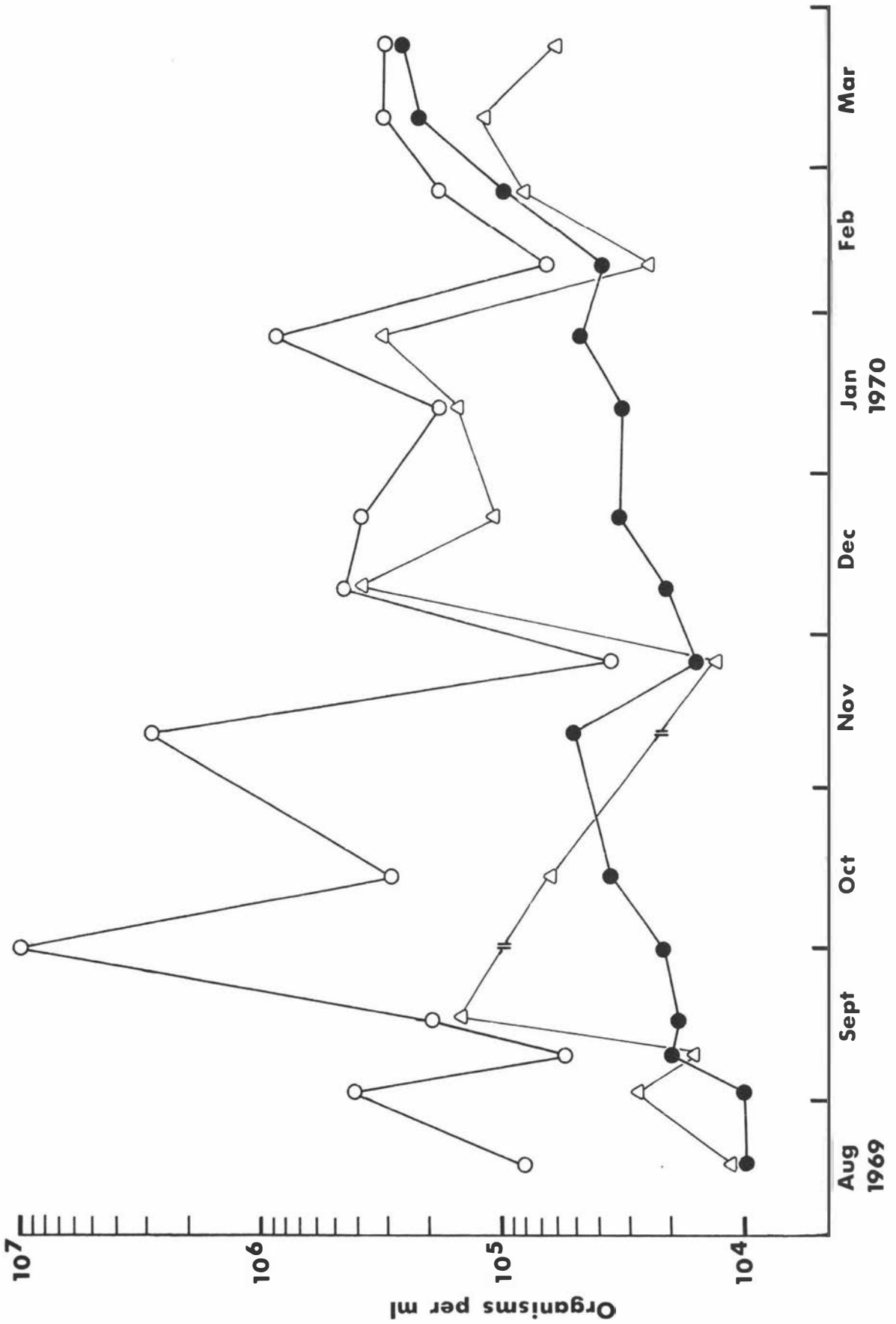


Fig. 16. Overall flavour acceptability scores of 6-month-old Cheddar cheeses made during the 1969-70 dairying season using single-strain starters HP (○), C<sub>13</sub> (●), AM<sub>2</sub> (△) and AM<sub>1</sub> (▲). Overall flavour acceptability was scored on a scale of 1 to 9. Cheeses with very serious flavour defects scored 1, and cheeses of exceptionally desirable flavour scored 9.



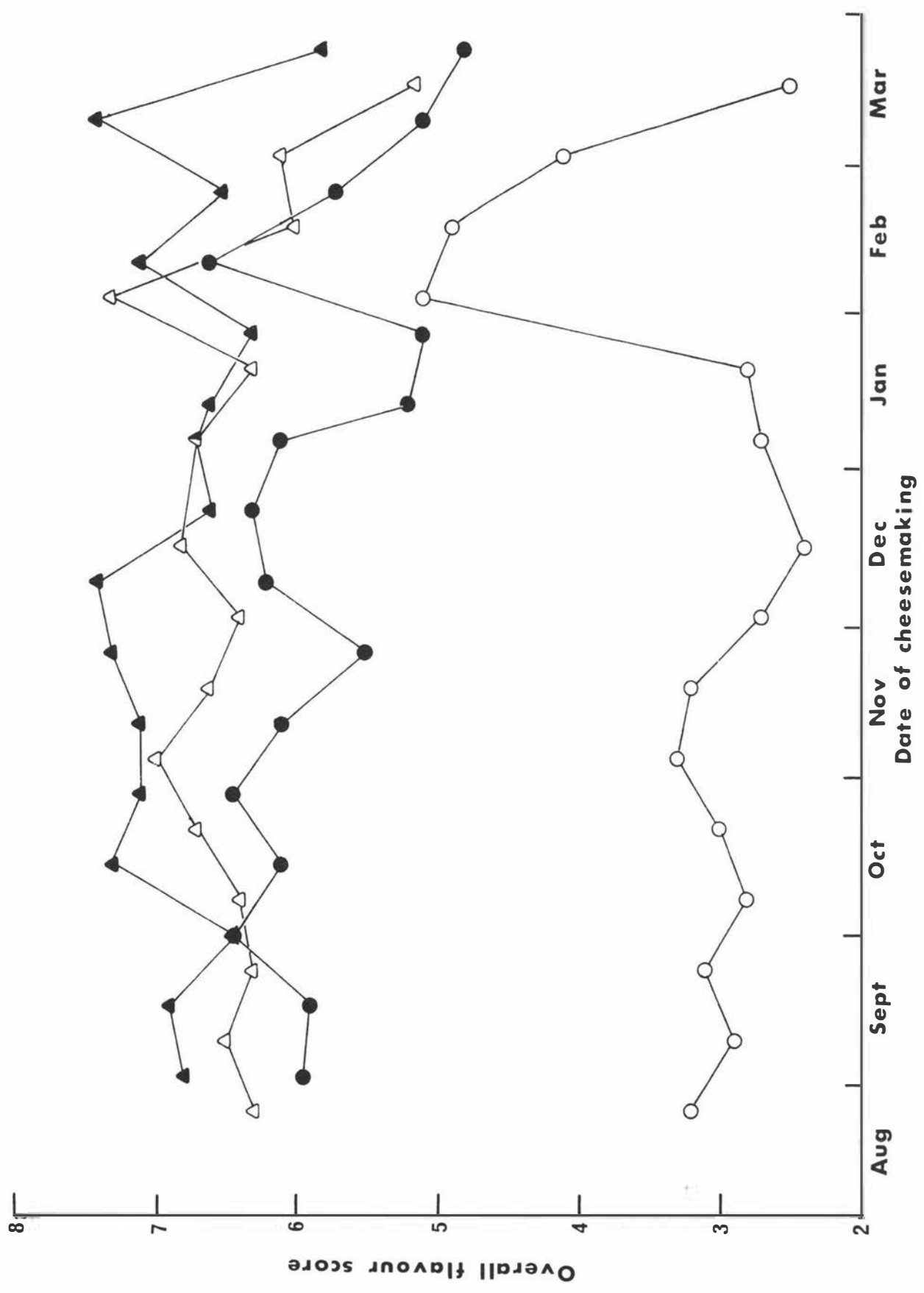
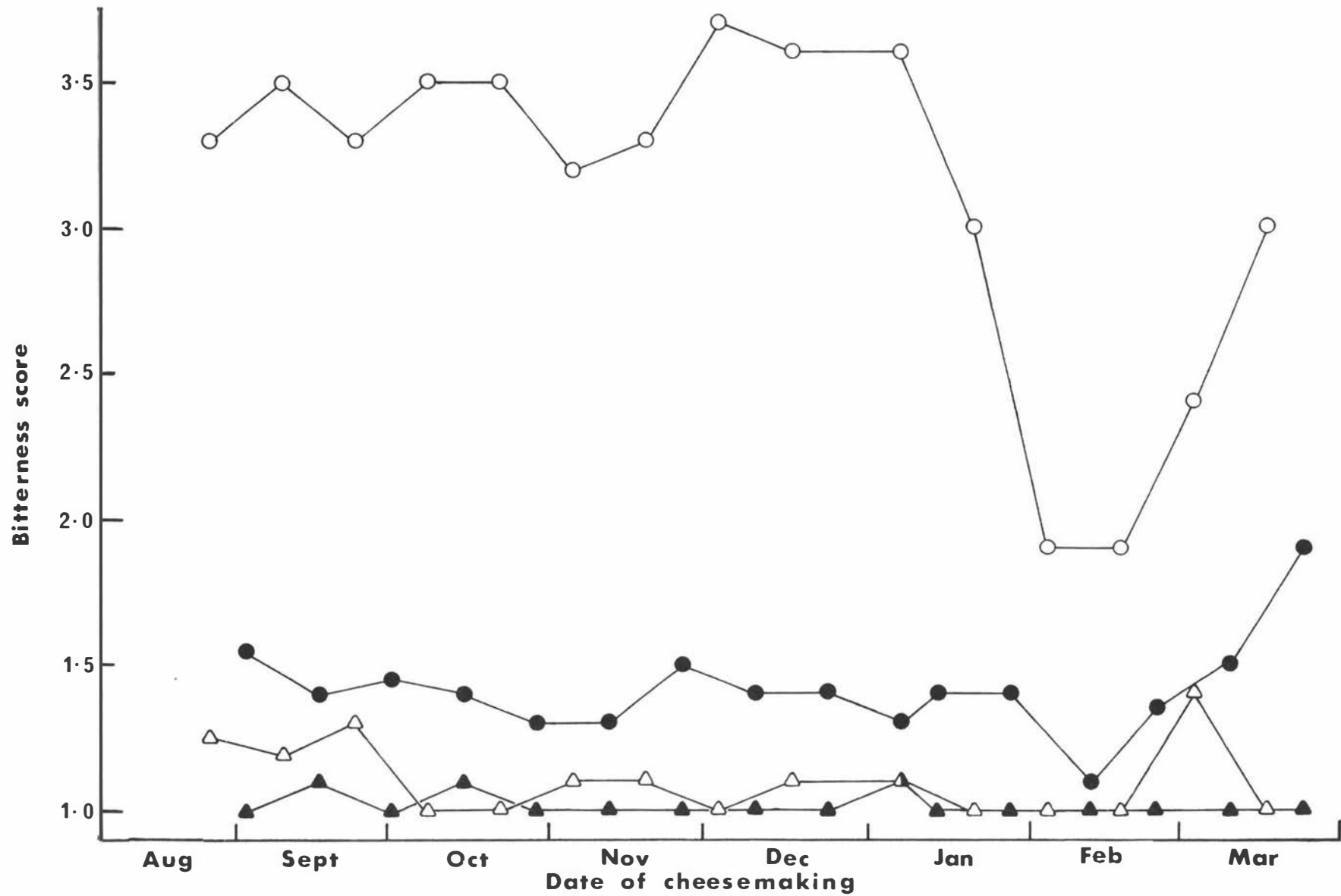


Fig. 17. Bitterness scores of 6-month-old Cheddar cheeses made during the 1969-70 dairying season using single-strain starters HP (○), C<sub>13</sub> (●), AM<sub>2</sub> (△), and AM<sub>1</sub> (▲). Bitterness was scored on a 1 to 5 scale - 1 corresponded to the absence of bitterness, and 5 to extremely intense bitterness.



Cheeses made with  $C_{13}$  were also generally good-flavoured (average 5.85, range 6.6 to 4.8) and the bitterness scores were low (average 1.42, range 1.1 to 1.9). Cheeses made with HP were characteristically bitter (average 3.11, range 3.7 to 1.9) and scored poorly on overall score (average 3.25, range 5.1 to 2.4).

Comparison of the scores for overall flavour (Fig. 16) with the scores for bitterness (Fig. 17) indicates that, in general, they are inversely related. As the intensity of the bitter flavour increased, the overall flavour acceptability decreased, and vice versa.

The overall flavour scores of cheeses made with any one starter strain varied between makes during the season, and during the last part of the season tended to drop slightly for all strains (Fig. 16). However, these variations in flavour scores could not be associated with variations in the bacteriological quality of the milk (compare Figs 15 and 16).

The bitterness scores of cheeses made with strains  $AM_1$  and  $AM_2$  remained practically constant throughout the season (Fig. 17). Low levels of bitterness were detectable in cheeses made with strain  $C_{13}$  towards the end of the season. Bitterness in cheeses made with HP fluctuated slightly more from one make to another during the season, and dropped considerably during February. During this and part of the preceding month the chemical composition of the milk was affected by an unusually dry spell of weather, and acid production by many of the starters was considerably inhibited. However, none of the variations in bitterness scores corresponded to variations in the milk flora (compare Figs 15 and 17).

CONCLUSIONS

The flora of the milk used for cheesemaking was shown to vary some 300-fold during the 1969-70 season. The total numbers and types of bacteria were probably influenced by such factors as the ambient temperature and the amount of milk-stone that accumulated in the milking machine during the season. The total numbers of proteolytic organisms also varied greatly and they frequently comprised about half of the raw milk flora. However, most were killed by pasteurization and their influence on cheese flavour would depend, therefore, on the heat stability of their enzymes.

The flavour of the cheese appeared to be unaffected by the considerable variations in the milk flora, and it must be concluded, therefore, that the strain of starter used in cheesemaking is the most significant factor controlling the development of Cheddar cheese flavour.

### VIII. MANUFACTURE AND ANALYSES OF SELECTED CHEESES

Earlier results showed considerable differences between starter strains with regard to their growth in PSM, subsequent survival in the presence of NaCl, and in their proteolytic and phosphatase activities. To ascertain the extent to which these features influence cheese flavour, cheeses were made from strains that consistently gave good-flavoured cheese (AM<sub>1</sub>, AM<sub>2</sub> and US<sub>3</sub>) or bitter cheese (HP and Z<sub>8</sub>). Cheeses were also made with strain ML<sub>1</sub> which has generally been regarded as a "non-bitter" starter (Emmons et al., 1962a).

#### METHODS

Manufacture of cheeses. The bacteriological quality of the milk both before and after pasteurization was routinely assessed by plate counting on SMCA. The total counts of the raw milk were c.  $10^5$  organisms/ml (30% proteolytic) and c.  $2 \times 10^4$  (1% proteolytic) in the pasteurized milk. These counts were within the range found during the 1969-70 season to have no influence on the development of cheese flavour.

A preliminary series of cheeses was made using starters US<sub>3</sub>, HP, ML<sub>1</sub> and Z<sub>8</sub>. Following analysis of these cheeses, a further series was made using starters AM<sub>1</sub>, AM<sub>2</sub>, HP and ML<sub>1</sub>.

The amount of starter used to inoculate the cheesemilk was varied according to the starter strain used. A greater amount of the "slow" (AM<sub>1</sub>, AM<sub>2</sub>, ML<sub>1</sub> and US<sub>3</sub>) compared to the "fast" starters (HP and Z<sub>8</sub>) was used. For the first series of cheeses, rennet was added at the level of 22 ml per 100 l milk, but in

the second series of cheeses the level was reduced to 18 ml per 100 l milk according to the recommendations of Lawrence & Gilles (1970).

After overnight pressing the cheeses were film-wrapped and matured at 13°C for 14 days, and then subsequently at 7°C. This corresponds to standard practice in New Zealand.

Counts of starter during cheesemaking and maturation. During the making process, starter counts were determined in the cheese-milk immediately after inoculation, in the curd at "draining" and immediately prior to "salting". Counts in the cheese were determined after overnight pressing and at intervals during the subsequent maturation up to 16 weeks.

The count in the cheesemilk immediately after inoculation with starter was determined as for the plate counting of milk cultures described earlier (see p. 39), blending for 30 sec. A 2 min period of blending was necessary to adequately homogenize 20 g samples of curd or cheese in 80 ml of 2% sodium citrate solution for plate counting. To prevent undue warming of these samples during blending, the blender and citrate were chilled to between 0 to 4°C. A 1:1 dilution of this homogenate was then made in 0.1% peptone diluent to give a  $10^{-1}$  dilution, and subsequent dilutions were made decimally in peptone. One ml amounts of suitable dilutions were plated in triplicate to two series of plates. One series was poured with agar in the usual way to give counts of total populations.

Counts of non-starter organisms were found from the second series of plates using a slight modification of the bacteriophage method of Robertson (1960a). Agar and broth media are

normally supplemented with  $\text{Ca}^{++}$  ions to ensure adequate phage infection of the bacterial cells, and is commonly supplied as  $\text{CaCl}_2$ . However, Ca is liable to precipitate in the presence of anions such as phosphate, resulting in cloudy media. To overcome this Das & Marshall (1967) used calcium borogluconate (CaBG) as the source of  $\text{Ca}^{++}$  ions in their media for staphylococcal phages. Preliminary experiments here indicated its value with lactic streptococcal phage systems. Consequently, 100 ml amounts of agar for pouring the second series of plates, each containing 1 ml of cheese dilution and 0.25 ml of the appropriate phage preparation, were tempered to  $44^\circ\text{C}$  and supplemented with 1 ml of sterile 1.0 M solution of CaBG (Fay & Baker, veterinary grade).

Phages specific for starter strains used in cheesemaking were prepared according to the method of Whitehead & Bush (1957) and were routinely checked by plating to ensure freedom from bacteria. The phage titres of the preparations were determined by the method of Lowrie & Pearce (1971) and the effectiveness of the preparations in specifically preventing the growth of the homologous bacterial strain routinely checked by plating pure broth cultures, one series with and a second series without the phage preparation.

The counts of the starter streptococci in the curd and cheese samples were determined as the difference between the total counts obtained from the first series of plates, and the counts of non-starter bacteria obtained from the second series of plates which had been treated with phage.



Routine analyses at 14 days. The cheeses were examined at 14 days to determine the moisture, NaCl and fat contents, and the pH according to standard procedures (British Standards Institution, 1963).

Proteolysis during maturation. The course of proteolysis during the maturation of the cheeses was followed in terms of the increase of "tyrosine" by the use of Folin-Ciocalteu reagent. Protein in 5 ml of the one-fifth dilution of cheese homogenate prepared for plate counting was precipitated with 10 ml of 12% TCA. Tyrosine in 5 ml of the TCA filtrate was determined, as described previously, by the Hull method, observing the precautions of Citti et al. (1963).

Determination of free amino acids. The cheeses were examined at intervals during maturation to determine the quantities of free amino acids present. TCA filtrates of the cheeses, prepared as described in the preceding section, were deep-frozen at  $-15^{\circ}\text{C}$  until it was convenient to have all the samples analyzed at once. Ten ml of TCA filtrate was extracted with an equal volume of ether to remove TCA, and evaporated to dryness on a rotary evaporator. The dry material was then redissolved in a small measured volume of distilled water, the precise volume being chosen so that conveniently measurable peaks were obtained on the chart of the amino acid analyser when a 0.1 ml samples was used for the analysis.

Identification and quantitative measurement of amino acids was obtained using a Beckman 120 C Automatic Amino Acid Analyzer with Beckman custom spherical ion exchange resin. The short

column for basic amino acids contained resin type PA 35 to a height of 8 cm. The elution buffer pH was 5.25. The long column for acidic and neutral amino acids contained resin type UR 30 to a height of 58 cm. Buffer, pH 3.25, was used as eluant, being replaced after 90 min by a second buffer at pH 4.30. The flow rate for all buffers was 68 ml/hr, and the flow rate for ninhydrin was 34 ml/hr. Calibration constants for the amino acids were obtained from calibration runs using a standard amino acid mixture (Pierce Chemical Co., Illinois, U.S.A.). Peaks obtained from the cheese samples were identified from standard elution times, and quantitated by the height-width method of measurement. Amino acids can be estimated this way with an accuracy of  $\pm 3\%$  for the major peaks (Moore & Stein, 1963).

Determination of acid phosphatase activity in cheese. The levels of acid phosphatase in cheeses made with single-strain starters were determined using the assay procedure described before (p. 70). The sample consisted of a blended one-fifth dilution of cheese sample, prepared as for plate counting, with the addition of merthiolate to a final concentration of 0.03% to prevent bacterial growth. Since nothing was known about suitable inactivating treatments for the phosphatases of the starter strains, it was not possible to include a control cheese sample in which phosphatase activity had been inactivated. Hence, as a control for the assay, 2.0 ml of distilled water was substituted for the cheese sample.

Flavour assessments. A panel of 6 to 10 experienced judges assessed the cheeses for Cheddar flavour and such off-flavours as

bitterness, astringency, fruitiness and sharpness. The intensities of these flavours were scored on a scale of 1 to 5, and the desirability of the overall cheese flavour on a scale of 1 to 9 as described previously (see p. 79).

## RESULTS

Manufacturing and analytical data. The manufacturing and analytical data for the cheeses have been included (Tables V and VI) since it is difficult to assess the significance of bacteriological findings unless these data are known.

The salt-in-moisture values of the first series of cheeses (I) varied from 3.55% for ML<sub>1</sub> to 4.25% for HP. Thus the effect of NaCl on the survival of the starter organisms and proteolysis in the cheese might not be strictly comparable between the different cheeses. A second series of cheeses (II) was made, keeping the making of the cheeses as constant as possible. The salt-in-moisture values of this series of cheeses were all similar (Table VI) and close to the average value of 4.5% obtained in commercial cheeses.

Starter populations during cheesemaking. To compensate for the fact that the moisture contents of the milk and curd differ from that of the cheese after "pressing", the actual counts obtained as per ml of milk or per gram of cheese curd have been expressed as per gram of curd having a moisture content equal to that of the finished cheese. The factor used to adjust the counts was obtained from determination of the moisture contents of the milk, the curd at "draining", and

Table V. Manufacturing details for cheeses.

Cheesemaking series	I	I	I	I	II	II	II	II
Starter strain	HP	ML <sub>1</sub>	US <sub>3</sub>	Z <sub>8</sub>	AM <sub>1</sub>	AM <sub>2</sub>	HP	ML <sub>1</sub>
% starter inoculum	2	2½	3	2	3½	2½	1½	3½
Rennet (ml/100 l milk)	22	22	22	22	18	18	18	18
Time (hr:min) Set to Dry	2:50	3:00	2:55	2:35	2:35	2:30	2:30	2:40
"    Dry to Salt	2:35	3:10	3:15	2:35	2:55	2:35	2:00	2:50
Total make time (hr:min)	5:25	6:10	6:10	5:10	5:30	5:05	4:30	5:30
Acidity (%) at Run	0.14	0.14	0.15	0.14	0.16	0.15	0.15	0.17
"    "    Dry	0.16	0.15	0.16	0.16	0.17	0.16	0.17	0.17
"    1 hr after Dry	0.33	0.21	0.24	0.34	0.30	0.29	0.34	0.29
"    at Salt	0.73	0.63	0.60	0.74	0.63	0.63	0.60	0.60
% salt added to curd	2½	2	2½	2½	2½	2½	2½	2½
Temperature (°C) Set	32	32	32	32	32	32	32	32
"    Cook	38	37	37	38	38	38	38	38
% moisture of curd, at Dry	51.6	53.7	56.1	51.3	56.2	51.4	57.5	56.8
at Salt	40.2	42.3	41.3	40.5	42.1	41.3	40.4	40.6

Table VI. Routine analyses of cheeses at 14 days.

Cheesemaking series	I	I	I	I	II	II	II	II
Starter strain	HP	ML <sub>1</sub>	US <sub>3</sub>	Z <sub>8</sub>	AM <sub>1</sub>	AM <sub>2</sub>	HP	ML <sub>1</sub>
Moisture %	33.8	36.6	35.6	34.0	35.3	34.1	33.9	34.3
Fat %	37.5	35.5	36.5	37.0	35.5	36.0	36.5	37.8
NaCl %	1.44	1.30	1.34	1.39	1.67	1.55	1.62	1.62
pH	4.92	4.92	4.95	4.94	5.01	4.98	4.96	4.99
Salt-in-moisture %	4.25	3.55	3.75	4.08	4.73	4.55	4.78	4.72

immediately prior to salting (Table V), and the moisture contents of the finished cheeses (Table VI). The moisture contents of the milk used for all cheeses was 87% (i.e. solids content 13%) at the time of inoculation. In this way a true indication of the increase of starter numbers can be obtained, rather than an increase due to a concentration effect as moisture is progressively lost from the curd.

With all strains, the maximum populations were attained during cheesemaking (Figs 18 and 19). Dawson & Feagan (1957) similarly found that maximum populations of starter were attained during making. However, considerable differences were found in the population trends of the starters during the cheesemaking process (Table VII). The greatest increase in cell numbers occurred during the first  $2\frac{1}{2}$  to 3 hr of cheesemaking to the stage after the whey had been run off ("Dry"). During this period, the starter populations underwent from  $1.1 \times 10^8$  cell divisions for strain  $AM_1$  (representing 0.55 doublings of the population) to  $9.8 \times 10^8$  cell divisions (representing 3.77 population doublings) for strain  $Z_3$  (Table VII). During the next  $2\frac{1}{2}$  to 3 hr of cheesemaking to the time immediately prior to the addition of salt to the curd ("Salt"), total viable starter populations in the cheeses made with strains  $AM_2$ ,  $AM_1$  and  $ML_1$  (series II) dropped by 84, 26 and 21% respectively. In spite of this, however, acid production continued. In the other cheeses, cell division continued although generally not as rapidly as during the earlier part of cheesemaking, representing up to 1.15 more doublings of the populations.

The extent of acid production in all the cheeses at "Dry"

Table VII. Starter populations during cheesemaking\*.

Starter strain	Cheese-making series	Counts X 10 <sup>-8</sup>			No. of cell divisions X 10 <sup>-8</sup>			population doublings		
		in milk after inoculation (O)	in curd at Dry (D)	in curd at Salt (S)	cell divisions X 10 <sup>-8</sup>			0 - D	D - S	0 to maximum count
					0 - D	D - S	0 - S			
AM <sub>1</sub>	II	2.0	3.1	2.2	1.1	-	1.1	0.55	-	0.55
AM <sub>2</sub>	II	1.2	2.9	0.47	1.7	-	1.7	1.17	-	1.17
HP	I	0.72	4.3	7.9	3.6	3.6	7.2	2.49	1.15	3.37
HP	II	0.63	4.4	6.6	3.8	2.2	6.0	2.75	0.51	3.29
ML <sub>1</sub>	I	1.3	7.4	13.2	6.1	5.8	11.9	2.42	0.78	3.27
ML <sub>1</sub>	II	1.3	11.0	8.7	9.7	-	9.7	3.09	-	3.09
US <sub>3</sub>	I	1.5	5.7	6.9	4.2	1.2	5.4	1.95	0.21	2.18
Z <sub>8</sub>	I	0.75	10.6	18.9	9.8	8.3	18.1	3.77	0.78	4.59

\*The differing moisture contents of milk and curd samples have been taken into account, and all counts expressed in terms of colony-forming units/g of curd having a moisture content equal to that of the finished cheese.

was approximately the same, and similarly at "Salt" for all except two of the cheeses (i.e. all except those made with HP and Z<sub>0</sub> in the first series) (Table V). It is readily apparent, therefore, that the strains differed markedly during cheesemaking in the quantities of acid produced per cell division.

In contrast, the differences between the strains during their growth to pH 5.2 in PSM at 35°C were less than during their growth in cheesemaking to the time when the curd was salted at c. pH 5.2. In PSM they underwent from  $1.8 \times 10^8$  cell divisions (4.8 population doublings) for strain ML<sub>1</sub>, to  $6.1 \times 10^8$  cell divisions (5.4 population doublings) for strain Z<sub>0</sub> (Table II).

It would seem, therefore, that the cheesemaking temperatures of up to 38°C are above the optima for cell division of most of the starter strains. Of the strains used in these cheesemaking trials, Z<sub>0</sub> appeared to be the least affected by the higher temperatures of cheesemaking, and the three strains consistently giving good-flavoured cheese (AM<sub>1</sub>, AM<sub>2</sub> and US<sub>3</sub>) the most markedly affected. This is also consistent with the heat sensitivity of strain AM<sub>2</sub> observed earlier (see p. 36).

Dorn & Rahn (1939) have pointed out that the rate of lactose fermentation (production of lactic acid) by lactic streptococci may be greatest at a temperature above the optimum for cell division. Thus, the differences between the starters in the amounts of acid formed per cell division particularly during cheesemaking probably also reflect the relative sensitivities of the strains to the temperatures of cheesemaking.

Survival of starter. Since the salt-in-moisture levels in the first series of cheeses were low, a direct comparison of the



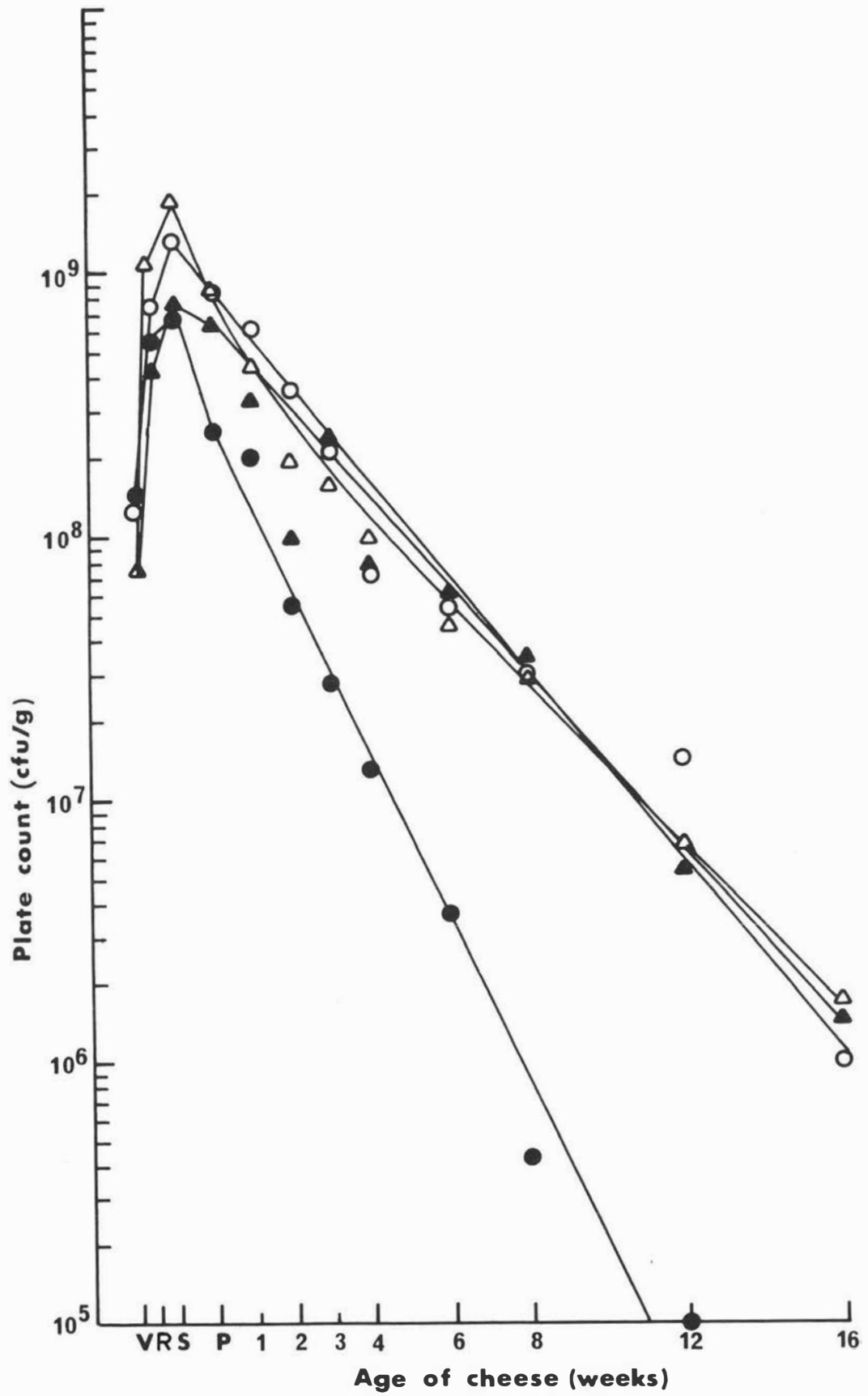
starter survival in the two series of cheeses must be made cautiously. In the first series of cheeses, populations of strains HP, ML<sub>1</sub> and Z<sub>2</sub> all declined at similar and approximately exponential rates, and were still present in relatively high numbers (c. 10<sup>6</sup> cfu/g) at 16 weeks (Fig. 18). By contrast, strain US<sub>3</sub> decreased at nearly twice the rate to <10<sup>5</sup> cfu/g at 12 weeks.

In the second series of cheeses, strains ML<sub>1</sub> and HP declined at approximately comparable rates to between 3 and 7 X 10<sup>6</sup> cfu/g at 8 weeks (Fig. 19). This rate was slightly greater than in the first series of cheeses, possibly as a result of the higher level of NaCl. Strains AM<sub>1</sub> and AM<sub>2</sub>, which had already started to decline markedly even before salt was added to the curd, continued to decline at a rapid rate to <10<sup>5</sup> cfu/g at 6 weeks. This was about twice the rate at which US<sub>3</sub> had declined, but the possible effects of differences in NaCl levels as well as the higher cooking temperature (38°C) used in the AM<sub>1</sub> and AM<sub>2</sub> cheeses compared with the US<sub>3</sub> cheese (37°C) should be noted.

In the investigation of the survival of the starters in PSM in conditions simulating some of those in cheese, strains AM<sub>1</sub>, AM<sub>2</sub> and US<sub>3</sub> tended to decrease more in 14 days than the other strains tested, even in the absence of NaCl (see p. 50). In the presence of NaCl, strains AM<sub>1</sub> and AM<sub>2</sub> also survived relatively poorly. Such laboratory experiments are, therefore, able to give a general indication of the ability of the starter strains to survive in the cheese.

The non-starter counts of the cheeses at 2 weeks were in the range 10<sup>5</sup> to 10<sup>6</sup> organisms per gram, and showed little

Fig. 18. Starter populations during the making and ripening of Cheddar cheeses made with  $ML_1$  (○),  $US_3$  (●),  $Z_8$  (△) and HP (▲). To account for differing moisture contents during the making process, the counts in the milk in the vat at the time of inoculation (V), and in the curd at Run (R) and immediately prior to the addition of salt (S) have been expressed in terms of colony-forming-units (cfu)/g of curd having a moisture content equal to that of the finished cheese. 'P' corresponds to the time at which the cheeses were removed from the press approximately 24 hr after 'V'.



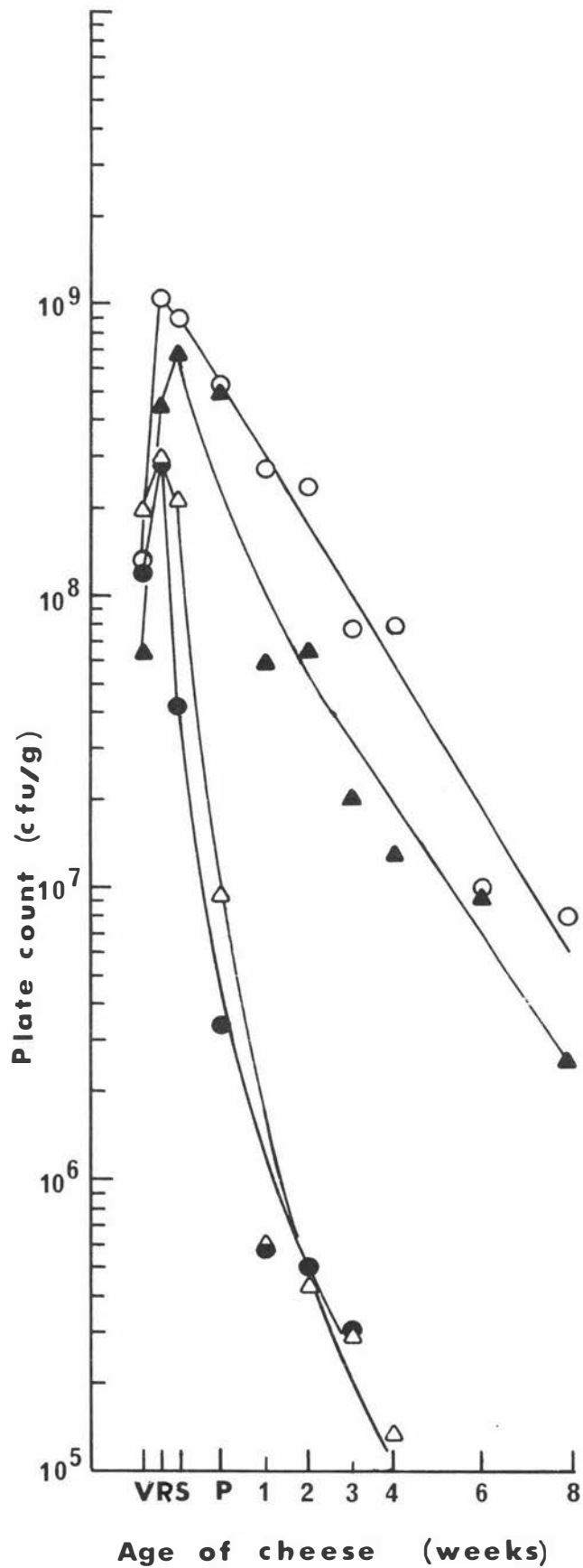


Fig. 19. Starter populations during the making and ripening of Cheddar cheeses made with strains ML<sub>1</sub> (○), AM<sub>2</sub> (●), AM<sub>1</sub> (△) and HP (▲). Abbreviations: as for Fig. 18.

tendency to increase beyond  $c. 5 \times 10^6$  at 16 weeks. Thus, the non-starter organisms predominated the microflora of the  $AM_1$  and  $AM_2$  cheeses after 2 to 4 weeks, and after about 8 weeks in the  $US_3$  cheese. In the other cheeses the starter streptococci remained predominant much longer until 12 to 16 weeks.

Acid phosphatase. The first series of cheeses, which had been made with starters varying greatly in phosphatase activity, as determined in PSM culture, was examined for acid phosphatase activity at 8, 16 and 26 weeks. The greatest levels of activity were found in the HP cheese (521, 492 and 610 units/g at 8, 16 and 26 weeks respectively). Slightly lower levels were found in the  $ML_1$  cheese (374, 304 and 505 units/g at 8, 16 and 26 weeks), and in the  $US_3$  cheese (287, 285 and 435 units/g at 8, 16 and 26 weeks). The cheese made with  $Z_0$  had the least acid phosphatase activity (87, 92 and 167 units/g at 8, 16 and 26 weeks). (1 unit of activity liberated 0.001 micromole PNP/hr under the conditions of assay.)

In the PSM cultures grown at  $35^\circ C$  to pH 5.2, the greatest level of acid phosphatase activity was exhibited by strain HP, and this level was 2 to 3 times that of strains  $US_3$  and  $ML_1$ . In contrast, strain  $Z_0$  had practically no acid phosphatase activity. Although the differences between the levels of acid phosphatase in cheeses made with these four starters were not as great as in PSM, there was nevertheless a tendency for acid phosphatase activity in the cheese to reflect the starter strain used in cheesemaking. The lesser differences of activity in the cheeses compared to the PSM cultures could reflect differences in the maximum populations attained (compare Tables IV and VII), and,

in addition, the activity contributed by the native acid phosphatase of milk which would have been unaffected by the pasteurization of the milk prior to cheesemaking, and by such non-starter flora as the lactobacilli which may increase during ripening.

Proteolysis. In both series of cheeses, the level of proteolysis detected in the curd immediately before the addition of NaCl was dependent upon the starter strain used in cheesemaking (Table VIII). Strains AM<sub>1</sub> and US<sub>3</sub>, which exhibited low levels of proteolysis in PSM, also exhibited less proteolysis in the cheese curd than other more proteolytic strains. Subsequent proteolysis in the cheeses could not, however, be related to the starter strains. Proteolysis was most rapid during the first 2 to 3 weeks of cheeseripening (Fig. 20a), probably because the cheeses were stored for the first 14 days at 13°C and subsequently at 7°C. In the first series of cheeses, proteolysis proceeded at different rates, and a relationship which tended towards linearity, especially after 12 weeks, was found between proteolysis in these cheeses and their salt-in-moisture levels (Fig. 20b). The variations, particularly up to 12 weeks, probably reflect partly the extent of proteolysis by the starter strain used in cheesemaking superimposed on the proteolysis by rennet. In general, however, the trends were similar to those obtained with rennet on its own at different levels of NaCl (Fig. 12). In all of the second series of cheeses, which had essentially equivalent levels of salt-in-moisture, proteolysis proceeded at approximately equivalent rates similar to the rate of proteolysis in the Z<sub>8</sub> cheese of the first series.

Table VIII. Proteolysis in cheese curd samples immediately prior to salting.

Starter	Cheese-making series	Proteolysis*
HP	I	0.036
ML <sub>1</sub>	I	0.043
US <sub>3</sub>	I	0.022
Z <sub>8</sub>	I	0.055
AM <sub>1</sub>	II	0.020
AM <sub>2</sub>	II	0.032
HP	II	0.036
ML <sub>1</sub>	II	0.037

\* mg tyrosine/5ml filtrate obtained after precipitating protein in 5 ml of a one-fifth dilution of cheese curd with 10 ml 12% TCA.

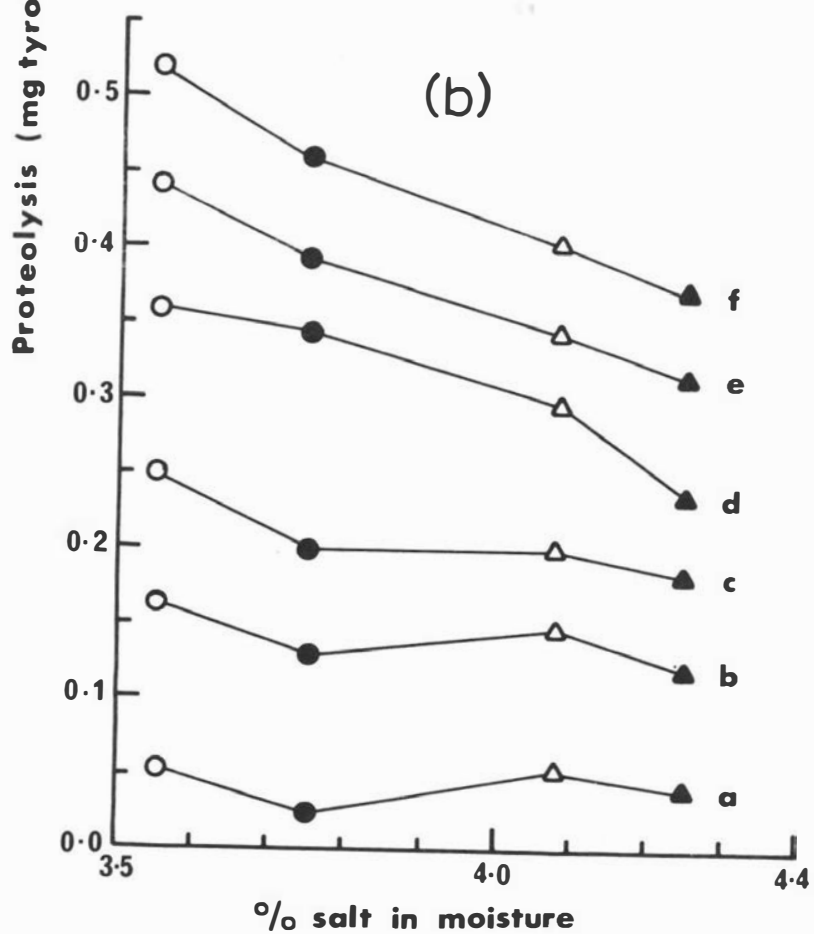
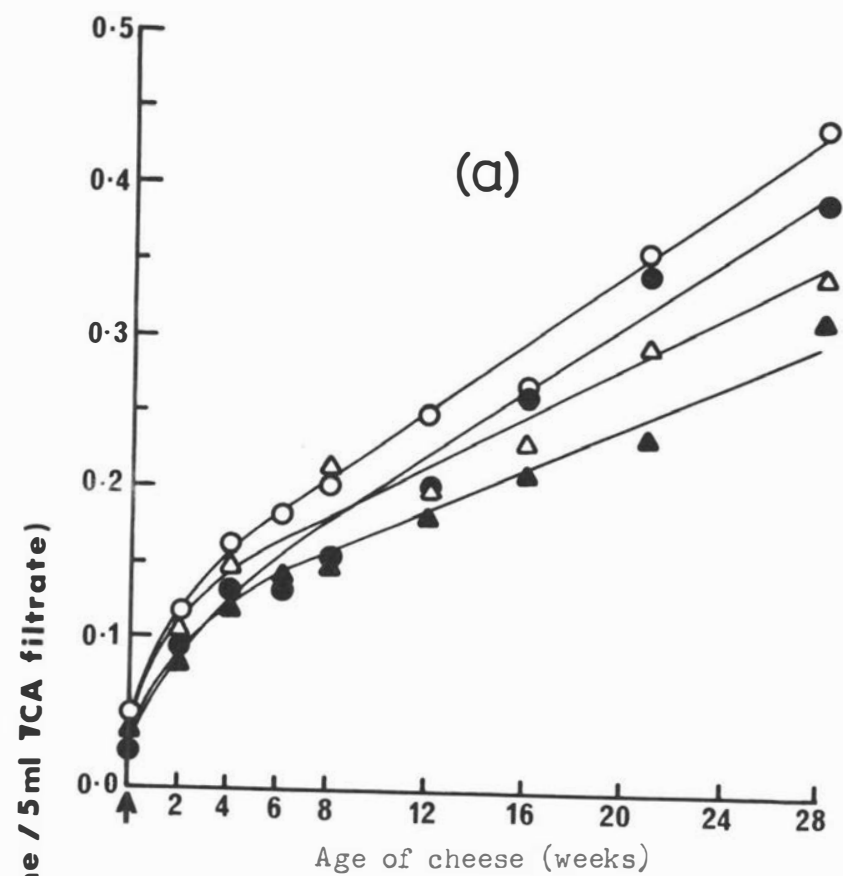




Fig. 20. Proteolysis in cheeses made with starter strains ML<sub>1</sub> (○), US<sub>3</sub> (⊙), Z<sub>6</sub> (Δ) and HP (Δ). Proteolysis was determined with Folin-Ciocalteu reagent as the level of "tyrosine" in the filtrate obtained after precipitating protein in 5 ml of a one-fifth dilution of cheese with 10 ml of 12% trichloroacetic acid.

(a) Proteolysis during cheeseripening. Cheeses were stored at 13°C for the first 14 days, and subsequently at 7°C. Arrow indicates values after pressing, e. 24 hr after the start of cheesemaking.

(b) Relationship of proteolysis at various stages during ripening to the salt-in-moisture levels of the cheeses (see Table VI). Ages of cheeses: a, 24 hr; b, 4 weeks; c, 12 wk; d, 21 wk; e, 28 wk; f, 36 wk.

These determinations of proteolysis in the cheeses tend to substantiate the suggestion made earlier (p. 64) that the starter organisms effect more proteolysis than the rennet during the earlier stages of cheesemaking, but that rennet proteolysis subsequently predominates, largely masking the continued activity of the starter.

Amino acid analyses of the cheeses. Amino acid analyses were carried out on the first series of cheeses which had been scored for good flavour (US<sub>3</sub>), bitterness (HP) and the "burnt" flavour (ML<sub>1</sub>). The cheeses were examined at 24 hr, 3 and 6 months. The latter two analyses coincided with the 3- and 6-month flavour assessments of the cheeses. The bitter Z<sub>8</sub> cheese was also examined at 3 months of age. A starter-free cheese made with gluconic acid lactone (GAL) according to the method of Perry & McGillivray (1964) using pasteurized milk and 22 ml of rennet/100 l milk was examined for free amino acids at 24 hr and again at 3 months.

A typical resolution of the amino acids found in the cheeses is shown in Fig. 21, and quantitative details of the analyses in Table IX. Free cysteine and tryptophan, which are present in casein in only very small amounts, were not detectable in any of the cheeses.

At 24 hr, the total levels of free amino acids in the cheeses made with ML<sub>1</sub>, HP and US<sub>3</sub> were low (0.4, 0.3 and 0.2 mg/g of cheese respectively). These values increased most rapidly in the ML<sub>1</sub> cheese to reach 9.6 mg/g at 3 months and 10.9 mg/g at 6 months. In the US<sub>3</sub> cheese the level increased to 4.1 mg/g at 3 months and was practically the same at 6 months (4.0 mg/g).

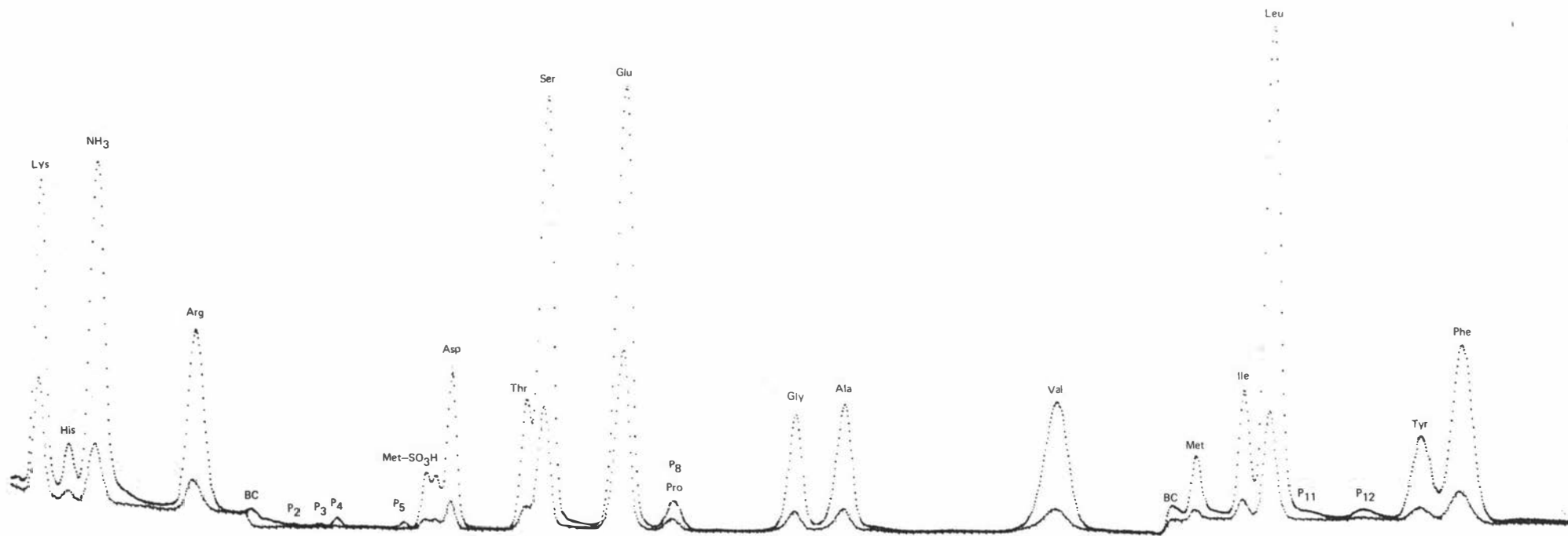


Fig. 21. Chart trace from the Amino Acid Analyzer of the free amino acids in a 6-month-old Cheddar cheese made with starter strain US<sub>3</sub>. Abbreviations: Lys, lysine; His, histidine; Arg, arginine; Met-SO<sub>3</sub>H, methionine sulphoxides; Asp, aspartic acid; Thr, threonine; Ser, serine; Glu, glutamic acid; Pro, proline; Gly, glycine; Ala, alanine; Val, valine; Met, methionine; Ile, isoleucine; Leu, leucine; Tyr, tyrosine; Phe, phenylalanine; BC, buffer change; P, probably low MW peptides.

Table IX. Free amino acids in cheeses during the course of ripening. The cheeses were made with the single-strain starters HP, ML<sub>1</sub>, US<sub>3</sub> and Z<sub>0</sub>, or with gluconic acid lactone (GAL) instead of starter. The concentrations of the free amino acids are expressed as µg/g of cheese.

Amino acids	Age of cheese											
	24 hr				3 months					6 months		
	GAL	HP	ML <sub>1</sub>	US <sub>3</sub>	GAL	HP	ML <sub>1</sub>	US <sub>3</sub>	Z <sub>0</sub>	HP	ML <sub>1</sub>	US <sub>3</sub>
Lys	3.3	45.3	55.3	13.9	72.4	144	1030	437	154	435	911	290
His	-	19.5	23.6	-	-	41.6	154	73.0	-	54.9	45.7	43.8
Arg	4.2	29.6	43.6	21.7	6.7	80.7	137	765	62.4	88.4	62.1	294
MetSO <sub>3</sub> H	-	-	-	-	-	39.7	292	81.8	99.6	59.5	166	114
Asp	-	17.4	17.0	17.3	-	37.4	210	109	28.8	94.6	254	128
Thr	-	-	-	6.7	-	16.1	-	-	33.7	69.2	255	106
Ser	1.3	17.0	15.3	13.8	-	85.3	921	474	82.3	257	1100	457
Glu	1.4	73.3	107	68.0	41.2	286	2260	654	234	744	2540	779
Pro	-	37.8	43.1	17.5	-	69.6	284	-	-	125	177	-
Gly	0.6	5.6	1.9	1.8	8.4	25.4	118	50.6	28.1	64.2	169	70.4
Ala	0.6	9.9	11.9	5.2	11.4	34.2	286	87.5	36.8	106	343	107
Val	-	10.0	14.6	5.1	11.0	63.1	574	139	77.4	200	555	215
Met	-	-	-	-	-	13.4	142	27.3	30.4	36.4	241	50.2
Ile	-	2.6	2.5	3.0	-	13.3	62.4	54.0	11.3	39.6	154	99.1
Leu	5.0	28.3	37.0	16.3	146	198	1660	656	173	619	2210	685
Tyr	-	21.5	30.5	4.7	11.7	65.9	217	128	43.4	144	504	169
Phe	6.7	19.2	31.4	20.0	112	164	1250	396	125	406	1240	365
Totals:	23	337	435	215	421	1378	9597	4132	1220	3543	10927	3972

\* Abbreviations: see Fig. 21.

The free amino acid level in the HP cheese increased to 1.4 mg/g by 3 months, and to 3.5 mg/g at 6 months. The level in the Z<sub>6</sub> cheese at 3 months was 1.2 mg/g. In PSM in the presence of NaCl, strain ML<sub>4</sub> was found to be more proteolytic than strains HP and US<sub>3</sub>. The finding that the relative proteolytic activities of these strains were reflected in the levels of free amino acids in the cheeses at 24 hr was not, therefore, unexpected. At 3 and 6 months, the ML<sub>4</sub> cheese still exhibited the highest levels of free amino acids compared to the HP and US<sub>3</sub> cheeses. Since the inhibition of starter proteolysis would be greater at a higher level of NaCl, the differing salt-in-moisture levels of these cheeses (Table VI) might also account for some of the observed differences.

However, although the total amounts of free amino acids in the cheeses differed, there was a marked similarity in the proportions in which the individual free amino acids occurred relative to the total amounts of free amino acids, and independent of the starter strain used in cheesemaking (Figs 22 and 23). Although arginine occurred in a relatively greater proportion in the US<sub>3</sub> cheese than in the other cheeses at 3 and 6 months, this was not apparent in the 24 hr analysis (Table IX).

Relative to the proportions in which the amino acids are known to occur in casein, the proportions in the cheeses of the free amino acids proline, isoleucine and aspartic acid were low, and the proportions of free leucine and phenylalanine high. This is in agreement with the findings of Mabbitt (1955) with Cheddar cheese, and Ali (1960) with Edam cheese, that proline and aspartic acid were found in lower proportions, and leucine in higher proportions than in casein.

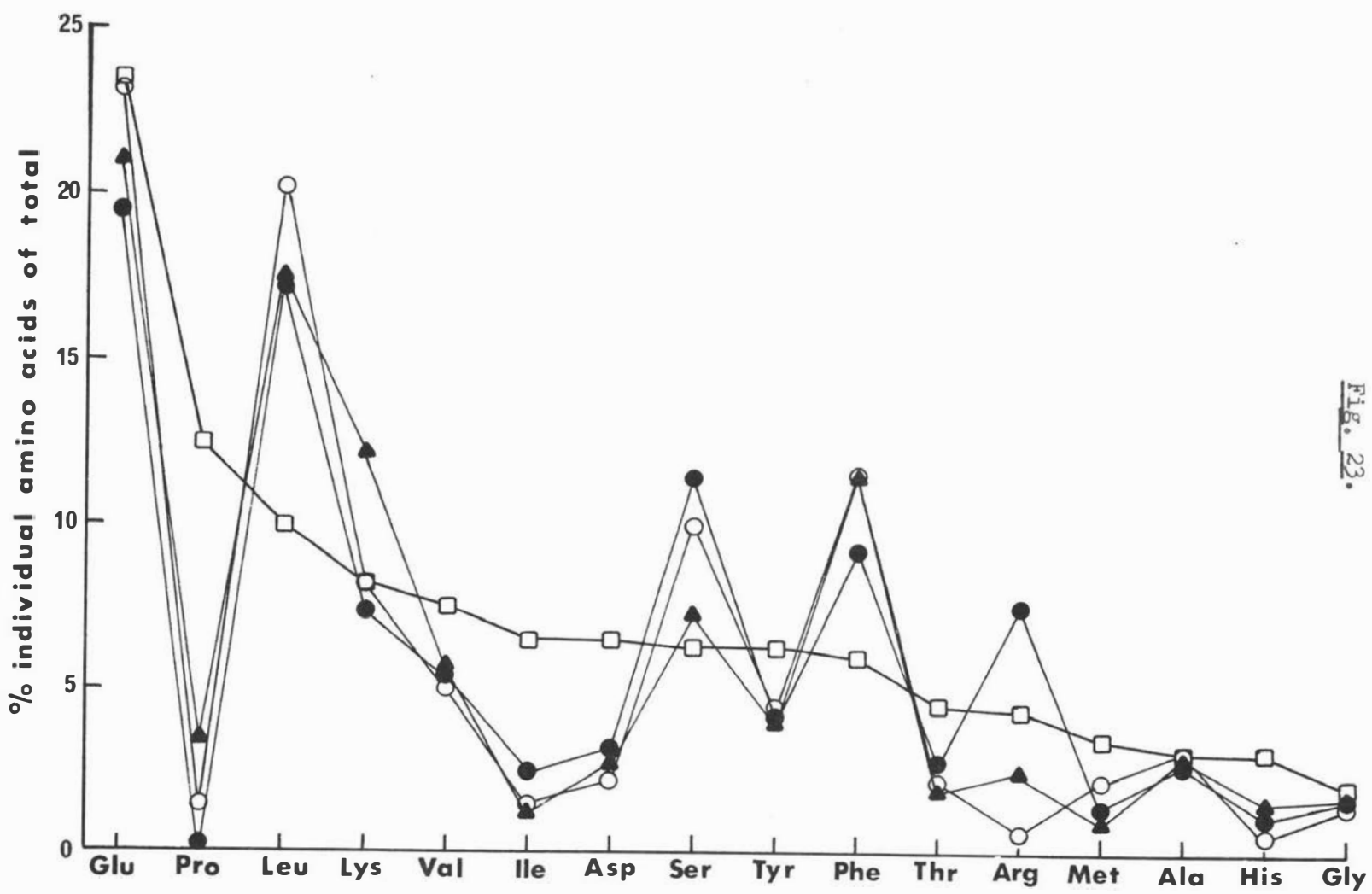
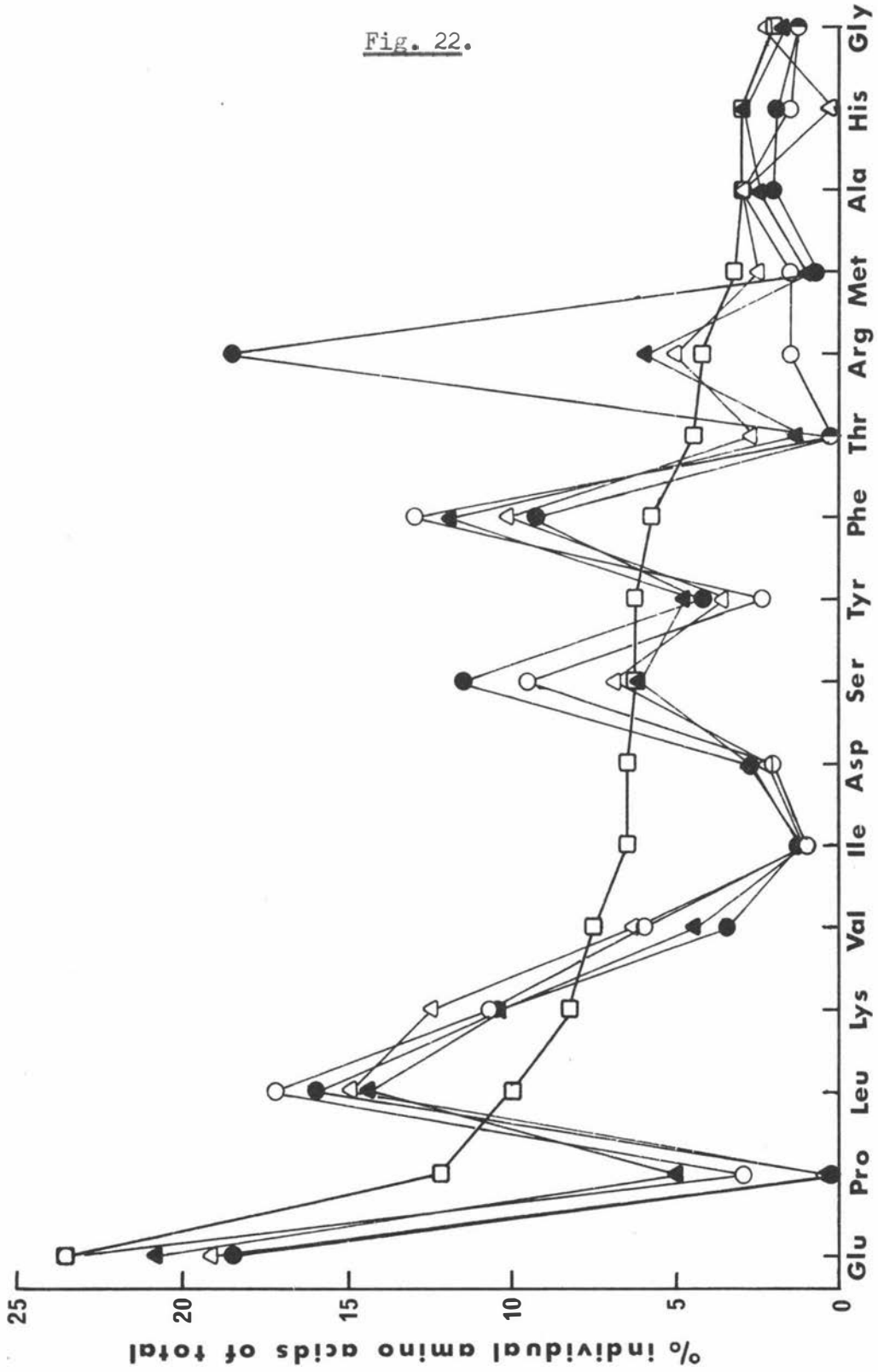


Fig. 23.

Fig. 22.



In contrast to the starter cheeses, the total amount of free amino acids in the CAL cheese was low, increasing from 0.02 mg/g cheese at 24 hr to 0.42 mg/g cheese at 3 months (Table IX), and the range of amino acids found was limited. At 3 months, leucine, phenylalanine and lysine predominated, forming 78% of the total. The balance was made up of lesser amounts of glutamic acid, tyrosine, valine, glycine and arginine. Although it has been suggested that amino acids are not formed by rennet proteolysis (Reiter et al., 1969) the low level of free amino acids and the predominance of leucine, phenylalanine and lysine in this cheese suggests that in fact slow but specific proteolysis does take place during the relatively long period of cheeseripening. The presence of leucine and phenylalanine in particular is consistent with the known specificity of rennet action (Bang-Jensen et al., 1964). This specific proteolysis by the rennet in addition to the relatively non-specific proteolysis by the starter proteinases might account for the relatively high proportions of leucine and phenylalanine found in the starter cheeses.

Flavour assessments. The first series of cheese made with starters HP, ML<sub>1</sub>, US<sub>3</sub> and Z<sub>8</sub> was tasted at 3, 4, 5 and 6 months (Table X). The cheese made with US<sub>3</sub> consistently possessed a greater level of typical Cheddar flavour than the other cheeses. Off-flavours such as bitterness and astringency were absent at all stages.

Cheeses made with HP and Z<sub>8</sub> were similar in many respects. Both were definitely bitter at 3 months, and the intensity of bitterness increased only slightly during further maturation. The scores for Cheddar flavour, and particularly the overall



Table X. Flavour scores of the first series of cheeses. The cheeses were tasted by a panel of 6 to 10 experienced judges whose scores were averaged. The overall acceptability of the flavour of the cheeses was scored on a scale of 1 to 9; on this scale a cheese with very serious flavour defects scored 1, an average cheese 5, and a cheese of exceptionally good flavour 9. The remaining flavours were scored on a 1 to 5 scale as follows: 1, flavour absent; 2, possibly present; 3, definitely present; 4, predominant flavour; 5, very intensely flavoured.

Strain: Flavour	Age of cheeses															
	3 months				4 months				5 months				6 months			
	ML <sub>1</sub>	HP	Z <sub>8</sub>	US <sub>3</sub>	ML <sub>1</sub>	HP	Z <sub>8</sub>	US <sub>3</sub>	ML <sub>1</sub>	HP	Z <sub>8</sub>	US <sub>3</sub>	ML <sub>1</sub>	HP	Z <sub>8</sub>	US <sub>3</sub>
Cheddar	2.4	2.6	2.3	3.0	2.6	2.4	2.4	3.0	2.6	2.6	2.4	2.9	2.6	2.5	2.5	3.4
Bitter	2.0	2.6	2.7	1.0	2.6	3.1	3.0	1.4	2.4	4.3	3.3	1.1	2.3	2.9	3.2	1.1
Astringent	1.4	1.9	1.6	1.0	1.4	1.1	1.1	1.1	1.4	1.9	1.6	1.0	2.2	2.0	2.0	1.2
Fruity	1.1	1.0	1.3	1.3	1.8	1.0	1.1	1.3	2.1	1.0	1.0	1.4	2.5	1.3	1.2	1.9
Sharp	1.1	1.3	1.0	1.1	1.0	1.3	1.1	1.1	1.4	1.0	1.0	1.0	1.2	1.2	1.2	1.3
Other	1.9	1.6	1.4	1.4	1.9	2.0	1.9	1.5	1.4	1.6	1.6	1.9	2.4	2.0	1.9	1.3
Overall accept- ability	3.7	3.3	3.1	5.6	3.5	3.5	3.4	4.9	2.6	3.9	3.3	5.9	3.5	3.8	3.6	6.6

flavour acceptability, were low.

The  $ML_1$  cheese similarly scored poorly for Cheddar flavour and overall flavour acceptability. A low level of bitterness was detectable at all stages, and there was also a tendency to astringency particularly at 6 months. However, the most common off-flavour associated with the  $ML_1$  cheese was variously described by the tasters as "burnt" or "caramel". This confirms the results of numerous other flavour assessments of  $ML_1$  cheeses carried out at this Institute (Lawrence & Pearce, 1972).

The second series of cheeses made with  $AM_1$ ,  $AM_2$ , HP and  $ML_1$  was tasted only at 8 weeks (Table XI). Although the development of flavour in these cheeses was not as full as in more mature cheese, characteristic differences in flavours associated with the different starters could nevertheless be detected. Cheeses made with  $AM_1$  and  $AM_2$  both scored better than those made with HP and  $ML_1$  for both Cheddar flavour and the overall acceptability of cheese flavour. Bitterness was marked in the HP cheese, less intense in the  $ML_1$  cheese, and absent from the  $AM_1$  and  $AM_2$  cheeses.

Contribution of free amino acids to the flavour of the cheeses. The importance of amino acids in cheese flavour will depend to a great extent on their concentration in the cheeses. The amino acids valine, leucine, isoleucine, tyrosine and phenylalanine are considered to have a bitter flavour when tasted on their own (Mulder, 1952).

Table XI. <sup>\*</sup> Flavour scores of the second series of cheeses at 8 weeks.

Flavour	Cheese made with strain			
	AM <sub>1</sub>	AM <sub>2</sub>	HP	ML <sub>1</sub>
Cheddar	2.6	2.5	2.0	2.1
Bitterness	1.6	1.2	2.6	2.1
Astringency	1.4	1.4	1.7	1.5
Fruity	1.3	1.7	1.2	1.7
Sharp	1.1	1.0	1.0	1.1
Other	2.0	2.3	2.4	2.0
Overall acceptability	5.2	5.0	3.5	4.2

\* See Table X for scales.

Matoba et al. (1970) reported that the threshold for detecting the bitter flavour of the bitter amino acids was a concentration of 0.1% in pure solution. However, it is likely that the thresholds would be higher in cheese on account of the presence of such other compounds as the fat and casein. The total concentrations of the bitter amino acids in the HP, ML<sub>1</sub>, and US<sub>3</sub> cheeses at 6 months were 0.14, 0.47 and 0.15% (w/v) respectively, and at 3 months 0.05, 0.38 and 0.14% (w/w) respectively, and 0.05% in the Z<sub>0</sub> cheese. At these levels the contribution of the bitter-tasting amino acids to bitterness in cheese seems

## D I S C U S S I O N

There is little doubt that the starter streptococci used in Cheddar cheesemaking are responsible for the development of typical cheese flavour and for specific flavour defects such as bitterness.

Good-flavoured cheese is always produced when S. cremoris strains AM<sub>1</sub>, AM<sub>2</sub> or US<sub>3</sub> are used, and cheeses characterized by bitterness and lower consumer acceptability are almost always produced when such strains as HP and Z<sub>8</sub> are used. This investigation was undertaken to determine the features of starter strains associated with good or poor flavour development in the cheese. An attempt was also made to differentiate the roles of starter streptococci, other micro-organisms and rennet, particularly with regard to the possible influence of their relative proteolytic activities on the acceptability of the cheese and the formation of bitterness.

The present investigation has demonstrated that good-flavoured cheese is characteristically made when starters are used which possess either one or both of the following features: (i) low rate of cell division at the temperature of cheesemaking which results in relatively low numbers of cells being produced; (ii) low proteolytic activity as determined in PSM containing 4 to 5% NaCl.

Growth of starters. The starter strains exhibited characteristic differences in the populations they attained and the number of doublings of the populations that occurred during cheesemaking. Populations of the "non-bitter" strains AM<sub>1</sub>, AM<sub>2</sub> and US<sub>3</sub> doubled between 0.6 to 2 times compared to between 3.3 to 4.6 times for the "bitter" strains HP and Z<sub>8</sub>. Cell division

of all strains proceeded more rapidly during the first  $2\frac{1}{2}$  to 3 hr of cheesemaking. During the next  $2\frac{1}{2}$  to 3 hr of cheesemaking, after the maximum temperature had been attained, the populations of strains  $AM_1$  and  $AM_2$  declined up to 10-fold, and the population of  $US_3$  remained virtually stationary. In spite of this, acid production by these strains continued. In contrast, cells of the "bitter" strains HP and  $Z_8$  continued to divide, although at a slightly lower rate, during the latter stages of cheesemaking.

Since the rate of cell division will limit the extent of bacteriophage replication, these differences between the "bitter" and "non-bitter" starters in their rates of cell division during cheesemaking may explain the earlier report by Lawrence & Pearce (1968) that the former strains tended to be more sensitive to bacteriophage infection than the latter strains.

Similar differences were also found in the populations attained and the number of cell divisions undergone by the "non-bitter" and "bitter" strains grown to pH 5.2 in PSM at  $35^\circ\text{C}$ , but these differences were not as marked as those found during cheesemaking. The number of population doublings varied between 4.2 for  $AM_2$  to 5.4 for  $Z_8$ . The differences in populations cannot be attributed to variations in chain lengths between different strains as the chains were reduced to approximately equal-sized colony-forming-units by blending of the cultures prior to standard pour plate counting. The difference between the growth of the starters in PSM and during cheesemaking emphasizes the important effect that raising the temperature from 35 to  $38^\circ\text{C}$ , the maximum temperature reached during cheesemaking, has on the growth of the "non-bitter" starters, such as  $AM_2$ , which are particularly heat-sensitive.

Proteolysis by starters. Proteolytic breakdown in skim milk cultures varied considerably according to the strain. Gordon & Speck (1965a) considered that "non-bitter" strains of cheese starters were less proteolytic in skim milk cultures than the "bitter" strains. In this investigation, proteolysis by three "non-bitter" strains, AM<sub>1</sub>, E<sub>8</sub> and US<sub>3</sub>, during growth to pH 5.2 in PSM at 35°C was relatively low compared to the "bitter" starters, but this was not true of three other "non-bitter" strains, AM<sub>2</sub>, ML<sub>1</sub> and R<sub>1</sub>.

The addition of 4 or 5% NaCl, levels commonly found in the moisture of cheese, to PSM cultures had practically no effect on the amount of proteolysis effected by the least proteolytic strains, AM<sub>1</sub>, E<sub>8</sub> and US<sub>3</sub>, but the amount of proteolysis by the more active strains was markedly reduced on extended incubation at 13°C. Thus proteolysis by the "non-bitter" strain AM<sub>2</sub> was reduced in the presence of NaCl to a level similar to that of the other "non-bitter" strains, AM<sub>1</sub>, E<sub>8</sub> and US<sub>3</sub>.

The "non-bitter" strain ML<sub>1</sub> was highly proteolytic, even in the presence of NaCl. Although cheese made with this strain has generally been described as non-bitter (Emmons et al., 1962a; Jago, 1962), cheeses made with ML<sub>1</sub> at this Institute have frequently been judged by the taste-panels to be slightly bitter. More particularly, however, an off-flavour variously described as "burnt" or "caramel" is also reported (Lawrence & Pearce, 1972). This seems to be a characteristic off-flavour associated with cheese made with this strain, and for this reason it is unwise to regard strain ML<sub>1</sub> as a typical "non-bitter" starter.

Although strain R<sub>1</sub> has also been considered to be a "non-bitter" starter (Czulak & Shimmin, 1961; Emmons et al., 1962a),

its similarity in many respects to HP in the present investigation suggested that it should be a "bitter" strain. This has in fact been confirmed in recent trials at the Institute where R<sub>1</sub> cheeses were found to be consistently bitter.

One conclusion that follows from these results could be of importance in the manufacture of lactic acid casein. By using only those starter strains with low proteolytic activity, the potential yield losses due to proteolysis during the coagulation period would be reduced to a minimum.

Specificity of starter proteinases. Amino acid analyses of a good-flavoured cheese and cheeses having either bitter or "burnt" off-flavours indicated that a wide range of free amino acids was liberated due to proteolysis. Although the total quantities of free amino acids varied between the cheeses, the proportions which the individual free amino acids formed of the totals were similar in all the cheeses. This indicates clearly that the proteinases of the starter strains used for making these cheeses differing in flavour exhibited practically no differences with regard to the peptide bonds cleaved. Other investigators have also shown that although lactic streptococci may differ between strains in the quantities of amino acids formed in milk cultures they could not be distinguished on the basis of specificity (Doležálek, 1966; Miller & Kandler, 1967).

Survival of starter in relation to proteolysis. Proteolysis in the PSM cultures in the simulated cheese environment systems continued whether the starters died out rapidly (strain C<sub>13</sub>) or survived well (strain ML<sub>8</sub>). Moreover, although the survival of HP was better in the presence of 5 than 4% NaCl, proteolysis was

slightly less at the higher NaCl level. This evidence, although not conclusive, does not support the suggestion by Anders & Jago (1964) that the rapid death of the starter during cheeseripening is responsible for limiting peptidase activity which in turn would thus enhance the development of bitterness. On the contrary, those starters such as AM<sub>1</sub>, AM<sub>2</sub> and US<sub>3</sub> which died out rapidly in the cheese were those which characteristically produced the least levels of bitterness.

Bitterness. At the start of this investigation it was considered possible that the bacteriological quality of the cheesemilk, particularly with regard to the incidence of proteolytic organisms, might have some influence on the development particularly of off-flavours such as bitterness. Feagan & Dawson (1959), for example, attributed bitterness in some Australian Cheddar cheeses to the influence of certain types of bacteria which predominated in the milk supply. However, under normal New Zealand conditions the proteinases of the milk flora have been shown in this investigation to play an insignificant role in influencing flavour development.

Czulak (1959) postulated that bitterness in cheese resulted from the accumulation of bitter peptides due to the action of rennet, and the use of starters with low or impaired proteolytic activity which were unable to further degrade these peptides (Fig. 24). If this hypothesis were correct, one would expect that the "non-bitter" strains such as AM<sub>1</sub>, AM<sub>2</sub> or US<sub>3</sub> would either be more proteolytic or be highly specific in their ability to degrade bitter peptides. However, in the present investigation the "non-bitter" starters were found to be less proteolytic than



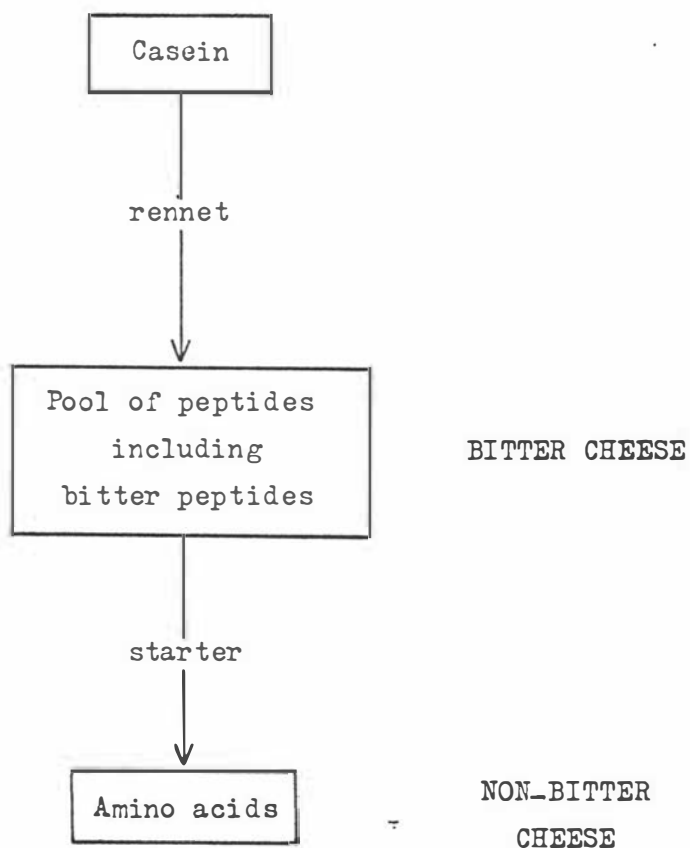


Fig. 24. Role of rennet and starter in the formation of bitterness according to the hypothesis of Czulak (1959).

the "bitter" starters, and the specificity of casein breakdown in cheese made with both the "bitter" and "non-bitter" starters was very similar.

Although Stadhouders (1962) also considered that the ability of the starters to break down rennet peptides was a specific one, he considered that the rate of acid production by the starter during cheesemaking could also influence the development of bitterness by determining the amount of rennet retained in the curd, since bitterness was frequently associated with high concentrations of rennet. However, no differences could be found in this investigation between the electrophoresis patterns of casein breakdown in a bitter cheese made with HP, and a non-bitter cheese made with AM<sub>2</sub>. This suggested that the level of rennet retained in the curd of cheeses made with these different starters must have been essentially the same.

Schormüller (1968) considered that the phosphatase activities of the organisms important in cheesemaking were essential for the degradation of the phosphopeptides derived from the proteolysis of casein. One might expect, therefore, that the proteolytic and phosphatase activities of the starters would be related, but this was not found in the present investigation.

Lawrence & Gilles (1970) found that cheese made with high levels of rennet exhibited a greater intensity of bitterness when "bitter" starters were used in cheesemaking. However, with three "non-bitter" starters, AM<sub>1</sub>, AM<sub>2</sub> and US<sub>3</sub>, a 3-fold increase in the normal amount to 66 ml/100 l of milk did not affect the level of bitterness. The fact that bitterness was not detected under these circumstances suggested that either the rennet peptides were not bitter or that if they were they were rapidly degraded

by the proteinases of the starter streptococci. This latter alternative, however, would not be consistent with the relatively low proteolytic activity or the absence of specificity of the proteinases of these "non-bitter" strains.

The following scheme, outlined in Fig. 25 is, therefore, suggested to explain the role of rennet and the different starter strains in determining whether or not bitterness is formed in cheese. The first stage of rennet proteolysis results in the formation of high MW, predominantly non-bitter, peptides. "Non-bitter" starters with low proteolytic activity do not degrade these precursor high MW peptides at a fast enough rate for the concentration of lower MW peptides including bitter peptides to exceed the threshold at which bitterness can be detected. Hence, increasing the quantity of precursor high MW peptides by employing higher levels of rennet has no effect on the level of bitterness in cheeses made with these starters. With the "bitter" starters, which have relatively more proteolytic activity, the precursor high MW peptides are broken down more rapidly and a greater accumulation of bitter peptides results. Increasing the rennet level increases the supply of these precursors and proteolysis by these "bitter" starters will lead to an increased level of bitter peptides being formed. Starters such as ML<sub>1</sub>, having greater proteolytic activity, probably degrade both the precursors and the bitter peptides more extensively. The characteristic "burnt" flavour associated with this strain may be due to the relatively large amount of free amino acids formed.

Relationship of starter populations to proteolytic activity.

As the "non-bitter" and "bitter" starter strains appear to differ

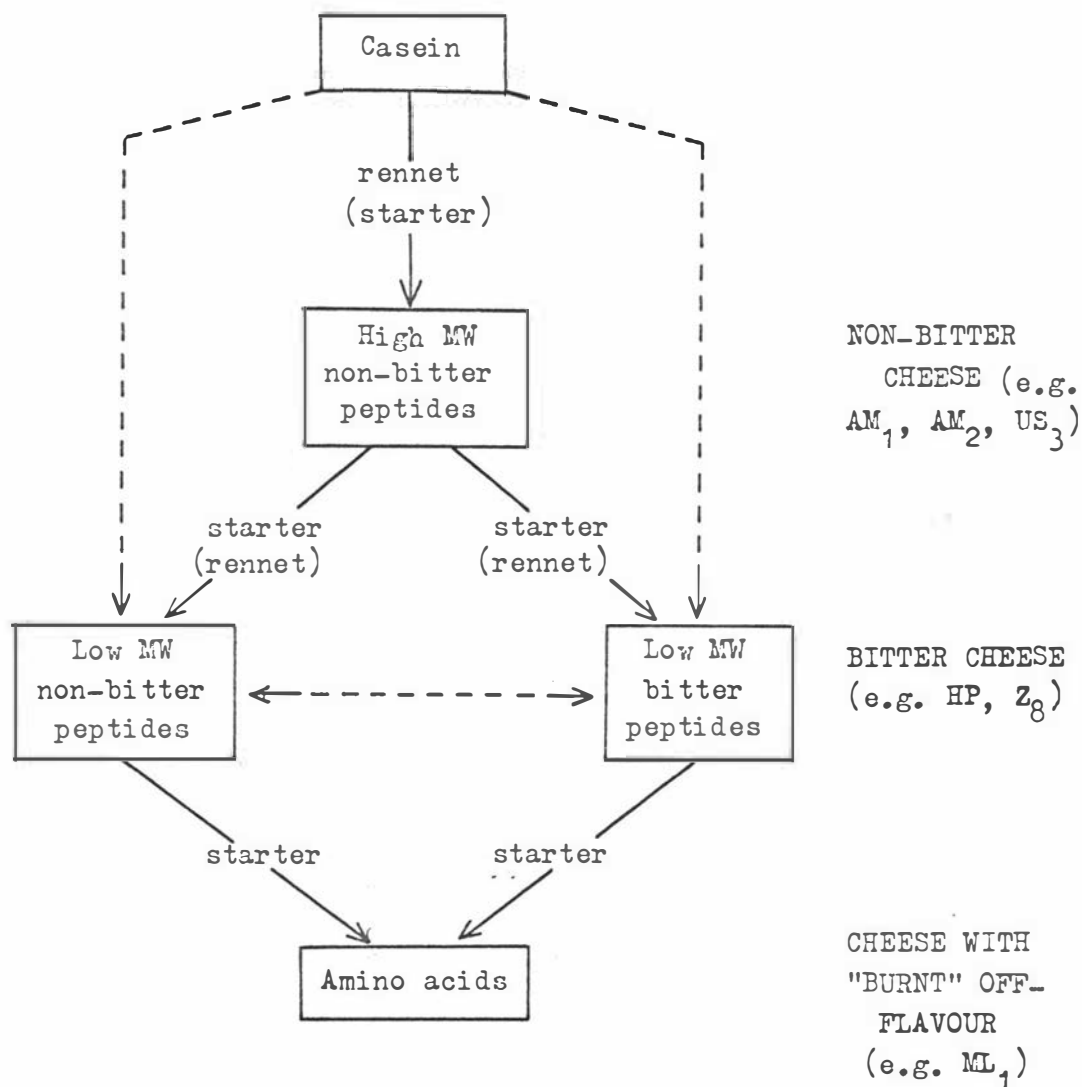


Fig. 25. The suggested major pathways of casein breakdown by the proteinases of rennet and starter streptococci that lead to the formation of flavours in Cheddar cheese associated with the use of specific starter strains. Proteinases in parentheses probably play a relatively minor role. Dotted lines: possibly minor pathways by either rennet or starter.

in their proteolytic activities only in the quantity of breakdown products formed and not in the nature of the products formed, it is likely that the level of starter proteinases formed during cheesemaking could be important.

Bacterial enzymes can generally be described either as extracellular or cell-bound. The cell-bound enzymes can be further classed as truly intracellular (cytoplasmic) or surface (membrane)-bound. Of 30 strains of starter streptococci grown on Standard Methods caseinate agar (see Appendix I), only two strains, which are infrequently used in cheesemaking, consistently formed white zones due to proteolysis extending beyond the colony, indicative of a diffusible extracellular proteinase. Some of the other strains formed traces of zones due to proteolysis, but only directly beneath the colony. This tends to indicate that the proteinases of the commonly-used cheese starter strains are cell-bound rather than extracellular, in agreement with the findings of Baribo & Foster (1952).

As cell-bound enzymes increase synchronously with the cells as they divide, the maximum level of enzyme formed in a culture is likely to be closely related to the maximum cell population. It is most likely, therefore, that the level of starter proteinases formed in cheese is determined by a combination of two factors: (i) the maximum number of cells formed during cheesemaking, and (ii) the relative proteolytic activity of the specific starter strain used.

The role of the proteolytic activities of starter in influencing the overall acceptability of flavour, and the intensity

of flavour defects such as bitterness, leads one to postulate that a considerable amount of control can be exerted on cheese flavour merely by regulating the population and, hence, the level of proteinase attained during cheesemaking. A decrease in the populations of "bitter" strains such as HP and Z<sub>8</sub> during cheesemaking should reduce the level of proteinase, and consequently decrease the tendency to bitterness in cheese. However, increasing the populations of typically "non-bitter" strains with low proteolytic activity per cell (e.g. AM<sub>1</sub> and US<sub>3</sub>) might not affect flavour significantly, as the level of proteinase might still not be sufficient to induce bitterness.

The reduction in the intensity of bitterness in cheese made using a "non-bitter" starter in association with a "bitter" starter is well established (Emmons et al., 1962b; Lawrence & Pearce, 1968). In this situation the maximum level of "bitter" starter attained during cheesemaking would be reduced on account of the presence of the "non-bitter" starter. Consequently the level of proteinase derived from the "bitter" strain would be correspondingly reduced, thereby reducing the intensity of bitterness in the cheese.

It might also be expected that increasing the populations of typically "non-bitter" starters having a higher level of proteolytic activity per cell (e.g. AM<sub>2</sub>) may lead to enough enzyme being formed to bring about bitterness.

Effect of NaCl on proteolysis and bitterness. Lawrence & Gilles (1969) considered that the development of bitterness in cheese made with specific "bitter" starters was determined by the salt-in-moisture level and, to a lesser extent, upon the pH of the cheese at 14 days. Almost without exception they found that

bitterness occurred in cheese made with HP when the salt-in-moisture level was  $<4.3\%$ , but was generally absent when the salt-in-moisture level was  $>4.9\%$ . This would be consistent with the findings in the present investigation that proteolysis by both rennet and starter is inhibited by NaCl. Although the overall proteolysis in cheeses was also related to their salt-in-moisture levels, it seems unlikely that the comparatively small variations of salt-in-moisture levels from  $<4.3$  to  $>4.9\%$  would affect the development of bitterness, as much as, for example, one more doubling of the starter population during cheesemaking with a corresponding doubling in the level of starter proteinase.

Relationship of the pH of the cheese to the incidence of bitterness. There have been conflicting reports on the significance of the relationship of the pH of the cheese to the incidence of bitterness. Czulak (1959) found that bitterness occurred most frequently in cheese having a  $\text{pH} < 5.0$  in the first week, but Dawson & Feagan (1960) failed to find any correlation between pH and bitterness. Emmons et al., (1962a) showed that some "bitter" starter strains were more significantly affected by pH than others. Lawrence & Gilles (1969) found that the pH of cheeses made with "bitter" HP starter was only of importance in influencing the bitterness of the cheese when the salt-in-moisture levels were within the range 4.3 to 4.9%.

A likely explanation of these conflicting reports can be made in terms of the population and associated enzyme levels of the starters. With the "fast" starters a significant amount of cell division takes place during the formation of acid in cheese-

making. Since the final pH of the cheese depends on the pH attained during cheesemaking (Dolby, 1941) it is likely that a more acid cheese at 14 days would indicate that a higher population of starter had been attained during cheesemaking. With the associated higher level of starter proteinase, bitterness or a greater intensity of bitterness would be more likely to develop.

The "non-bitter" starters practically cease dividing in the latter half of cheesemaking, although acid production continues. Hence, a relatively low pH of a cheese made with a "non-bitter" starter would not necessarily indicate that a high population of starter had been attained during cheesemaking. In this situation one would thus not expect a relationship between the pH of the cheese and the incidence of bitterness.

Conclusions. The development of good flavour and the absence of flavour defects such as bitterness in Cheddar cheese are largely determined by the starter strain and the amount and type of rennet used. Variations in the bacteriological quality of the milk normally have no detectable influence on cheese flavour.

The determination of the precise blend of components which makes up typical Cheddar flavour is likely to prove extremely difficult. However, the present investigation indicates that differences in the typical flavours associated with the use of specific starter strains can be explained very largely in terms of quantitative differences in proteolytic activity rather than in differences in specificity of proteolytic breakdown of casein or casein degradation products.

Good-flavoured cheese is associated with a low level of starter proteolysis and this in turn may be associated with



relatively low numbers in the curd. More extensive proteolysis by starter results in the production of bitterness, while "burnt" flavours probably reflect a further degree of proteolytic degradation and the accumulation of high levels of amino acids. The increase in intensity of bitterness in cheeses made using "bitter" starters and high levels of rennet may result from the production of greater amounts of precursors by the rennet.

NaCl inhibits proteolysis by both starter and rennet, and high salt-in-moisture levels in the cheese may, to a limited extent, control the development of bitterness.

The level of starter proteinase in the cheese appears to be the most important factor determining the intensity of bitterness in the cheese. This level is determined by a combination of two factors: (i) the amount of proteinase per cell; and (ii) the maximum starter population attained during cheesemaking, which appears to be a reflection of the ability of the starter to divide at the temperatures used in cheesemaking. The temperature in turn might also partly govern the survival characteristics of the starter, but it is unlikely that the rate at which the starter die per se is important in determining the rate of proteolysis.

The decreased bitterness in cheese associated with the pairing of "bitter" and "non-bitter" starters probably reflects a reduced population of the "bitter" strain. However, it should also be practicable to reduce the populations of the "bitter" starters by employing higher temperatures during cheesemaking to retard cell division and, to a lesser extent, by salting the curd at an earlier stage of cheesemaking.

Thus, limiting excessive cell division of the starter during cheesemaking has important practical implications in reducing the incidence or intensity of bitterness in Cheddar cheese.

## R E F E R E N C E S

- Alford, J.A. & Frazier, W.C. (1950). *J. Dairy Sci.* 33, 107.
- Ali, I.A.M. (1960). *Meded. LandbHogesch., Wageningen* 60 (4), 1.
- American Public Health Association (1967). "Standard Methods for the Examination of Dairy Products," 12th ed. New York: American Public Health Association.
- Amunstad, O. (1950). *Medd. Statens Mejeriförsök* 28, 170 (via *Chem. Abstr.* (1951) 45, 6765c).
- Anders, R.F. & Jago, G.R. (1964). *J. Dairy Res.* 31, 31.
- Anders, R.F. & Jago, G.R. (1970). *Aust. J. Dairy Technol.* 25, 68.
- Bang-Jensen, V., Foltmann, R. & Rombauts, W. (1964). *C. r. Trav. Lab. Carlsberg* 34, 326.
- Baribo, L.E. & Foster, B.M. (1952). *J. Dairy Sci.* 35, 149.
- Baughman, R.W. (1964). Ph.D Thesis. Ann Arbor, University Microfilms.
- Bills, D.D., Morgan, M.E., Libbey, L.M. & Day, E.A. (1965). *J. Dairy Sci.* 48, 1168.
- Bingham, D.W. & Zittle, C.A. (1963). *Archs Biochem. Biophys.* 101, 471.
- Flock, R.J. & Weiss, A.B. (1956). "Amino acid handbook," Springfield, Ill: Thomas.
- Breed, R.S., Murray, E.C.D. & Smith, N.R. (1957). In "Bergey's Manual of Determinative Bacteriology," 7th ed. Baltimore: Williams & Wilkins.
- British Standards Institution (1963). B.S. 770. "Methods for the chemical analysis of cheese." London: British Standards Institution.

- Brown, R.W., Sandvik, O., Scherer, R.K. & Rose, D.L. (1967).  
J. gen. Microbiol. 47, 273.
- Citti, J.E., Sandine, W.E. & Elliker, P.R. (1963). J. Dairy Sci.  
46, 337.
- Citti, J.E., Sandine, W.E. & Elliker, P.R. (1965). J. Dairy Sci.  
48, 14.
- Cohen, J.O. & Smith, P.E. (1964). J. Bact. 88, 1364.
- Creamer, L.K. (1970). N.Z. J. Dairy Sci. Technol. 5, 152.
- Creamer, L.K., Lawrence, R.C., Gilles, J. & Humphries, M.A.  
(1970). N.Z. J. Dairy Sci. Technol. 5, 35.
- Czulak, J. (1959). Aust. J. Dairy Technol. 14, 177.
- Czulak, J. & Shimmin, P. (1961). Aust. J. Dairy Technol. 16, 96.
- Das, N.K. & Marshall, R.T. (1967). Appl. Microbiol. 15, 1479.
- Davies, W.L., Davis, J.S., Dearden, D.V. & Mattick, A.T.R. (1937).  
J. Dairy Res. 8, 92.
- Dawson, D.J. & Feagan, J.T. (1957). J. Dairy Res. 24, 210.
- Dawson, D.J. & Feagan, J.T. (1960). Aust. J. Dairy Technol. 15, 65.
- de Man, J.C., Rogosa, M. & Sharpe, M.E. (1960). J. appl. Bact.  
23, 130.
- Dolby, R.M. (1941). N.Z. J. Sci. Technol. A 22, 289.
- Dolby, R.M., Creamer, L.K. & Elley, E.R. (1969). N.Z. J. Dairy  
Technol. 4, 46.
- Doležálek, J. (1966). XVII Int. Dairy Congr. D, 523.
- Doolittle, R.F. & Armentrout, R.W. (1968). Biochemistry, N.Y.  
7, 516.

- Dorn, F.L. & Rahn, O. (1939). Arch. Mikrobiol. 10, 6.
- Druce, R.G. & Thomas, S.R. (1959). J. appl. Bact. 22, 52.
- Elliker, P.R., Anderson, A.W. & Hannesson, G. (1956). J. Dairy Sci. 39, 1611.
- El-Negoumy, A.M. (1970). J. Dairy Res. 37, 437.
- Emmons, D.P., McGugan, W.A., Elliott, J.A. & Morse, P.M. (1962a). J. Dairy Sci. 45, 332.
- Emmons, D.P., McGugan, W.A., Elliott, J.A. & Morse, P.M. (1962b). J. Dairy Sci. 45, 595.
- Feagan, J.T. & Dawson, D.J. (1959). Aust. J. Dairy Technol. 14, 59.
- Folin, O. & Ciocalteu, V. (1927). J. biol. Chem. 73, 627.
- Forbes, D. (1968). J. Dairy Res. 35, 399.
- Fox, P.F. (1969). J. Dairy Sci. 52, 1214.
- Fox, P.F. & Walley, B.F. (1971). J. Dairy Res. 38, 165.
- Franklin, J.G. & Sharpe, M.E. (1963). J. Dairy Res. 30, 87.
- Frazier, W.C. & Rupp, P. (1928). J. Bact. 16, 65.
- Fryer, T.F., Reiter, B. & Lawrence, R.C. (1967). J. Dairy Sci. 50, 388.
- Garvie, E.I. (1959). J. Dairy Res. 26, 227.
- Goel, M.C. & Marth, E.H. (1969). J. Dairy Sci. 52, 1941.
- Gordon, D.F. Jr & Speck, M.L. (1965a). J. Dairy Sci. 48, 499.
- Gordon, D.F. Jr & Speck, M.L. (1965b). Appl. Microbiol. 13, 537.
- Hammer, B.W. (1948). "Dairy Bacteriology," 3rd ed. New York: Wiley.
- Hammer, B.W. & Olson, H.C. (1941). J. Milk Technol. 4, 83.

- Harper, W.J. (1959). *J. Dairy Sci.* 42, 207.
- Harris, W.C. & Hammer, B.W. (1940). *J. Dairy Sci.* 23, 701.
- Harwalkar, V.R. & Elliott, J.A. (1955). *J. Dairy Sci.* 48, 784.
- Harwalkar, V.R. & Elliott, J.A. (1971). *J. Dairy Sci.* 54, 12.
- Hattowska, H. (1970). XVIII Int. Dairy Congr. 1E, 521.
- Hull, M.E. (1947). *J. Dairy Sci.* 30, 881.
- Jacquet, J., Villette, O. & Richou, R. (1956). *Chem. Abstr.* 51, 548g.
- Jago, G.R. (1962). *Aust. J. Dairy Technol.* 17, 83.
- Jespersen, N.J.T. (1966). XVII Int. Dairy Congr. E, 465.
- King, D.H. & Koburger, J.A. (1969). *J. Dairy Sci.* 52, 1715.
- Kishonti, E. & Sjöström, G. (1970). XVIII Int. Dairy Congr. 1E, 501.
- Klimovsky, I., Zvyagintsev, V., Gudkov, A. & Medvedeva, Z. (1970). XVIII Int. Dairy Congr. 1E, 119.
- Kosikowski, T.V. & Vocquet, G. (1958). F.A.O. agric. Stud. No. 38, 141.
- Kristoffersen, T. (1967). *J. Dairy Sci.* 50, 956.
- Kristoffersen, T. & Gould, I.A. (1960). *J. Dairy Sci.* 43, 1202.
- Lawrence, R.C. (1963). *J. Dairy Res.* 30, 225.
- Lawrence, R.C. (1967). *Dairy Sci. Abstr.* 29, 59.
- Lawrence, R.C. & Gilles, J. (1969). *N.Z. J. Dairy Technol.* 4, 189.
- Lawrence, R.C. & Gilles, J. (1970). *N.Z. J. Dairy Sci. Technol.* 6, 30.

- Lawrence, R.C. & Pearce, L.E. (1968). N.Z. J Dairy Technol. 2, 137.
- Lawrence, R.C. & Pearce, L.E. (1972). N.Z. J Dairy Sci. Technol. (In press).
- Lawrence, R.C. & Sanderson, W.B. (1969). J. Dairy Res. 36, 21.
- Ledford, R.A., O'Sullivan, A.C. & Nath, K.R. (1966). J. Dairy Sci. 49, 1098.
- Lightbody, L.G. (1961). Qd J. agric. Sci. 19, 109.
- Lowrie, R.J. & Pearce, L.E. (1971). N.Z. J Dairy Sci. Technol. (In press).
- Mabbitt, L.A. (1955). J. Dairy Res. 22, 224.
- Mabbitt, L.A. (1961). J. Dairy Res. 28, 303.
- Mabbitt, L.A., Chapman, H.R. & Sharpe, M.E. (1959). J. Dairy Res. 26, 105.
- Mabbitt, L.A. & Zielinska, M. (1956). XVI Int. Dairy Congr. 2(2), 323.
- Malveaux, F.J. & San Clemente, C.L. (1969). J. Bact. 97, 1209.
- Marandon, J.L. & Oeding, P. (1966). Acta. pathol. microbiol. scand. 67, 149.
- Matoba, T., Hayashi, R. & Hata, T. (1970). Agric. biol. Chem. 34, 1235.
- McGugan, W.A., Fowsam, S.G., Elliott, J.A., Emmons, D.B., Reiter, B. & Sharpe, M.E. (1968). J. Dairy Res. 35, 237.
- McDonald, I.J. (1964). J. Dairy Res. 31, 147.
- Miller, I. & Kandler, O. (1967). Milchwissenschaft 22, 608.
- Moore, S. & Stein, W.H. (1963). In "Methods in Enzymology" Vol. VI, p. 819. Eds. Colowick, S.P. & Kaplan, N.O. New York: Academic Press.

- Morgan, M.E. & Nelson, F.E. (1951). *J. Dairy Sci.* 34, 446.
- Morris, C.S. & Edwards, M.A. (1947). *Nature, Lond.* 160, 434.
- Mulder, H. (1952). *Neth. Milk Dairy J.* 6, 157.
- Naylor, J. & Sharpe, M.E. (1958a). *J. Dairy Res.* 25, 92.
- Naylor, J. & Sharpe, M.E. (1958b). *J. Dairy Res.* 25, 431.
- Ohmiya, K. & Sato, Y. (1970). *Agric. biol. Chem.* 34, 1463.
- Orla-Jensen, S. (1942). "The Lactic Acid Bacteria," 2nd ed. Copenhagen.
- Pearce, I.E. & Limsowtin, G. (1969). New Zealand Dairy Research Institute, 41st Annual Report, p. 36. Palmerston North, New Zealand.
- Peebles, M.M., Gilliland, S.E. & Speck, M.L. (1969). *Appl. Microbiol.* 17, 805..
- Perry, K.D. (1961). *J. Dairy Res.* 28, 221.
- Perry, K.D. & McGillivray, W.A. (1964). *J. Dairy Res.* 31, 155.
- Peterson, M.H., Johnson, M.J. & Price, W.V. (1948). *J. Dairy Sci.* 31, 55.
- Postgate, J.R. (1969). In "Methods in Microbiology" Vol. 1. Eds. Norris, J.R. & Ribbons, D.W. New York: Academic Press.
- Raadsveld, C.W. (1953). *XV Int. Dairy Congr.* 2, 676.
- Reiter, B., Fryer, T.F., Pickering, A., Chapman, H.R., Lawrence R.C. & Sharpe, M.E. (1967). *J. Dairy Res.* 34, 257.
- Reiter, B., Fryer, T.F., Sharpe, M.E. & Lawrence, R.C. (1966). *J. appl. Bact.* 29, 231.
- Reiter, B., Sorokin, Y., Pickering, A. & Hall, A.J. (1969). *J. Dairy Res.* 36, 65.



- Richardson, B.C. & Creamer, L.K. (1970). N.Z. J Dairy Sci. Technol. 5, 166.
- Robertson, P.S. (1960a). J. Dairy Res. 27, 1.
- Robertson, P.S. (1960b). J. Dairy Res. 27, 161.
- Robertson, P.S. (1964). J. Dairy Res. 31, 297.
- Robertson, P.S. (1966). Dairy Inds 31, 305.
- Robertson, P.S. & Perry, K.D. (1961). J. Dairy Res. 28, 245.
- Rowlands, S.J. (1938). J. Dairy Res. 2, 42.
- Sandvik, O. & Fossum, K. (1965). Am. J. vet. Res. 26, 357.
- Sasaki, R. & Nakae, T. (1959). Dairy Sci. Abstr. 22, 739.
- Sato, Y. & Ohniya, K. (1966). Agric. biol. Chem. 30, 731.
- Schormüller, J. (1968). Adv. Fd Res. 16, 231.
- Sellars, A. (1969). N.Z. J Agric. 112(4), 106.
- Shahani, K.M. (1966). J. Dairy Sci. 49, 907.
- Sharpe, M.E. & Franklin, J.G. (1962). VIIIth Int. Congr. Microbiol. Abstracts, p. 46.
- Sharpe, M.E. & Fryer, T.F. (1965). Lab. Pract. 14, 697.
- Sherwood, I.R. (1939). J. Dairy Res. 10, 426.
- Sherwood, I.R. & Whitehead, H.R. (1934). J. Dairy Res. 5, 208.
- Silverman, G.J. & Kosikowski, F.V. (1955). J. Dairy Sci. 38, 950.
- Slade, H.D. & Slamp, W.C. (1956). J. Bact. 71, 624.
- Smith, K.L. (1965). J. Dairy Sci. 48, 741.
- Stadhouders, J. (1962). XVI Int. Dairy Congr. B, 353.

- Stadhouders, J., de Vries, E. & Mulder, H. (1959). XV Int. Dairy Congr. 2, 709.
- Sullivan, J.J. & Jago, G.R. (1970a). Aust. J. Dairy Technol. 25, 111.
- Sullivan, J.J. & Jago, G.R. (1970b). Aust. J. Dairy Technol. 25, 141.
- Suzuki, S.K., Hastings, E.C. & Hart, E.B. (1909-10). J. biol. Chem. 7, 431.
- Swenczuk, A. & Malozzyk, M. (1969). Eur. J. Biochem. 9, 63.
- Taylor, M.K. (1967). Dairy Inds 32, 278.
- Thatcher, F.S. & Clark, D.S. (1968). "Microorganisms in Foods: Their Significance and Methods of Enumeration." University of Toronto Press.
- Thomas, S.B., Bruce, R.G., Peters, G.J. & Griffiths, D.G. (1967). J. appl. Bact. 30, 255.
- Thomas, T.D. & Batt, R.D. (1969). J. gen. Microbiol. 50, 367.
- Tittsler, R.P., Sanders, G.P., Lochry, H.R. & Sager, O.S. (1948). J. Dairy Sci. 31, 716.
- Torriani, A. (1960). Biochim. Biophys. Acta 38, 460.
- Tourneur, C. (1970). XVIII Int. Dairy Congr. 1E, 138.
- Twomey, A. & Crawley, W.E. (1969). N.Z. J. Dairy Technol. 4, 185.
- Tuckey, S.L. & Ruehe, H.A. (1940). J. Dairy Sci. 23, 517.
- Vadehra, D.V. & Boyd, J.C. (1963). J. Dairy Sci. 46, 599.
- Valkeris, D.G. & Price, W.V. (1959). J. Dairy Sci. 42, 264.
- van der Zant, W.C. & Nelson, F.E. (1953). J. Dairy Sci. 36, 1104.
- van der Zant, W.C. & Nelson, F.E. (1954). J. Dairy Sci. 37, 795.

- van Slyke, L.L. & Hart, E.B. (1902). N.Y. (Geneva) Agr. Expt. Sta., Bull. 226.
- Vedamuthu, E.R., Sandine, W.E. & Elliker, P.R. (1966). J. Dairy Sci. 42, 151.
- Wallmark, G. & Finland, M. (1961). Proc. Soc. exp. Biol. Med. 106, 73.
- Whitehead, H.R. & Bush, E.J. (1957). J. Dairy Res. 24, 381.
- Williamson, W.T., Tove, S.R. & Speck, M.F. (1964). J. Bact. 87, 49.
- Willis, A.T. & Hobbs, G. (1959). J. Path. Bact. 77, 511.
- Wilson, H.L., Hall, S.A. & Rogers, L.A. (1945). J. Dairy Sci. 28, 187.
- Witter, L.D. (1961). J. Dairy Sci. 44, 983.
- Yamamoto, T. & Yoshitake, M. (1962). XVI Int. Dairy Congr. B, 395.
- Zaadhof, K.-J. & Terplan, G. (1967). Arch. Lebensmittelhyg. 18, 27 (via Dairy Sci. Abstr. 30, 978).

## A P P E N D I X I

AN IMPROVED MEDIUM FOR THE DETECTION OF  
PROTEOLYTIC ORGANISMS IN TOTAL COUNTS

SUMMARY An improved medium for the detection of proteolytic organisms has been developed by the addition of caseinate (1% w/v), citrate (0.015 M) and calcium ions (0.02 M) to Standard Methods Agar. Its greater sensitivity compared with existing milk agar media is related to its ability to detect the first stage of casein breakdown as shown by the formation of a white zone of precipitation. This results from the deposition of insoluble para-caseins, mainly para-K-casein, which is readily detected in the transparent medium. The extent of proteolysis exhibited by an organism is reflected both by the size and type of precipitation zone forming round the colony. No significant difference was found between total counts of raw milk samples measured on the new medium and Standard Methods Agar. The new medium can thus be used for the simultaneous determination of proteolytic and total bacterial counts.

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INTRODUCTION

Most investigators have come to the conclusion that the hydrolysis of casein in an opaque "milk agar" is the most suitable indicator of proteolytic organisms (Druce & Thomas, 1959; Willis & Hobbs, 1959; Brown, Sandvik, Scherer & Rose, 1967; Zaadhof & Terplan, 1967). However, it is apparent from the many attempts that have been made to improve the existing methods that a satisfactory medium has yet to be devised.

The American Public Health Association (1967) recommends the addition of 10% (v/v) of sterile skim milk to Standard Methods Agar, yet it has long been known that acid-forming bacteria can produce zones of clearing on such media. Two surveys (Frazier & Rupp, 1928; Lightbody, 1961) showed that 37 to 45% of the zones of clearing on milk agar were not due to proteolysis. The most serious disadvantage, therefore, associated with the milk agar method is the need to flood the medium with a protein precipitant to confirm that zones of clearing are due to proteolysis and not caused by acid formed by the organisms as a result of sugar fermentation. In an attempt to overcome some of these difficulties, Frazier & Rupp (1928) devised an opaque carbohydrate-free casein agar medium to eliminate clearing due to acid production. The limitation of this medium for organisms not requiring carbohydrate has prevented its general adoption.

Lawrence & Sanderson (1969) recently described a caseinate agar slide micro-technique for the quantitative estimation of proteinases. A small well cut in the caseinate agar is filled with the enzyme solution which then diffuses outwards, breaking down the caseinate to form a white precipitate which consists initially mainly of para-K-casein, together with small amounts of high molecular weight degradation products of  $\alpha_{S1}$ - and  $\beta$ -caseins. With highly active proteinases, subsequent breakdown of the white precipitate may occur to yield soluble fractions, with the formation of a white ring of precipitation and an inner clear area surrounding the well. The present work describes the adaptation of this principle to an agar medium for the simultaneous determination of both total and proteolytic counts of milk samples.

It is more sensitive than the milk agar method and has the further advantage that, as the addition of a protein precipitant is unnecessary, colonies can be picked off for further investigations.

#### MATERIALS AND METHODS

Standard methods caseinate agar. Standard Methods Agar (Baltimore Biological Laboratory Inc., Baltimore, Md., U.S.A.) was selected as a suitable general purpose, basal medium. Sodium caseinate (commercial grade, New Zealand Dairy Board, Wellington, New Zealand) was added as the substrate for proteolysis, sodium citrate as a buffer to prevent acid-forming organisms from precipitating casein at pH values  $< 5.0$ , and  $\text{Ca}^{++}$  to ensure the precipitation of the insoluble para-caseins resulting from proteolysis. The composition of the medium (g/l) was: Standard Methods Agar (SMA), 23.5 (pancreatic digest of casein, 5.0; yeast extract, 2.5; glucose, 1.0; agar, 15.0); sodium caseinate, 10.0; hydrated trisodium citrate, 4.41;  $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ , 4.38. The sodium caseinate was dispersed in half the 0.015 M sodium citrate solution using a Polytron blender (Kinematica GmbH., Lucerne, Switzerland) and added to the SMA, which had been rehydrated in the balance of the sodium citrate solution, before autoclaving at  $121^\circ\text{C}$  for 15 min. Finally 20 ml of sterile  $\text{CaCl}_2$  (1.0 M) solution was added to the molten agar. The complete medium (pH 6.6), Standard Methods caseinate Agar (SMCA), was mixed thoroughly by gentle agitation before being dispensed in 12 ml amounts in flat-bottomed, 8.7 cm diam Petri dishes to form a layer 2 mm thick which became transparent on cooling and solidifying. The plates were held at  $30^\circ\text{C}$  for 48 hr and stored at  $5^\circ\text{C}$  until required.

Plate counts. (a) Proteolytic organisms. Triplicate 0.1 ml samples of 10-fold dilutions in quarter strength Ringer's solution of the milk samples were spread on the surfaces of SMCA plates with sterile glass spreaders. The plates were allowed to dry for c. 15 min.

(b) Total and proteolytic counts were made by surface plating on 10% milk agar (MA) prepared according to the American Public Health Association (1967). Plates were poured, dried and inoculated as for SMCA.

(c) Total counts. SMA was used in the pour plate method (American Public Health Association, 1967) and the surface plate method. In the pour plate method, 12 ml of SMA was melted and cooled to 45°C before pouring. For surface plate counting, 12 ml were poured in Petri dishes and dried as for SMCA.

All plates were examined after 24, 48 and 72 hr at 30°C.

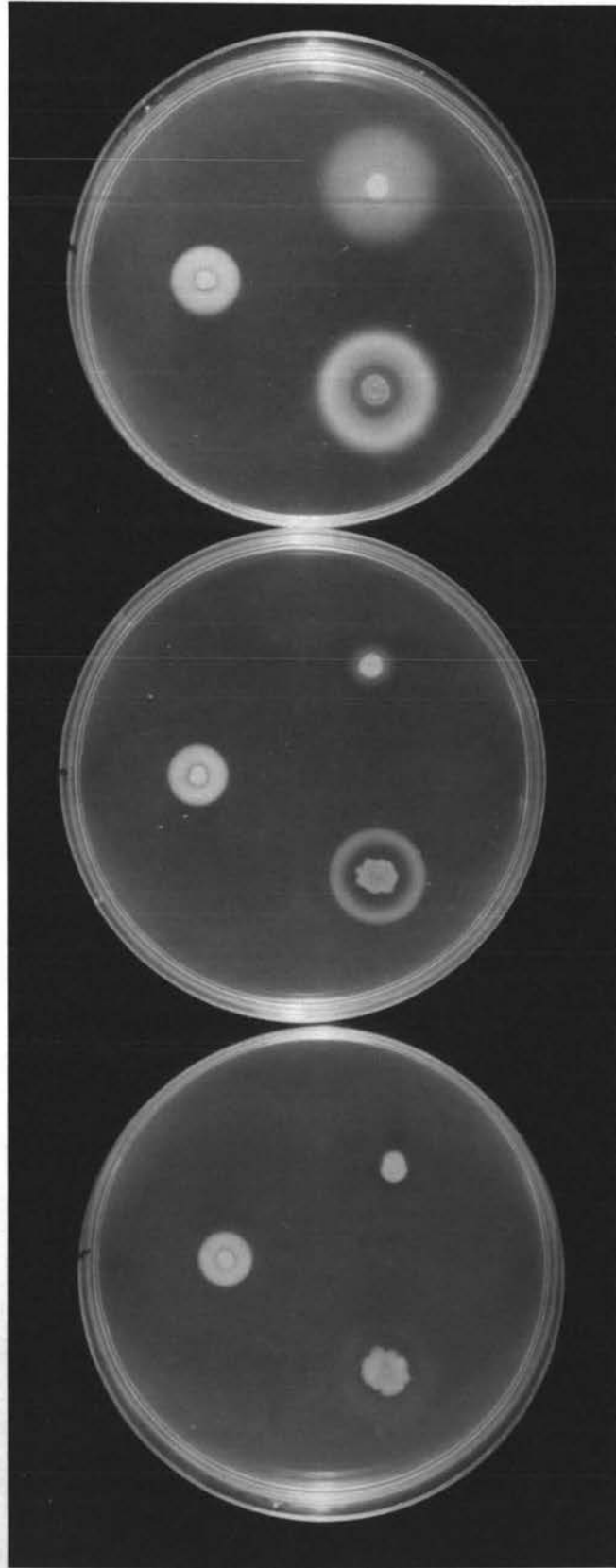
Interpretation of zones. Only organisms which formed white or off-white precipitates around the colony on SMCA were considered to be proteolytic. MA plates were flooded with acidified mercuric chloride solution to check that zones of clearing were due to proteolysis. Only those zones that remained clear were read as positive for proteolysis (Harrigan & McCance, 1966; American Public Health Association, 1967). On some occasions after flooding plates with acid HgCl<sub>2</sub> solution, zones that were initially clear became opaque but not as dense as the background (Table I). The difficulty of interpreting whether such zones are the result of proteolysis has been emphasized by Lightbody (1961).

Effect of calcium ion concentration. The precipitation of the white zone of para-caseins was in general dependent on  $\text{Ca}^{++}$  ion concentration. This was illustrated (Fig. 1) by Pseudomonas fragi (NCDC 752), Micrococcus freudenreichii (NCDC 1223) and Serratia marcescens grown at  $30^{\circ}\text{C}$  for 48 hr on SMCA without added  $\text{Ca}^{++}$ , and with the cation added at levels of 0.015 M and 0.02 M. M. freudenreichii formed a distinct white precipitate in the absence of added  $\text{Ca}^{++}$ , S. marcescens and Ps. fragi required  $\text{Ca}^{++}$  at concentrations of 0.015 and 0.02 M, respectively, before the para-caseins were precipitated. Increasing the  $\text{Ca}^{++}$  concentration generally resulted in zones of precipitation becoming larger and more intense, but at concentrations  $>0.025$  M the medium tended to become opaque. Flooding the caseinate agar medium containing no added  $\text{Ca}^{++}$  with a 0.1 solution of  $\text{CaCl}_2$  resulted in the immediate precipitation around the colony of a white zone of a size and type comparable to that formed on caseinate agar containing at the outset 0.02 M  $\text{CaCl}_2$ . This demonstrated that the effect of additional  $\text{Ca}^{++}$  was to precipitate the para-caseins formed as a result of proteolysis and not to increase the proteolytic activity of the organisms. The optimum amount of  $\text{Ca}^{++}$  required in SMCA for routine use varied slightly with each batch of caseinate, and needed to be predetermined.

Effect of temperature. SMCA could be incubated at temperatures up to  $45^{\circ}\text{C}$ . At higher temperatures the medium became opaque presumably as a result of the decreased solubility of calcium salts.



Fig. 1. Influence of  $\text{Ca}^{++}$  on the precipitation of paracaseins. On each plate of SMCA, Micrococcus freudenreichii is at the top with Serratia marcescens lower left, and Pseudomonas fragi lower right. The medium contained: (A) no additional  $\text{Ca}^{++}$ ; (B) 0.015 M  $\text{Ca}^{++}$ ; (C) 0.02 M  $\text{Ca}^{++}$ . Incubation, 48 hr/30°C.



C

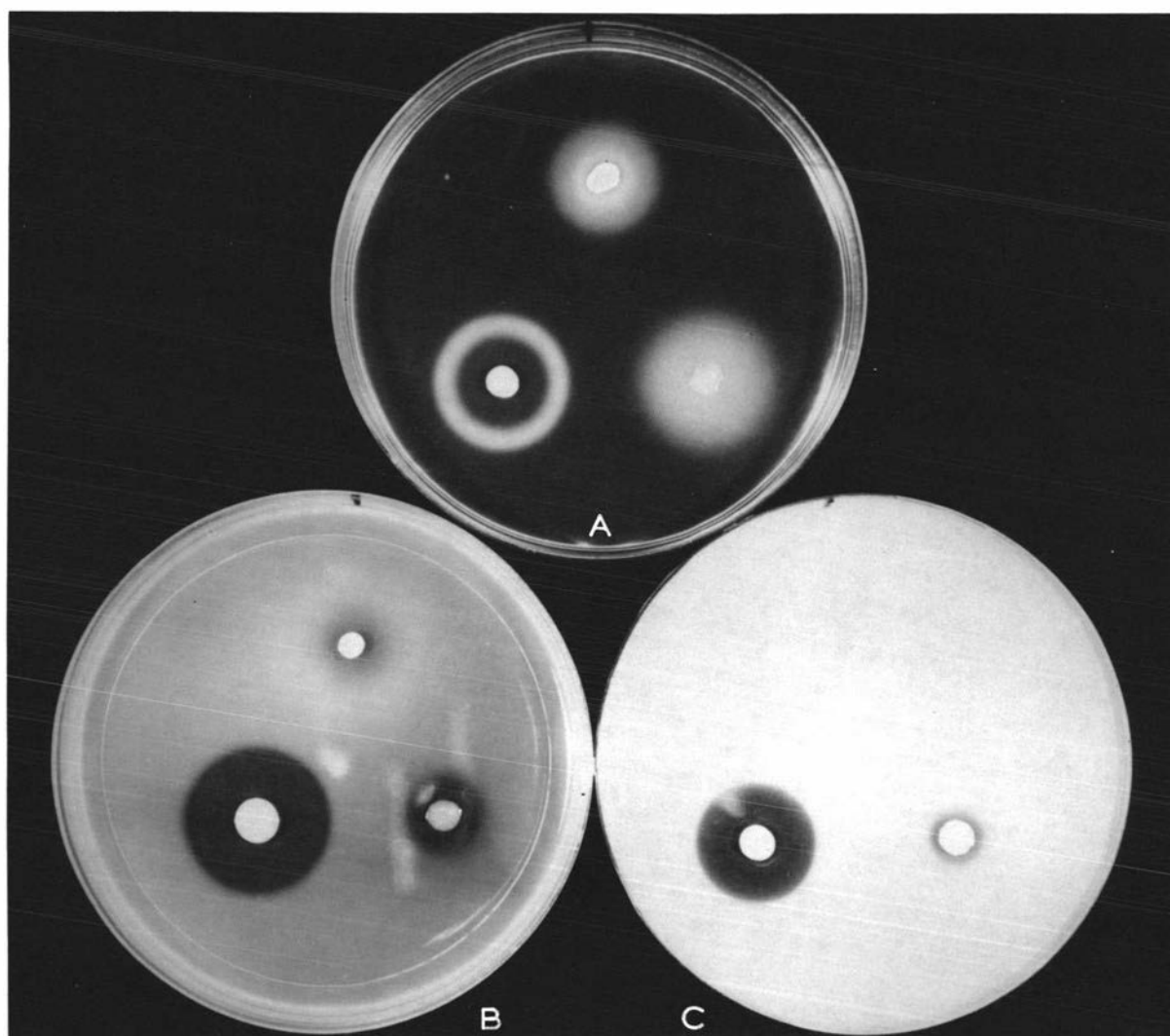
B

A

Proteolytic reactions of known organisms. Laboratory stock cultures were individually spot-inoculated with a platinum wire from Trypticase Soy Broth (BBL) cultures on SMCA and MA. Figure 2 illustrates the changes in SMCA due to the proteolytic action of Sarcina lutea, Staphylococcus aureus and Ps. fragi, and the effect that these organisms had on MA before and after flooding with acid  $HgCl_2$  solution. The zone of clearing that formed within the ring of precipitation around the colony of Sarc. lutea on SMCA indicated that extensive digestion of the caseinate and para-caseinate had taken place. The strongly proteolytic nature of this organism was confirmed on MA where the clear zone was not lost when flooded with acid  $HgCl_2$  solution. Staph. aureus caused a slight clearing on MA but flooding with acid  $HgCl_2$  solution resulted in the disappearance of the clear zone. On SMCA, however, a definite white zone of precipitation was formed and it had a characteristic intense inner region of precipitation adjoining the colony. Ps. fragi showed considerable proteolytic activity but, like Staph. aureus, the para-caseins were not further degraded to give an inner clear area. Nevertheless Ps. fragi was considered more active than Staph. aureus because it produced larger zones of precipitation. However, although the zone of precipitation formed by Sarc. lutea was slightly smaller than that formed by Ps. fragi, the inner clear zone of the former showed that more extensive degradation of the para-caseins had taken place.

Even weakly proteolytic organisms such as the lactic streptococci could form small white zones on SMCA. Three out of 30 strains of cheese "starter" streptococci gave zones after 3 days, and a further 6 strains after 10 days, at 30°C.

Fig. 2. Zones due to proteolysis formed on (A) SMCA by Staphylococcus aureus (above), Sarcina lutea (lower left) and Pseudomonas fragi (lower right), and the effect of these organisms on Milk Agar (B) before, and (C) after flooding with acidified  $\text{HgCl}_2$  solution. Incubation, 60 hr/30°C.



Proteolytic reactions of isolates from raw and pasteurized

milks. Pure cultures of organisms isolated from milk were inoculated on SMCA and MA. SMCA was more sensitive than MA for demonstrating the proteolytic activity of organisms from raw and pasteurized milks (Table I). The raw milk contained a higher proportion of relatively fast growing mesophiles that exhibited more rapid and extensive proteolytic activity on SMCA than did those from pasteurized milk (Table II). A large proportion of the latter formed only small white zones on SMCA. A high proportion of the psychrotrophs from raw milk grew rapidly at 30°C and displayed extensive proteolytic activity, 27 of the 60 isolates forming clear zones not precipitated by acid HgCl<sub>2</sub> on MA (Table I). On SMCA, 21 of the 27 cleared the central part of the white precipitate. The remaining 6 resembled *Ps. fragi* in forming uniform zones of precipitation on SMCA. Clear zones on MA that became opaque again either completely or partially on the addition of acid HgCl<sub>2</sub> solution were formed by 129 organisms (Table I). Of these, 23 of the 56 raw milk mesophiles and 59 of the 71 pasteurized milk mesophiles, but neither of the two psychrotrophs, caused precipitation in SMCA at 30°C. All of the 36 isolates which were confirmed proteolytic on MA, and all but one of the 53 isolates that were entirely unreactive on MA were confirmed proteolytic on SMCA. The results obtained at 22°C were essentially the same as those described above, although some organisms took > 3 days to form zones of precipitation on SMCA.

At 5°C, 55 of the 60 psychrotrophs had formed zones of precipitation on SMCA by 10 days. The zones at this temperature

Table I. Reactions on Milk Agar and Standard Methods Caseinate Agar<sup>1</sup>  
of mesophiles and psychrotrophs isolated from milk.

Class of isolates <sup>2</sup>	No. examined	Reactions on MA			No. giving zones on SMCA <sup>6</sup>	
		none <sup>3</sup>	Clear zone reprecipitated by HgCl <sub>2</sub> <sup>2-5</sup>			
			completely <sup>3</sup>	partially <sup>4</sup>	unaffected <sup>5</sup>	
Raw milk mesophiles	78	16	45	11	6	45
Pasteurized milk mesophiles	80	6	57	14	3	67
Raw milk psychrotrophs	60	31	1	1	27	58

<sup>1</sup>After 72 hr incubation at 30°C; <sup>2</sup>random isolates obtained from plate counting, raw milk (total count  $5 \times 10^5$  organisms/ml) and the same sample of milk after pasteurization at 71°C/15 sec (total count  $3 \times 10^4$  organisms/ml); <sup>3</sup>nonproteolytic by standard interpretation; <sup>4</sup>doubtful proteolytic reaction (Lightbody, 1961); <sup>5</sup>confirmed proteolytic by standard interpretation; <sup>6</sup>confirmed proteolytic (see text).

Table II. Rate at which zones of precipitation were formed at 30°C on Standard Methods Caseinate Agar by proteolytic isolates\* from raw and pasteurized milks.

Class of isolates	No. (and %) of isolates with zone formed within		
	24 hr	48 hr	72 hr
Raw milk mesophiles	27 (60%)	43 (95%)	45 (100%)
Pasteurized milk mesophiles	7 (10%)	49 (73%)	67 (100%)
Raw milk psychrotrophs	54 (93%)	58 (100%)	58 (100%)

\* From Table I.

Table III. Comparison of counts on 15 raw milk samples.

Medium	SMCA	SMA	SMA
Counting method	SP	SP	PP
Mean count ( $\times 10^{-5}$ )/ml	1.117	1.114	1.082
S. E.	$\pm 0.04$	$\pm 0.04$	$\pm 0.04$

No significant difference between means,  $P > 0.2$ .

PP, pour plating; SP, spread plating.



tended to be rather faint but holding at 37°C for 1 hr intensified the precipitates. In contrast, only 26 of the 60 psychrotrophs gave confirmed clear zones on MA at 5°C.

Suitability of SMCA for total plate counts and the simultaneous enumeration of proteolytic organisms. The results obtained from surface plate counting with SMCA of 15 raw milk samples received at the Institute during 1968-69 were compared with those obtained on SMA, and those in pour plate counts on SMA (Table III). The correlation of counts between the two techniques and between the two media were good, the correlation coefficients in each case being 0.98.

The suitability of SMCA in enumerating both the total and proteolytic microorganisms in milk samples was limited by the density of the colonies on the plate. Up to 80 colonies/plate could be read for proteolytic and nonproteolytic organisms (Fig. 3). Merging of the zones of precipitation made it difficult to count plates containing more organisms, especially after incubation for 72 hr. When determining the incidence of proteolytic organisms in crowded plates, it was found useful to do an initial count at 48 hr, with slower developing proteolytic colonies, again after a further period of incubation.

The recognition of zones of proteolysis on SMCA was much easier than on MA plates. A comparison of these two media when used to determine the total and proteolytic counts of 10 raw milk samples confirmed that SMCA detected a much greater proportion of proteolytic organisms in the total count than MA (Table IV).

Fig. 3. Proteolytic (P) and nonproteolytic (NP) colonies clearly differentiated on a SMCA plate used to determine the total and proteolytic count of raw milk. Incubation, 48 hr/30°C.

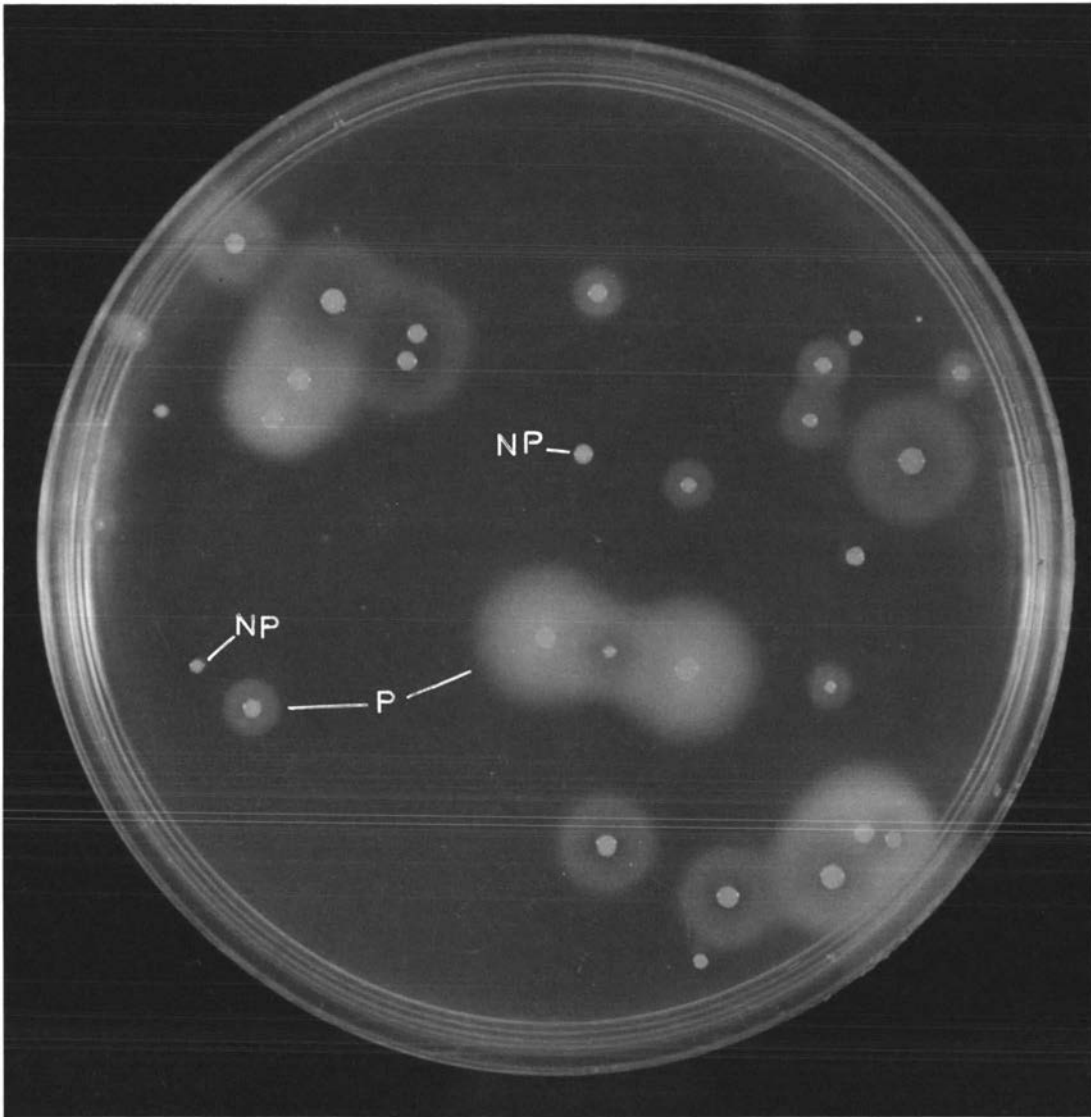


Table IV. Proportion (%) of confirmed proteolytic organisms of the total counts of raw milk samples as determined by plating on Standard Methods Caseinate Agar and Milk Agar\*.

Sample No.	% of proteolytic organisms in milk on	
	SMCA	MA
1	59	5
2	63	11
3	49	3
4	20	2
5	68	12
6	26	5
7	65	20
8	29	5
9	21	7
10	35	5

\* Incubation, 30°C/72 hr.

DISCUSSION

The criticisms by Lightbody (1961) of the use of MA as an indicator of proteolysis have been substantiated in this investigation, particularly with regard to the difficulty in the interpretation of clear zones which become only partially opaque when flooded with acidified  $HgCl_2$  solution. The buffered SMCA medium described here affords a much more sensitive and reliable test for proteolysis than do milk agars. Its greater sensitivity in determining the proteolytic ability of organisms is a direct consequence of its ability to detect the very first stage in casein breakdown, the formation of the white precipitate of para-caseins. Organisms which are extensively proteolytic can further break down the precipitate nearest to the colony to soluble components with the formation of an inner transparent zone and an outer white ring. The clear zones on MA, on the other hand, reflect only this more complete breakdown to the soluble components and the first stage of proteolysis cannot be detected against the opaque background. It was not surprising, therefore, that many of the less proteolytic organisms could be detected on SMCA but not on MA. The presence of large numbers of weakly proteolytic organisms would obviously be important in the quality of products such as cheese, milk powders and casein which must sometimes be stored for long periods.

SMCA also has the advantage of being free of the false-positive zones of the milk agars caused by the fermentation of lactose, as it is well buffered against the maximum amount of acid that could be formed from the glucose it contains. There was no significant difference found between the total bacterial

counts of raw milk samples obtained on SMA and SMCA. The level of citrate (0.015 M) in SMCA is well below the 10 mg/ml which McDonald (1957) found to cause inhibition of the caseolytic activity of lactic streptococci.

The difference in patterns of the white zones obtained on SMCA with different microbial proteinases appears to be due mainly to the relative sensitivity to precipitation by calcium ions of the high molecular weight products formed in the initial stages of the caseinate breakdown. This is in agreement with the observations of Lawrence & Sanderson (1969). The relative areas of the outer white and inner transparent zones give an indication of the ability of the proteinase not only to carry out the initial breakdown of the caseins to para-caseins but also to further degrade these products. The pattern of zones obtained on SMCA appears to have considerable potential as a means of differentiating species and strains of staphylococci (Appendix II). This is of particular interest in view of the recent attempts to use proteolytic activity as an aid to taxonomy (Sanvik & Fossum, 1965; Brown *et al.*, 1967).

Taylor (1967) proposed a method of assessing raw milk quality by counting the bacteria growing in a thin film of milk sample adhering to a plain water-agar base. The crowding of colonies on the plates which occurs even in the case of comparatively good quality milk samples, makes it difficult, in our experience, to distinguish the three types of zones formed by proteolytic, acid-proteolytic and acid-forming organisms. However, the technique of inoculating plates by flooding with a

milk sample and decanting the excess as described by Taylor can be satisfactorily applied to SMCA plates (Appendix III), and it gives a good indication of milk quality in terms of the proportion of proteolytic organisms of the total flora.

#### REFERENCES

- American Public Health Association (1967). "Standard Methods for the Examination of Dairy Products", 12th ed. New York: American Public Health Association.
- Brown, R.W., Sandvik, O., Scherer, R.K. & Rose, D.L. (1967). *J. gen. Microbiol.* 47, 273.
- Druce, R.G. & Thomas, S.B. (1959). *J. appl. Bact.* 22, 52.
- Frazier, W.C. & Rupp, P. (1928). *J. Bact.* 16, 65.
- Harrigan, W.F. & McCance, M.E. (1966). "Laboratory Methods in Microbiology". London: Academic Press.
- Lawrence, R.C. & Sanderson, W.B. (1969). *J. Dairy Res.* 36, 21.
- Lightbody, L.G. (1961). *Qd. J. agric. Sci.* 18, 109.
- McDonald, I.J. (1957). *Can. J. Microbiol.* 3, 411.
- Sandvik, O. & Fossum, K. (1965). *Am. J. vet. Res.* 26, 357.
- Taylor, M.M. (1967). *Dairy Inds* 32, 278.
- Willis, A.T. & Hobbs, G. (1959). *J. Path. Bact.* 77, 511.
- Zaadhof, K.-J. & Terplan, G. (1967). *Arch. Lebensmittelhyg.* 18, 27 (via *Dairy Sci. Abstr.* 30, No. 978).

## A P P E N D I X II

THE TYPING OF COAGULASE-POSITIVE STAPHYLOCOCCI BY  
PROTEOLYTIC ACTIVITY ON CASEINATE-AGAR, WITH  
SPECIAL REFERENCE TO PHAGE NONTYPABLE STRAINS

SUMMARY The specificities of the proteinases produced by 136 strains of coagulase-positive staphylococci on a buffered caseinate-agar medium at 37°C were investigated. Five well-defined groups of staphylococci could be differentiated by the patterns of precipitation zones obtained as a result of proteolysis. Within these five groups, slight variations in zone types were observed. The type of zone produced by any one strain was, however, highly reproducible, suggesting that the proteinases produced by staphylococci are strain-specific. Strains could be further differentiated by the patterns of precipitation obtained at 30°C. The differences in zone types on caseinate-agar can be used as an adjunct to standard typing procedures, being particularly valuable in epidemiological investigations and in the differentiation of phage "nontypable" strains. In this survey, 63 of the strains investigated could not be typed by the international set of typing phages but could be readily differentiated in terms of their proteolytic activities at 37 and 30°C. No correlation was found between proteolytic activity, phage type, origin of the strains or the production of enterotoxins A, B or C.

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INTRODUCTION

Epidemiological studies of staphylococci require methods for identifying and associating strains. The principal method used is phage typing, although as many as 15 to 30% of isolates may be nontypable by phage at routine test dilutions. In certain



environments, still larger percentages of phage nontypable strains have been reported (Cohen & Smith, 1964). Therefore, it is not surprising that attempts have been made to develop more comprehensive methods of differentiating strains of staphylococci and micrococci by such means as the serological typing of their proteolytic enzymes (Brown, Sandvik, Scherer & Rose, 1967; Sandvik & Fossum, 1965). After the finding that the pattern of precipitation zone(s) obtained on buffered caseinate-agar medium appeared to be specific for a particular strain of organism (Appendix I), 136 strains of coagulase-positive staphylococci, including 63 phage nontypable strains, were examined on caseinate-agar to determine if they could be readily differentiated in this manner. The types of precipitation zones produced fell into five well-defined groups, and these have been compared with phage types, the ability of the strains to produce enterotoxins, and the source of the staphylococci.

#### MATERIALS AND METHODS

Strains. One hundred and thirty-six strains of coagulase-positive staphylococci comprising 83 clinical, 25 phage-propagating, 25 bovine isolates and 3 strains obtained from M.S. Bergdoll were maintained as slope cultures on Trypticase Soy Agar (BBL). Details of 89 of the strains have been reported previously (Jarvis & Lawrence, 1970), and the remaining 47 were phage nontypable strains of clinical origin.

Proteolytic activity. Standard methods caseinate agar (SMCA) plates were prepared as previously described (see Appendix I). Stock cultures of the strains were streaked onto Trypticase

Soy Agar and incubated at 37°C for 24 hr. By using a straight wire, a single colony of each strain was then picked off and pinpoint inoculated onto each of two SMCA plates. One plate was incubated at 37°C and the duplicate plate at 30°C. The size and type of zone formed due to proteolysis was examined after 48 and 72 hr of incubation.

Phage type and enterotoxigenicity. The strains were phage-typed by the method of Blair and Williams (1961). Eighty-nine representative strains were cultured by a cellophane-over-agar technique as previously described (Jarvis & Lawrence, 1970). The supernatants were tested for enterotoxins A, B and C using a microslide gel diffusion technique described by Zehren and Zehren (1968).

## RESULTS

Types and sizes of zones due to proteolysis. All the 136 strains of staphylococci studied grew well on SMCA and to approximately the same extent as judged by colony diameters. The types of precipitation zones produced by the strains after 48 hr at 37°C could be grouped, as shown in Table I, into four general groups (groups A to D correspond to Figs 1A-1D respectively). A further classification, group E (Fig. 1E), was necessary for 11 strains, 4 of which produced no proteolysis at all and 7 of which formed small zones so close to the colony as to be immeasurable.

Within each of groups A to D, the type of precipitation zone(s) varied slightly. The type of zone produced by any one strain, however, was highly reproducible. These zones result from the precipitation of para-caseins, mainly para-K-casein

Table I. Source and phage type of 136 strains of coagulase-positive staphylococci in relation to proteolytic activity measured as zone diameter and zone type on Standard Methods Caseinate Agar<sup>1</sup>.

Source	Phage group <sup>2</sup>	No. of strains with proteolytic activity <sup>3</sup>			No. of strains with zone types <sup>4</sup>				
		High	Medium	Low	A	B	C	D	E
Bovine	III	16	1	2	0	16	1	0	2
	UC	1	3	1	0	0	2	2	1
	NT	0	0	1	0	0	0	1	0
Clinical	I	4	0	0	0	2	1	1	0
	II	3	1	0	0	0	3	1	0
	III	1	2	0	0	1	1	1	0
	IV	3	3	0	0	1	2	3	0
	UC	1	4	0	0	0	4	1	0
	NT	30	15	16	4	20	13	20	4
Phage propagating	I	2	1	1	0	0	2	1	1
	II	4	1	0	0	1	2	2	0
	III	6	4	2	0	0	4	6	2
	IV	0	0	1	0	0	0	0	1
	UC	2	0	0	0	2	0	0	0
	Misc	1	0	0	0	0	0	1	0
M.S. Bergdoll	III	1	1	0	0	0	1	1	0
	NT	1	0	0	0	1	0	0	0

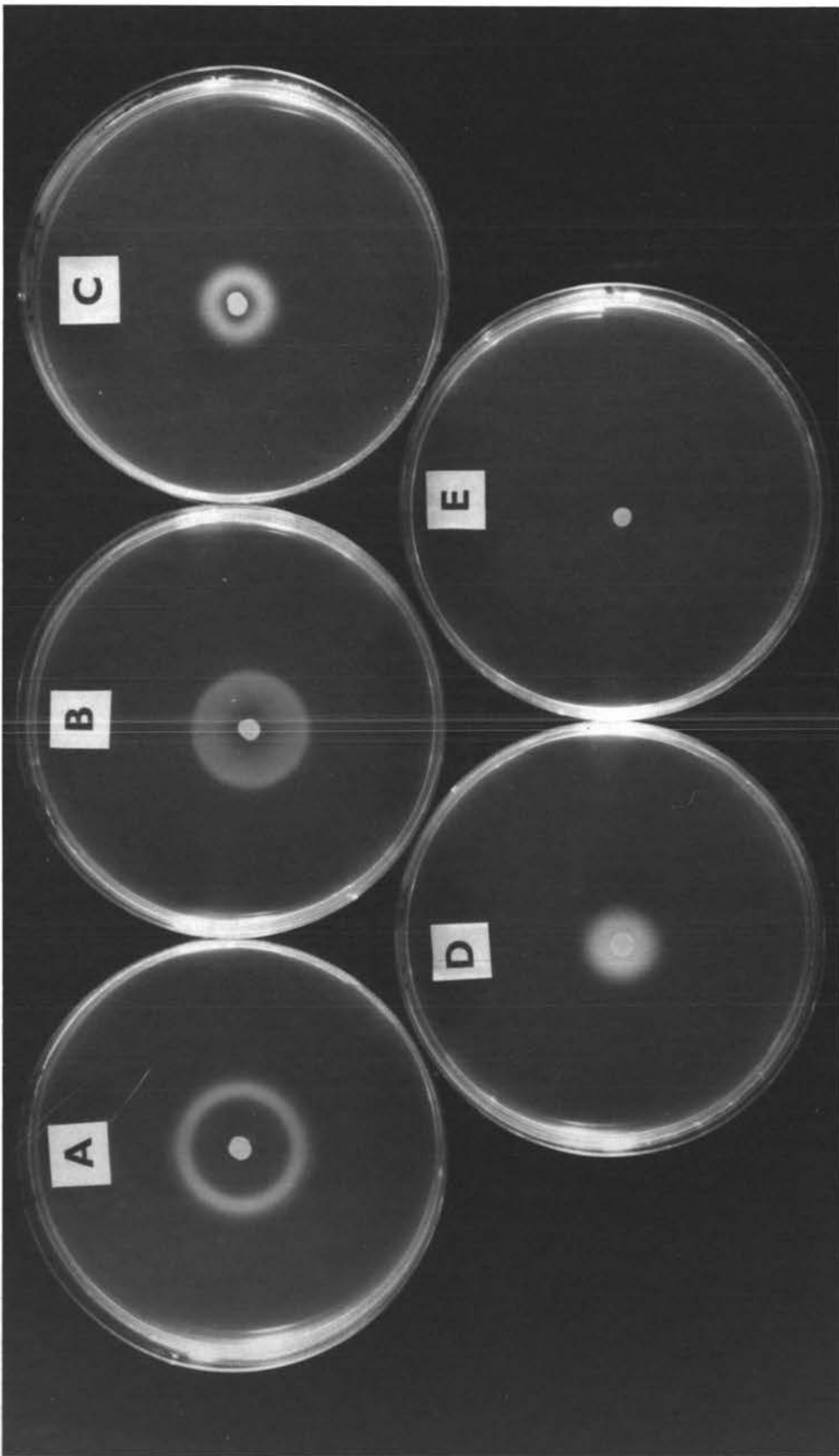
<sup>1</sup> Incubation was at 37°C for 48 hr.

<sup>2</sup> Abbreviations: UC, unclassifiable; NT, not phage typable.

<sup>3</sup> Diameter of zones: low activity, 0 to 9.9 mm; medium activity, 10 to 14.9 mm; high activity,  $\geq 15.0$  mm.

<sup>4</sup> See Fig. 1.

Fig. 1. Proteolytic zone types A to E formed on Standard Methods Caseinate Agar by different strains of coagulase-positive staphylococci. The plates were incubated at 37°C for 48 hr.



(Lawrence & Sanderson, 1969), and the differences in patterns of precipitation occur presumably because the para-caseins formed by different strains differ slightly both in their solubility in the presence of  $\text{Ca}^{++}$  ion and in their relative rates of further degradation (Lawrence & Creamer, 1969). This variation between strains in the same group was most marked for those in groups C and D.

The proteolytic activities of the strains were arbitrarily classified into three categories - low, medium and high - on the basis of the diameter of the zone of precipitation that formed round the colony after 48 hr at  $37^{\circ}\text{C}$  (Table I). This, however, does not take into account the specificity of some proteinases to break down further the initial precipitate of para-caseins to soluble components with the formation of an inner transparent zone and an outer white ring (Fig. 1A). Only the small number of strains (4 of 136) assigned to group A were active in this respect, however, and it is interesting to note that these strains were only weakly coagulase-positive. Sarcina lutea, a coagulase-negative micrococcus, has previously been reported to form a similar type of zone, but another coagulase-negative strain, Micrococcus freudenreichii, did not form a group A zone on SMCA (see Appendix I). The types of precipitation zones obtained were related in general to the diameters. The average diameters of the precipitation zones were 23.2, 20.5, 14.2 and 12.8 mm for groups A, B, C and D respectively, although there was considerable overlap.

Relationship between proteolytic activity and the source of the strains. Forty-two (51%) of the 83 clinical strains showed .

high proteolytic activity, 25 (30%) medium, and 16 (19%) low activity (Table I). Apart from 4 strains that were assigned to group A and another 4 to group E, the frequency with which the remaining 75 clinical strains were classified into groups B, C and D was approximately the same. There was thus no tendency for clinical strains to be related to any specific pattern of proteolytic activity, either in terms of the type or the diameter of the proteolytic zones. Furthermore, the phage types into which the clinical strains could be classified were not related to their proteolytic activity (Table I).

The phage-typable bovine strains examined tended to possess high proteolytic activity and to classify in group B (Table I). This tendency may be more apparent than real, however, since 13 of 17 highly proteolytic bovine strains and 13 of 16 group B strains were isolated from the same herd and were found to possess identical phage types. If these 13 bovine strains were identical, the above findings would emphasize how readily staphylococcal strains can be typed on caseinate-agar to trace a common origin.

Effect of temperature. While strains assigned to groups A and B at 37°C usually produced zones of a similar type at 30°C, strains from groups C and D, and to a lesser extent those from group E, usually produced zones of a different type. Group A and D strains generally formed larger zones at 37 than 30°C. About half of the group B strains formed only marginally larger zones at 37 than 30°C, the remainder producing approximately equivalent sized zones at both temperatures. Group C strains were more variable in their activities at 37 and 30°C, about half forming considerably bigger zones at 30°C. Growth was slow at 22°C and

zones of precipitation were faint, presumably because of the higher solubility of calcium salts at lower temperatures. However, the zones could be intensified by incubating the plates at 37°C for 1 hr.

It is apparent that comparison of the proteolytic activity of a strain at 37°C and its activity at 30°C affords a most valuable means of further differentiating strains, especially for those strains assigned to proteolytic groups C, D and E.

Effect of repeated subculture. To check that the type of zone due to proteolysis formed by a strain on SMCA was a stable feature, a single-colony isolate made from each of 12 strains was subcultured daily in Nutrient Broth at 30°C for 21 days. The cultures were inoculated onto SMCA plates on the first day, and subsequently every third day, and incubated at 30 and 37°C for 48 hr. No significant change was observed in the proteolytic activities of any of the strains, either in terms of the size or the type of zone produced.

Variation of proteolytic activity between colonies from a single strain. Stock cultures of 12 different strains were streaked on Trypticase Soy Agar and 10 colonies of each strain were isolated. Their proteolytic activities were determined by pinpoint inoculation onto SMCA plates and incubation at 30 and 37°C. The types of zones formed after 48 hr incubation by the 10 single-colony isolates of each strain were compared. No significant variation was detected within each group of isolates. This would indicate that proteinase activity is a comparatively stable characteristic of staphylococcal strains, in contrast to the reported inter-colony variations within a single strain in



such activities as hemolysin (Elek & Levy, 1954) and enterotoxin production (Sugiyama, Bergdoll & Dack, 1960).

Proteolytic activity and enterotoxin production. Neither the specificity of proteolytic action on SMCA as reflected by zone type nor the extent of proteolysis as indicated by the zone size could be correlated with the ability of 23 of 89 strains investigated to form enterotoxin A, B or C (Table II). If the enterotoxins formed by staphylococci are sensitive to the proteinase(s) also produced by the organisms, as described for a strain of Pseudomonas aeruginosa (Liu & Hsieh, 1969), one might expect that enterotoxigenicity would be associated with low proteolytic activity. Enterotoxigenic staphylococcal strains, on the contrary, exhibited a wide range of proteolytic activity as measured on SMCA. No tendency was found for enterotoxigenic strains to have low proteolytic activity or for non-enterotoxigenic strains to be highly proteolytic.

#### DISCUSSION

This investigation has shown that strains of coagulase-positive staphylococci can be readily differentiated into five well-defined groups by the type of proteolytic zones formed at 37°C on a buffered caseinate-agar medium. In epidemiological investigations, the routine purification of isolates on SMCA would give a presumptive differentiation of strains prior to standard phage typing or, alternatively, could be used as a means of further subdivision after phage typing. The finding that some strains forming similar zones at 37°C could often be distinguished by zone size or type at 30°C provides a further means of

Table II. Enterotoxigenicity of 89 strains of coagulase-positive staphylococci in relation to proteolytic activity measured as zone diameter and zone type on Standard Methods Caseinate Agar<sup>1</sup>.

Enterotoxins formed	No. of strains with proteolytic activity <sup>2</sup>			No. of strains with zone type <sup>3</sup>				
	High	Medium	Low	A	B	C	D	E
A	1	1	2	0	1	1	1	1
AB	3	1	0	0	0	3	1	0
B	6	3	1	0	2	5	3	0
BC	1	0	0	0	1	0	0	0
C	2	2	0	0	1	3	0	0
Total ent <sup>+</sup> strains (23)	13 (57) <sup>4</sup>	7 (30)	3 (13)	0	5 (22)	12 (52)	5 (22)	1 (4)
Total ent <sup>-</sup> strains (66)	37 (56)	21 (32)	8 (12)	0	23 (35)	17 (26)	20 (30)	6 (9)

<sup>1</sup>Incubation was at 37°C for 48 hr.

<sup>2</sup>As in Table I.

<sup>3</sup>See Fig. 1.

<sup>4</sup>Numbers in parentheses represent percentages.

differentiation.

The typing of coagulase-positive staphylococci in this way is especially useful in differentiating the phage nontypable strains. These have been reported to occur with a frequency as great as 30% (Cohen & Smith, 1964; Marandon & Oeding, 1966; Wallmark & Finland, 1961) and attempts have been made to classify such strains into subgroups according to the antigenic response of their proteinases. We have shown that the use of buffered caseinate-agar is a much simpler technique for differentiating proteinases and that all of 63 phage nontypable strains could be classified into five distinct proteolytic groups. The pinpoint technique of inoculation used is highly reproducible, and different isolates from the same strain form almost identical zones, both in size and type. From the epidemiological standpoint, typing by proteinase activity on SMCA may be able to resolve some of the questions left unanswered by phage typing. There is no doubt that the use of both typing systems would result in a better definition of epidemiological relationships.

#### REFERENCES

- Blair, J.E. & Williams, R.E.O. (1961). Bull. Wld Hlth Org. 24, 771.
- Brown, R.W., Sandvik, O., Scherer, R.K. & Rose, D.L. (1967). J. gen. Microbiol. 47, 273.
- Cohen, J.O. & Smith, P.B. (1964). J. Bact. 88, 1364.
- Elek, S.D. & Levy, E. (1954). J. Path. Bact. 68, 31.
- Jarvis, A.W. & Lawrence, R.C. (1970). Appl. Microbiol. 19, 697.
- Lawrence, R.C. & Creamer, L.K. (1969). J. Dairy Res. 36, 11.
- Lawrence, R.C. & Sanderson, W.B. (1969). J. Dairy Res. 36, 21.

- Liu, P.V. & Hsieh, H. (1969). *J. Bact.* 22, 406.
- Marandon, J.L. & Oeding, P. (1966). *Acta pathol. microbiol. scand.* 67, 149.
- Sandvik, O. & Fossum, K. (1965). *Am. J. vet. Res.* 26, 357.
- Sugiyama, H., Bergdoll, M.S. & Dack, G.M. (1960). *J. Bact.* 30, 265.
- Wallmark, G. & Finland, M. (1961). *Proc. Soc. exp. Biol. Med.* 106, 73.
- Zehren, V.L. & Zehren, V.F. (1968). *J. Dairy Sci.* 51, 635.

## A P P E N D I X III

THE RAPID SCREENING OF MILK SAMPLES  
FOR PROTEOLYTIC AND TOTAL BACTERIAL COUNTS

SUMMARY A technique has been developed which allows milk samples from individual suppliers to be screened rapidly but quantitatively for both proteolytic organisms and a total bacterial plate count. The sample is diluted 1,000-fold in sterile Ringer's solution and poured onto Standard Methods Agar to which caseinate (1% w/v), citrate (0.015 M) and calcium chloride (0.02 M) have been added. The excess diluted milk is drained off and the plates incubated at 30°C for 24 hr. A highly significant correlation ( $r = 0.87$ ) is found between the counts obtained in this way after 24 hr incubation and total plate counts carried out by standard procedures.

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INTRODUCTION

There are two aspects of the bacteriological quality of raw milk as received at the factory: (1) its bacterial content as a reflection of milk production methods and handling procedures between production and receipt, and (2) its bacterial content as a determinant of the suitability of the milk for processing. The increasing use of refrigerated vats on farms, alternate-day collection, transportation of raw milk over long distances, and bulk storage at factories has recently shifted the emphasis to groups of organisms such as the psychrotrophs, which are capable of growing at refrigeration temperatures. Twomey, Crawley & Derrick (1969) have confirmed in New Zealand

the results of Thomas, Bruce, Davies & Bear (1966) and many other workers, who showed that modern developments in dairy procedure cause a change in the bacterial flora of bulk milk and that the grading tests in current use, such as the methylene blue test, no longer have their original significance for discrimination between milk samples of various qualities. Psychotropic organisms may show little or no reducing activity towards methylene blue but most strains are highly proteolytic (see Appendix I). It is not generally appreciated that difficulty in processing milk may result if large numbers of proteolytic organisms are present in the raw milk (Franklin, 1969) or that yield losses in the whey due to solubilization of protein before processing may be substantial. Proteinases and some other enzymes produced by bacteria in the raw milk are also likely to survive heat treatment and thus may give rise to flavour defects in the produce on storage.

Since it is likely that most proteolytic organisms found in milk are external contaminants (Taylor, 1967) their numbers in raw milk have been used by Taylor and in this laboratory as an index of hygienic production. The development of a buffered caseinate medium for detecting proteolytic organisms (Appendix I) allows the determination not only of a total plate count by standard microbiological techniques but also gives the proportion of the total count that is proteolytic. The present investigation describes how this medium can be used as a rapid but quantitative screening test of milk supplies to give both an index of farm hygiene and of potential harmful effects on the processing and storage quality of dairy products.

Total and proteolytic counts by standard surface plate (SSP) method. Duplicate 0.1 ml samples of 10-fold dilutions in quarter strength Ringer's solution of the milk samples were spread on the surfaces of Standard Methods Caseinate Agar (SMCA) plates, prepared as described previously (see Appendix I), with sterile glass spreaders. The plates were allowed to dry for c. 15 min. All plates were incubated at 30°C for 48 hr.

Rapid screening procedure using a flooding technique. A 1:1000 dilution of milk sample was made by adding 0.1 ml milk to 100 ml sterile quarter strength Ringer's solution and the mixture then shaken thoroughly. The diluted sample was poured over the surface of the SMCA, and the excess decanted. The plate was replaced upside down in its lid, in which a circle of clean filter paper had been closely fitted. Any further excess sample drained into the filter paper. The plates were incubated at 30°C for 24 hr. Proteolytic organisms could be differentiated by white zones of precipitation round the colonies and thus determined simultaneously as a proportion of the total count.

## RESULTS

Reproducibility of counts. In preliminary work, 22 milk samples from a local commercial factory were examined for total and proteolytic counts using the flooding technique and the SSP method. Each sample was examined in duplicate by each method. The variation in counts between duplicates of the same sample using the flooding technique was no greater than the variation between duplicates using the SSP method.

One aspect of the flooding technique which could not be completely standardized was the variation in the volume of diluted milk sample which remained on the surface of the plate after the excess had been decanted. The volume adsorbed on the plates using 30 different diluted samples had a mean of 0.37 ml with a standard deviation of 0.085. Part of the variation could, however, be accounted for by the differing amounts of sample that remained on the plastic rim of the dish. In any case this variation was relatively small compared to the inherent errors in all microbiological tests and, as shown below, the correlation between the flooding technique and the SSP method was very good.

Correlation of flooding technique with SSP method. The proteolytic and total bacterial counts of 96 milk samples were estimated by both the flooding technique and the SSP methods with incubation periods of 24 and 48 hr respectively. The estimates of the correlations between the total counts obtained by the SSP method after 48 hr incubation and the flooding technique after 24 hr and after 48 hr were respectively 0.87 and 0.99 (see Fig. 1). Thus although more colonies were detected on the flooded plates after 48 hr, the difference between the correlation coefficients was not significant at the 10% probability level. The regression of the SSP counts on the flooding technique (FT) counts, shown by the solid line in Fig. 1, is given as:

$$\text{organisms/ml (SSP)} = (72 + 61.1 \times \sqrt{\text{count (FT)}})^2.$$

From Fig. 1, values were arbitrarily selected to give suitable standards. Ten-fold differences in counts on plates using the flooding technique were chosen since Davis (1969) has argued convincingly that only such differences in plate counts are



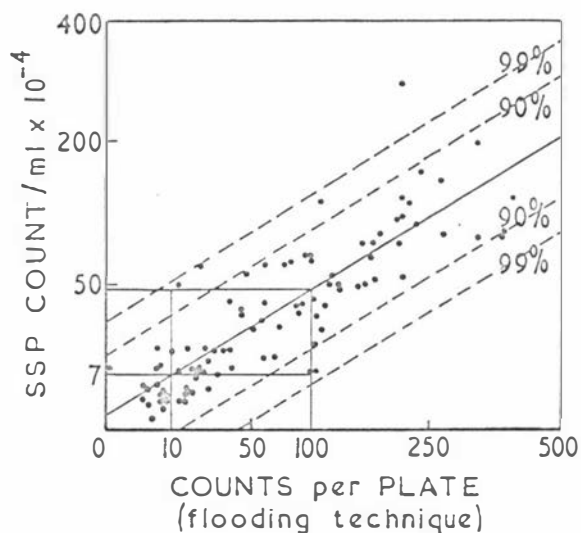


Fig. 1. Correlation diagram between ~~logarithm~~ of standard surface plate (SSP) count and count obtained on plate using the flooding technique (FT). The solid line represents the regression of SSP on the FT count. The dotted lines indicate the 90% and 99% confidence limits. Number of milk samples was 96.

significant when grading milks. Less than 10 colonies and greater than 100 colonies on a flooded plate correspond to counts of  $<70,000$  and  $>500,000$  organisms/ml respectively in the original milk sample (Fig. 1). Following the initial 1,000-fold dilution the flooding technique was used to grade milks as Finest ( $<10$  counts), First grade (10-100 counts) and Second grade ( $>100$  counts). In practice most milks could be graded at a glance (Fig. 2) and only very rarely was it necessary to count colonies. For a borderline case of a flooded plate containing exactly 10 colonies, for instance, it can be estimated that there was a 1% only chance that the milk was in fact Second grade milk and a 50% chance that it was First grade. Similarly, as the count increased above 100,

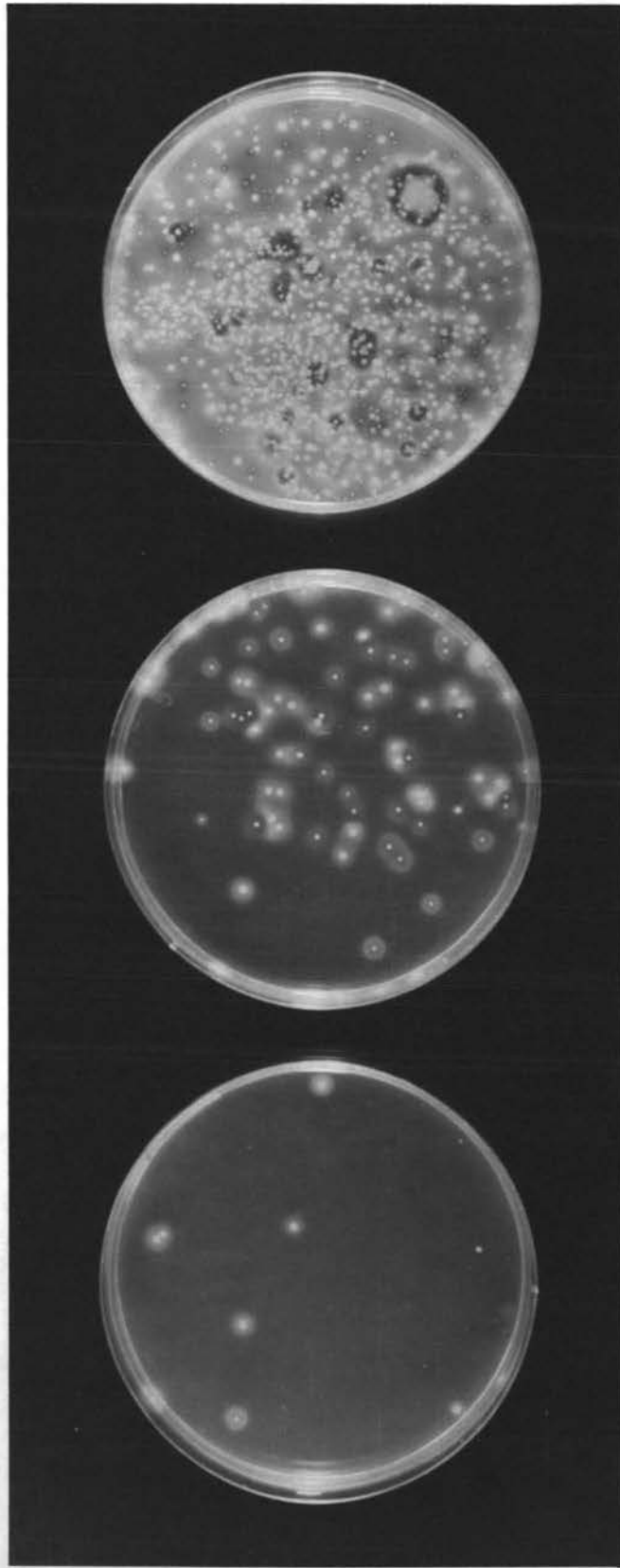
the chance of grading a good-quality milk as Second grade became negligible. It is also possible to calculate the probable errors in grading milks in the 10-100 range. Thus there was a 10% chance that milk giving a flooded plate count of 45 was really Finest and a 20% chance that the milk was really Second grade. Such errors, however, are no worse than if the SSP method were to be used.

In addition to the total counts, the proportion of organisms that were proteolytic could also be determined since these formed white zones of precipitation round the colonies. All but two of the colonies in Fig. 2A and all but one in Fig. 2B were proteolytic. The pattern of zones obtained gave an indication of those organisms which were extensively proteolytic since these can further break down the precipitate of para-caseins to soluble components with the formation round the colony of an inner transparent zone and an outer white ring (Lawrence & Sanderson, 1969).

#### DISCUSSION

Although agars containing milk powders were proposed in the 1920s in America for the detection of proteolytic bacteria, the media were troublesome to prepare and so the presence of proteolytic organisms as an indication of hygiene never gained popularity. Taylor (1967) returned, however, to this concept of assessing raw milk quality by counting the bacteria growing in a thin film of undiluted milk sample adhering to a plain water-agar base. The considerable crowding of colonies on the plates which occurs even in the case of comparatively good-quality milk samples makes it difficult, in our experience, to

Fig. 2. Milk quality determined on SMCA plates. The plates demonstrate milks which were graded on a total count basis (A) as Finest (< 10 counts), (B) as First grade (10 to 100 counts), and (C) as Second grade (> 100 counts) by the flooding technique. Proteolytic colonies are surrounded by a white zone. The plates were incubated at 30°C for 24 hr.



C

B

A

distinguish the three types of zones formed by proteolytic, acido-proteolytic and acid-forming organisms. However, the technique of inoculating SMCA plates by flooding with a diluted milk sample, and decanting the excess as described by Taylor, has proved very satisfactory. It gives a quantitative determination of milk quality, in terms of a total count and the proportion of proteolytic organisms, as rapidly as any of the modified dye reduction tests. The extent of proteolysis exhibited by an organism is reflected both by the size and type of precipitation zone forming round the colony.

Franklin (1969) has emphasized that even a low incidence of unsatisfactory individual farm supplies should not be tolerated since a good-quality bulk milk can be adversely affected by the addition of as little as 1% poor-quality milk. The survey carried out in the present investigation showed that the bacterial flora from individual farm milks varied enormously both in total count and the proportion that were proteolytic.

It is unlikely that a single test will ever be developed which will measure all the bacteriological qualities of raw milk. Furthermore, dairy laboratories in general are not equipped at present to carry out tests other than the most simple and inexpensive on a routine scale. The flooding technique is simple to carry out and since it is no less quantitative than the standard method plate count, the scale can be suitably adjusted to meet different requirements and to regulate the permitted number of samples coming within any one class. The standards suggested here have been formulated on the basis of a small number of samples from a single area and are only suggested as a basis for trials in other parts of the country.

The value of the test is that it gives a good picture of the general microbial condition of milk in terms of numbers and types of organisms, which may not be revealed by dye tests, particularly when counts are low. A grader can assess with great accuracy poor and good-quality milks as received at the factory without having to count the colonies, although a quantitative count can be carried out if considered necessary. The plates can be read after  $24 \pm 3$  hr, thus allowing more flexibility than one has with dye tests, and then stored in a cold room as visual proof for the supplier of the quality of his milk.

#### REFERENCES

- American Public Health Association (1967). "Standard Methods for the Examination of Dairy Products", 12th ed.  
New York: American Public Health Association.
- Davis, J.G. (1969). *Lab. Practice* 18, 839.
- Franklin, J.G. (1969). *J. Soc. Dairy Technol.* 22, 100.
- Lawrence, R.C. & Sanderson, W.B. (1969). *J. Dairy Res.* 36, 21.
- Taylor, Margaret M. (1967). *Dairy Inds* 32, 278.
- Thomas, S.B., Bruce, R.G., Davies, A. & Bear, J.S. (1966).  
*J. Soc. Dairy Technol.* 19, 161.
- Twomey, A., Crawley, W.E. & Derrick, Linda L. (1969). *N.Z. J Dairy Technol.* 4, 63.