PHYSIOLOGICAL CHANGES ASSOCIATED WITH THE APPEARANCE OF SLOW VARIANTS IN CULTURES OF STREPTOCOCCUS LACTIS

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A B S T R A C T

Streptococcus lactis C10 Slow, a variant of the normal 'fast' strain of S.lactis C10, was only capable of rapid and extensive growth in skim milk if casein hydrolysates were added. It was postulated, therefore, that the slow variant is defectively proteolytic.

A sensitive assay of proteolysis, based on the release of radioactivity from iodinated casein, was developed, checked for usefulness with known proteinases, then used to assay the streptococcal enzymes.

Fractionation of the two strains, by either mechanical cell disruption and differential centrifugation or by cell-wall digestion with a muraminidase, established that most of the cell-bound proteinases of the parent strain were surface-bound. This activity was virtually absent in the slow variant.

Partial characterization of the surface-proteinase(s) showed that maximum activity was exhibited at pH 6.0-6.8, in 0.05 M phosphate buffer, at 30-32°C. It was rapidly inactivated at 37°C, both when cell-bound and when free of the cells. Examination of a second pair of strains, S.lactis H1 Fast and S.lactis H1 Slow, indicated a difference in proteinase activity and localization similar to that found between the two S.lactis C10 strains.

It was concluded, on the basis of both nutritional evidence and enzymatic analyses, that the slow variant of S.lactis C10 is limited in skim milk by the supply of amino acids and that this is due to a defective surface-bound proteolytic activity.

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PREFACE

Rapid growth in milk, with the concomitant production of lactic acid, is the primary attribute of the single-strain Cheddar cheese starter. Variations in a strain's activity may be due to changes in the culture's environment or to changes in its capabilities. A clear example of the latter is seen in those strains which segregate slow variants. The variants are stable genetic derivations of the normal 'fast' cells and, when cultured as pure isolates, exhibit limited growth in milk. Their segregation at high frequency, together with their nutritional support by the parental cells, enables accumulation to occur to the point where they contribute the majority of the cells in the culture.

It has been suggested that slow variants are proteolytically deficient in comparison to the parental type. The relationship between starter proteolysis and activity in milk is unclear, but many strains are stimulated by the addition of nitrogenous hydrolysates to the medium.

This investigation was undertaken to examine the hypothesis that slow variants are defectively proteolytic. It was anticipated that the work would also provide useful information concerning the properties and cellular location of starter proteinases in general.

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INTRODUCTION

GENERAL

A small number of strains of Streptococcus lactis and Streptococcus cremoris (the lactic streptococci) are used as 'starters' in the manufacture of Cheddar cheese. Their primary role is to produce lactic acid; they also contribute to cheese flavour (Lowrie, Lawrence, Pearce & Richards, 1972).

A clear-cut difference in activity (rate and extent of acid production in milk) between the cells in some pure cultures of S. lactis was reported by Harriman & Hammer (1931). About 2% of the cells were 'slow coagulators'. The slow characteristic was stable and the variant cultures never segregated the parental 'fast' cells. Analysis of the non-protein nitrogen fraction of 'fast' and 'slow' milk cultures indicated less proteolysis in the latter, although proteolytic activity per cell was not determined. Subsequent work has confirmed: (1) the high frequency with which slow variants appear in susceptible cultures (Garvie & Mabbitt, 1956; Citti, Sandine & Elliker, 1965; Westhoff, Cowman & Speck, 1971); (2) the one-way nature of the transition (Garvie & Mabbitt, 1956; Citti et al., 1965); and (3) the correlation between 'slowness' and relatively low proteolytic activity (Citti et al., 1965; Westhoff et al., 1971). The last point has been the least convincingly shown and its examination forms the basis of the present investigation.

CARBOHYDRATE METABOLISM

The production of lactic acid by starters is the direct

result of energy-yielding carbohydrate catabolism and is the growth function usually measured when starter 'activity' is assessed. The lactic streptococci are part of a large group of micro organisms that carry out the homolactic fermentation. This process, analagous to muscle glycolysis, has been elucidated largely through the use of radioactive tracers and has been thoroughly reviewed (Wood, 1961; Kandler, 1961; Marth, 1962; Busse, 1966). The fate of lactose is as follows:

Most lactic streptococci (including S.lactis C10) accumulate lactose by a phosphoenolpyruvate-dependent phosphotransferase system (McKay, Miller, Sandine & Elliker, 1970), analagous to that of Staphylococcus aureus. The sugar, transported as a phosphate derivative, is split by a 6-phospho-β-galactosidase into glucose and galactose-phosphate, which then enter the Embden-Meyerof-Parnas pathway. The latter is summarized as follows: (Wood, 1961)

$$\text{glucose} + 2\text{ADP} + 2\text{P}_i \longrightarrow 2 \text{ lactate} + 2\text{ATP} + 2\ \text{H}_2\text{O}$$

The products from one mole of lactose are, theoretically, four moles of lactate and four moles of ATP. Analysis of the products of glucose metabolism by homolactic streptococci has shown that small amounts of acetate, formate, CO_2, ethanol, acetoin, and 2,3-butanediol may be formed in addition to lactic acid, their relative proportions depending on culture conditions, particularly the pH. At the least, lactate accounted for 90% of the recovered glucose carbon (Platt & Foster, 1958).

NUTRITIONAL REQUIREMENTS

The rate and extent of acid production by a given single-strain starter reflects its overall ability to grow in the

medium, an ability determined by a complex interaction between the available nutrients, the culture conditions, and the strain's capabilities. In the absence of constraints, such as inhibitors (e.g. penicillin in milk), suboptimal temperatures, and virulent bacteriophage, the activity is probably a function of the strain's ability to obtain essential amino acids from the medium.

Niven (1944) reported that S.lactis required niacin, biotin and pantothenate for growth in a medium complete but for the B vitamins. Thiamin and riboflavin were essential for 18 and 10 strains, respectively, of the 21 examined, while pyridoxine stimulated most. Amino acid requirements were extensive. All strains required valine, leucine, isoleucine, arginine and methionine for growth, but a response comparable to that obtained in the presence of casein hydrolysates required a minimum of 16 amino acids. The only acids without effect were tyrosine, norleucine, aspartic acid, threonine, hydroxyproline and tryptophane. The absolute requirement of all strains for glutamine and asparagine was controversial, Pollack & Lindner (1944) having reported that several strains of S.lactis could use the equivalent acids if they were included at sufficient concentration. Subsequent investigations have substantiated the findings of Niven, minor discrepancies being attributed to the use of different basal media, experimental conditions, and strains. Husain & McDonald (1957) found that S.lactis and S.cremoris required histidine in addition to the acids reported by Niven; however, excellent growth was obtained in the presence of only 12 amino acids, reflecting the basal medium which was improved by the inclusion of Tween 80 (a source of oleate) and acetate. Their finding that S.cremoris was more fastidious than S.lactis for

amino acids was confirmed by Reiter & Oram (1962).

MILK AS A MEDIUM

Bovine milk, the stock substrate for starter maintenance and cheesemaking, appears to contain ample concentrations of protein, lactose, and the essential vitamins (average levels in whole milk, per litre: protein, 35 g; lactose, 49 g; niacin, 0.94 mg; biotin, 31 µg; pantothenate, 3.46 mg; thiamin, 0.44 mg; riboflavin, 1.75 mg) (Webb & Johnson, 1965; Hartman & Dryden, 1965). All the essential amino acids, except methionine, have been found in the non-protein nitrogen fraction of milk (Block, 1951; Van der Zant & Nelson, 1954; Deutsch & Samuelson, 1959; Schwartz & Pallansch, 1962), but the concentrations are generally considered to be insufficient for optimal starter development. The evidence for this contention is indirect and generally unsatisfactory but forms the basis of the "proteinase-dependency" hypothesis. There have been numerous reports of the stimulation of lactic streptococci in milk, particularly of 'slow' strains, by nitrogenous extracts (pancreas extract, yeast extract, corn steep liquor, liver fraction L; see review by Reiter & Moller-Madsen, 1963). The effect of these additives has usually been attributed to peptides but the only complex whose stimulatory component has been identified ("pancreas extract") was effective because of its content of inosine, hypoxanthine, and adenine (Koburger, Speck & Aurand, 1963). The only convincing evidence for stimulation specifically by amino acids and/or peptides was given by Garvie & Mabbitt (1956) who demonstrated that peptone, casamino acids, and acid-hydrolysed casein stimulated both S. cremoris 924 and its slow variant in milk.

STARTER PROTEINASES AND 'SLOWNESS'

There is a sound a-priori basis for the hypothesis that starter growth in milk is dependent on milk protein degredation, and that slow strains and slow variants are special cases of cultures limited by this constraint. The evidence for the importance of starter proteinases in this process is scarce and difficult to assess, due mainly to the very low proteolytic activity of even the fastest of strains.

Proteinases have been purified from the 'soluble' and 'particulate' fractions of sonicated cell suspensions of both S.lactis 3 and its slow variant (Cowman, 1966; Westhoff, Cowman & Swaisgood, 1971; Westhoff, Cowman & Speck, 1971). Quantitative differences in proteolytic activity between the two strains, whether assessed from whole cells or from the fractions, were very low (ca. 1.5-fold). The variant 'intracellular' enzyme did, however, differ in many properties from the equivalent enzyme of the parent strain, and the authors were forced to conclude that the difference in specificity of the proteinase was responsible for the inability of the variant to grow extensively in milk (Westhoff & Cowman, 1971). Much of the published work concerning S.lactis 3 is unsatisfactory, although there can be no doubt that the proteinases have been thoroughly characterized as enzymes. The proteinase assay used was not tested for its sensitivity or reproducibility and specific activities of assayed fractions are rarely stated. In addition to this lack of data for the comparison of activities, the evidence for the localization of the proteinases, essential to a meaningful interpretation of their physiological function, is insufficient, consisting merely of whether or not the activity

remains with the pellet after centrifugation of disrupted cells (Cowman, 1966). The major criticism of the work, however, concerns the lack of essential information relevant to the procedures, a deficiency which precludes both the interpretation of the results and the extension of the methods to other strains.

AIM OF THE INVESTIGATION

The aim of this study was to examine the hypothesis that slow variants are relatively non-proteolytic in comparison to normal 'fast' strains. It was evident from assessment of previous attempts to characterize the proteinase enzyme systems of S. lactis that the work would require a very sensitive assay for proteinase and subsequently a critical determination of the localization of relevant enzymes in the organisms.

METHODS

MICROBIOLOGICAL METHODS

MEDIA

Skim milk. 100 g spray-dried skim milk powder (New Zealand Dairy Board, Wellington, N.Z.) was dissolved in 910 ml deionized water (45°C) and autoclaved at 10 pounds per square inch (psi) for 20 min. The medium contains 9.5% solids, the average level of milk solids in fresh skim milk (Dolby, Creamer & Elley, 1969). Each litre of skim milk contains approximately 1 g milkfat; 35 g protein; 46 g lactose; 8 g mineral salts and 1 g minor constituents (Export Purchase Handbook, 1971-1972, New Zealand Dairy Board). One bag of powder was the source of all the skim milk used in this investigation.

"Peptides". Exzymatic hydrolysate of casein (Nutritional Biochemicals, Cleveland, Ohio) was made up in deionized water and autoclaved at 15 psi for 15 min. The hydrolysate contains vitamins (riboflavin, thiamin, pantothenate, biotin and pyridoxine) in addition to peptides and amino acids.

"Amino acids". Hydrochloric acid hydrolysate of casein (Nutritional Biochemicals) was fortified by the addition of 1 µg L-tryptophane per mg, made up in deionized water and autoclaved at 15 psi for 15 min.

M16 Media:

Broth. (Lowrie & Pearce, 1971.) Solution A: beef extract (BBL), 5.0 g; polypeptone (BBL), 5.0 g; phytone (BBL), 5.0 g; yeast extract (BBL), 2.5 g; ascorbic acid, 0.5 g; sodium acetate trihydrate, 3.0 g; deionized water, 500 ml. The solution was adjusted to pH 7.2 with 2.0 M NaOH. Solution B: lactose, 2.0 g;

glucose, 2.0 g; deionized water, 200 ml. The solutions were autoclaved at 15 psi for 15 min., allowed to cool, then mixed to give the complete medium.

Diluent per litre: 100 ml M16 broth made up to 1000 ml with deionized water, autoclaved at 15 psi for 15 min.

Plate agar per litre: components of M16 broth plus 10 g agar (Davis, N.Z.), autoclaved at 15 psi for 15 min., cooled to 50°C, poured into plates. M16 plates were dried at 30°C for 30 min. before use for viable-count determinations.

Soft agar per litre: components of M16 broth plus 4.5 g agar (Davis, N.Z.), autoclaved at 15 psi for 15 min., cooled to 50°C, dispensed into tubes (46°C) in 2.5 ml lots.

M16 media were used for routine maintenance of cultures and for determinations of viable count and phage titre by the soft agar overlay technique.

T_5 broth. (T.D. Thomas, personal communication.)
Solution A: beef extract (BBL), 2.0 g; polypeptone (BBL), 5.0 g; phytone (BBL), 2.0 g; yeast extract (BBL), 2.0 g; disodium hydrogen phosphate, 8.5 g; potassium dihydrogen phosphate, 2.0 g; 1.0 M magnesium chloride hexahydrate, 1 ml; ascorbic acid (10%, w/v), 5 ml; deionized water, 50 ml. The solutions were autoclaved at 15 psi for 15 min., allowed to cool, then mixed to give the complete broth (pH (cool) ca. 7.2).

T_5 broth is a modification of M16. The increased buffering capacity allows greater cell densities and the exclusion of glucose prevents lactose-negative mutants of S.lactis from growing. It was the standard medium for preparation of cells for fractionation and proteinase assay.

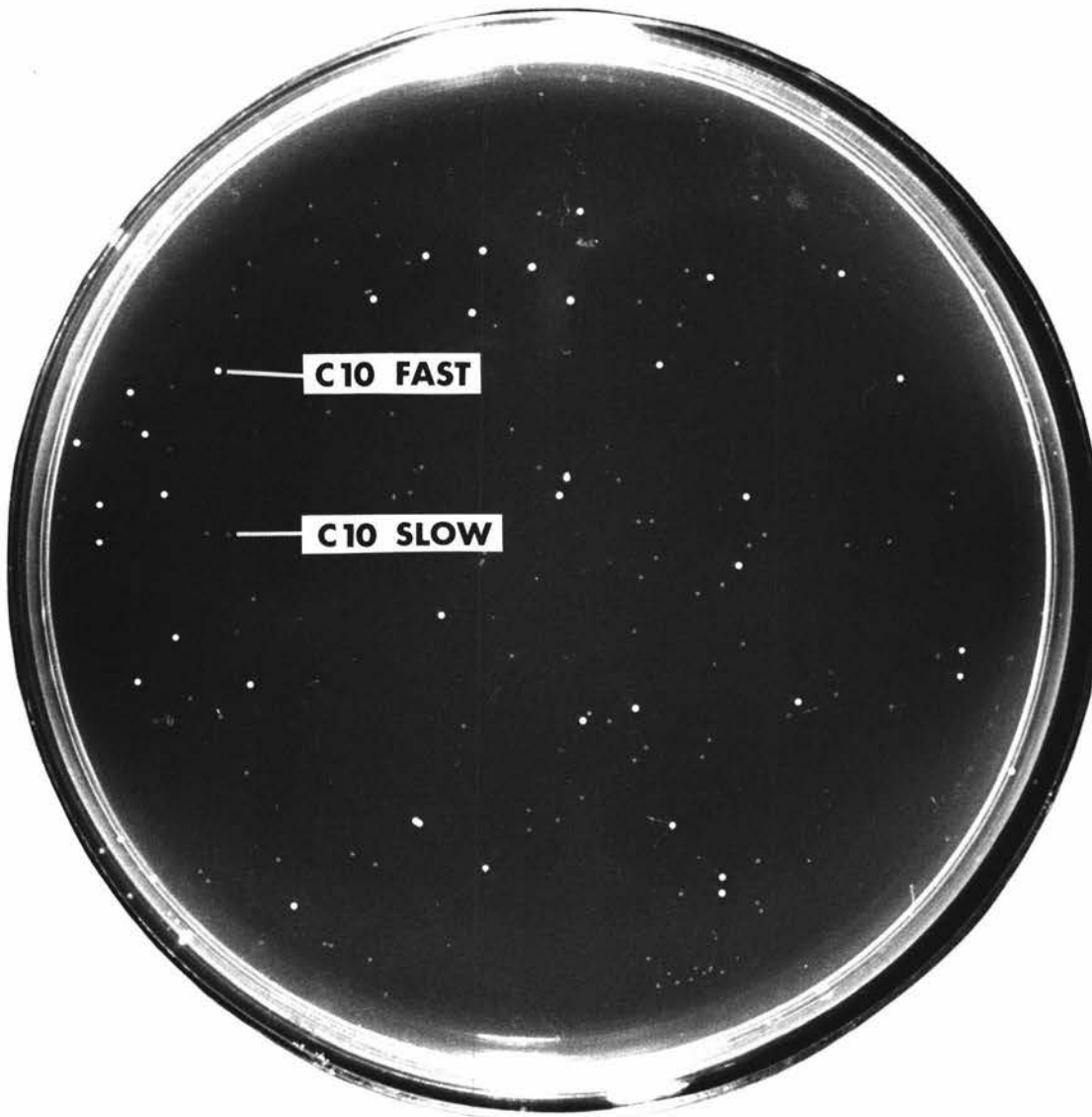
Citrated milk agar. (Brown & Howe, 1922; modified by L.E. Pearce.) Solution A: skim milk powder, 20 g; trisodium citrate dihydrate, 1.69 g; deionized water, 482 ml. The solution was left to stand at room temperature for 2 hours, adjusted to pH 6.8 with 5 M HCL, then autoclaved at 10 psi for 10 min. Solution B: agar (Davis, N.Z.), 15 g; deionized water, 500 ml; autoclaved at 15 psi for 15 min. After cooling to 50°C, the two solutions were mixed thoroughly and poured into plates.

Citrated milk agar was used initially to isolate the slow variant from S.lactis C10 Fast (Plate 1). Subsequently it was used to check culture identity.

BACTERIA AND BACTERIOPHAGE

Streptococcus lactis C10 and its homologous phage, phage c10, were obtained from Dr W.E. Sandine, Oregon State University, Portland, Oregon. A 1% inoculum of the strain, from either a 16-hour, 22°C skim milk culture, or a 16-hour, 22°C M16 broth culture, clotted skim milk within 18 hours at 22°C. The strain was designated S.lactis C10 Fast. A clotted skim milk culture of this strain was spread on citrated-milk-agar for single colonies and incubated at 30°C for two days. Several small clear colonies were present, among an excess of the large white, parental type (Plate 1). One of the small colonies was picked into skim milk and incubated overnight at 30°C. The culture was checked for activity by three successive subcultures in skim milk with 18-hour, 22°C incubations. The strain was designated S.lactis C10 Slow, based on its inability to clot skim milk within 18 hours at 22°C, when inoculated at 1% from a similar culture (Citti, Sandine & Elliker, 1965). The two strains of S.lactis C10 were indistinguishable when grown in

Plate 1. Differentiation of S.lactis C10 Fast and S.lactis C10 Slow on citrated-milk-agar.



M16 or T_5 broths and supported equal multiplication of phage c10 when used as indicator organisms in the assay of the phage stock.

Streptococcus lactis H1 Fast and S.lactis H1 Slow were obtained from the culture collection maintained by the New Zealand Dairy Research Institute. S.lactis H1 Slow was originally isolated from S.lactis H1 Fast by the use of citrated-milk-agar and was indistinguishable from the parent strain both with regard to growth in broths and to ability to support phage h1 multiplication (L.E. Pearce, personal communication).

CULTURE MAINTENANCE

Close attention was paid to culture maintenance (Table 1) because of the nature of the appearance of slow variants in fast cultures of S.lactis C10. On the original citrated-milk-agar plate used to identify the slow variant, about 1% of colonies had the variant phenotype. This very high frequency could result in a predominantly 'slow' culture if the 'pure' C10 Fast strain was subcultured indefinitely. Carvie (1959) reported that mixtures of normal cells and slow variants invariably appeared 'fast' in milk, presumably due to nutritional support of the variants by the normal cells.

The extent of subculturing was strictly limited, to minimize slow variant accumulation. The procedure given in Table 1 was adopted for both strains of S.lactis C10, although slow variants have never been observed to give rise to the parental type.

Deep-frozen stocks, the primary source of the organisms, were prepared by growing the strains for 5 hours at 30°C in skim milk, dispensing the cultures into 0.5 ml lots in sterile plastic vials, and covering the vials with liquid N_2. The stocks were

Table 1. Culture maintenance.

System	Medium	Temperature
deep-frozen stock	skim milk	-75°C
2-month intervals		
recovery culture	M16 broth	30°C (16 hours)
7-day intervals		
working stock	M16 agar	30°C (5 hours); 4°C (7 days)
working culture	M16 broth or skim milk	22°C (16 hours)
experimental system	T_5 broth or skim milk	30°C

stored under dry ice (-75°C). When required for the reinitiation of the culture procedure, they were thawed rapidly by immersion in a 37°C water bath.

PREPARATION OF CELLS FOR ASSAY AND FRACTIONATION

Skim milk, the only medium in which the "slowness" of S.lactis C10 Slow is expressed, is unsuitable for cell preparation because of the difficulty of harvesting. Attempts to construct an optically clear differential medium were unsuccessful.

T_5 broth, a well-buffered complex medium, supported rapid and extensive growth of both strains of S.lactis C10 (Figure 9) and was used to grow all cells assayed for proteinase and used in fractionations. Procedure: T_5 broth (usually 1500 ml) was inoculated at 0.5% with a 16-hour, 22°C M16 broth culture of either S.lactis C10 Fast or S.lactis C10 Slow, incubated at 30°C until the culture reached mid-logarithmic phase (E_{580} 0.5,[1] about 4 hours), chilled for 10 min., then centrifuged in the cold at 4080 x g for 2 min. The culture supernatant was discarded. Subsequent operations with the pellet are described under 'Fractionation Procedures'.

DETERMINATION OF VIABLE COUNT

A soft agar overlay technique was used to determine the number of viable units in skim milk and broth cultures of S.lactis C10; 0.1 ml of an appropriate dilution of the culture was added to 2.5 ml M16 soft agar and the mixture poured over M16 plate agar. Duplicate plates were prepared. After overnight incubation (30°C) the colonies were counted, the two

1 Turbidity measured at 580 nm in a Spectronic 20 (Bausch and Lomb).

counts averaged, and the count expressed as colony-forming-units per ml of original culture. S.lactis C10 occurs predominantly as pairs of cocci, so the viable counts represent approximately 50% of the actual number of viable cells.

DETERMINATION OF PHAGE TITRE

A stock suspension of phage c10 was assayed periodically, to check the identity of the two strains of S.lactis C10 by the soft agar overlay method of Adams (1950).

ANALYTICAL METHODS

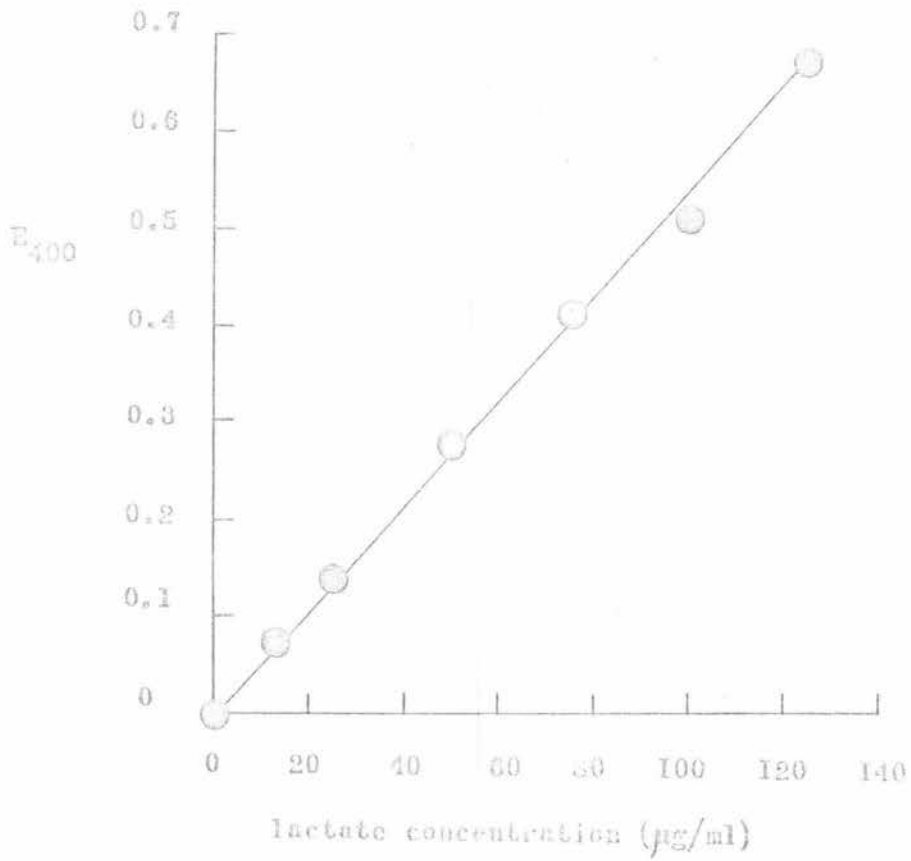
LACTATE DETERMINATION

The colorimetric method of Steinsholt & Calbert (1960) was used to measure lactate in both skim milk and broth cultures of S.lactis C10. The colour intensity of the lactate-ferric chloride complex was measured at 400 nm in a Spectronic 20. Analar lactic acid was used to construct standard curves (Figure 1), its concentration in the stock solution having been determined by titration to pH 7.9. The lactate values reported are means of duplicate determinations.

PROTEIN DETERMINATION

Protein was measured by its reaction with the Folin-Ciocalteau reagent (BDH), as described by Lowry, Rosebrough, Farr & Randall (1951). Casein (BDH 'Hammarsten'), dissolved in 0.1 M phosphate buffer, pH 7.6, was used as the standard protein. The blue colour was measured in a Klett-Summerson Colorimeter with the red filter.

I. Figure I (page I6). Replace with the following figure:



Determination of lactate by the method of Steinholt and Calbert (I960).

DRY-WEIGHT DETERMINATION

Cells were washed three times with phosphate buffer and resuspended in the same medium to a known volume. Samples (1 ml) were dried on milk bottle tops in a vacuum dessicator. Triplicate samples were processed and the mean dry weights corrected for the weight of buffer.

PROTEINASE ASSAY

INTRODUCTION

The lactic streptococci are only weakly proteolytic. When plated on well-buffered caseinate agar, the majority of cheese starters fail to form the zones of precipitated calcium paracaseinate indicative of proteolysis, unless incubation is continued for 10 days (Martley, Jayashankar & Lawrence, 1970). It was evident that a very sensitive proteinase assay was a prerequisite for meaningful localization experiments.

Proteinases of unknown specificity and multiplicity are generally assayed by their hydrolysis of haemaglobin or casein, after the methods of Anson (1938) and Kunitz (1947). Addition of trichloroacetic acid (TCA) to the incubated reaction mixture terminates the reaction and precipitates undigested substrate. The amount of low molecular weight products in the supernatant can be measured either directly as extinction at 280 nm or colorimetrically after reaction with the Folin phenol reagent (Anson, 1938), biuret reagent (Lapresle & Webb, 1960), or ninhydrin (Greenbaum & Sherman, 1962). This general method is very widely used but suffers from insensitivity. Any TCA-soluble amino acid-containing substance will contribute to the measured

"proteolysis" and in the case of complex biological samples, such as blood and bacterial lysates, the enzyme sample can contribute the major part of the measurement. A specific example of the difficulty that can be encountered with casein as a substrate has been given by Marrink & Gruber (1966) who found the substrate to contain ribonuclease. Hydrolysis of RNA in the enzyme sample (tissue extract) produced acid-soluble nucleotides whose absorbance at 280 nm contributed the majority of the "proteolysis".

The methods of Anson and Kunitz have been improved by labelling the complex substrates with radioactive isotopes. Measurement of the radioactivity in the acid-soluble fraction of the terminated reaction mixture eliminates the uncertainty associated with unmodified substrate. Only derivatives of the added substrate can be detected. Proteins labelled with radioactive iodine have been used to assay trypsin (Katchman, Zipf & Homer, 1960; Loken, Terrill & Mosser, 1961), chymotrypsin (Katchman et al., 1960), papain (Schneider, Ventrice & Weiner, 1965), blood proteinases (Heuson, 1959) and bacterial proteinases (Chaloupka, 1961).

PREPARATION OF PROTEINASE SUBSTRATE

Casein, labelled with iodine-125 by the method of Heuson (1959), was used as the substrate for streptococcal proteinases in this investigation.

Two g casein (BDH 'Hammarsten' casein) were dissolved in 24 ml 0.1 M phosphate buffer, pH 7.6, by heating in boiling water for 15 min. The suspension was cooled to room temperature and transferred to a magnetic stirrer in the fume cupboard. To the suspension were added 1.5 ml 0.1 M KI, 2 mci iodine-125

in 2 ml 0.3% sodium thiosulphate (IMS 1P, Radiochemical Centre, Amersham, England) and 1.5 ml 30-volume H_2O_2. The suspension was stirred gently for 60 min. at room temperature, then transferred to Visking dialysis tubing (18/32) and dialysed at 4°C against 0.1 M phosphate buffer, pH 7.6, to remove unbound iodine (six changes of buffer, at 12-hour intervals, to 9 litres). The dialysed casein was divided into small lots and stored at -20°C.

Three lots of iodinated casein were prepared during the investigation (Table 2). There were minor differences in the preparation of each lot, as follows:

Preparation 1: as above.

Preparation 2: the iodinated casein was dialysed initially against 0.1 M phosphate buffer, pH 7.6 (two changes, to 3 litres), then against 0.1 M KI (two changes, to 3 litres) and finally against 0.1 M phosphate buffer, pH 7.6 (two changes, to 3 litres). The extra step was an attempt to reduce the level of unbound iodine-125 in the protein suspension, but it was unsuccessful.

Preparation 3: the labelled protein was dialysed against 0.1 M phosphate buffer, pH 6.0 (four changes, to 8 litres).

Table 2. Preparation of proteinase-substrate

Preparation	Input of isotope	Recovery in casein, % [a]	Specific activity cpm. mg^{-1}	Substrate-blank, % total cpm
1	2 mci	100	3.5×10^5	0.5
2	2 mci	100	1.1×10^6	0.5
3	5 mci	50	4.3×10^5	0.5

a - assuming that the sum of the activities in the casein and in the dialysates equals 100% input.

Both the pH and the ionic strength of the buffer in which the substrate was suspended required modification during the work. Where this was required, 2 ml iodinated-casein were dialysed at 4°C against 2 litres of the appropriate buffer for 24 hours.

MEASUREMENT OF IODINE-125

Iodine-125 has a half-life of 60 days, decaying by orbital electron capture with the emission of k x-rays and weak gamma rays. The gamma emission is detected with a sodium iodide crystal. Iodine-125 was determined with a Packard Auto Gamma Spectrometer (containing the well detector) connected to a Packard Tri Carb Scintillation Spectrometer. Instrument settings: window, A-B, (A.70, B.200); Gain, 80%; high voltage, 925 v. One-ml samples, contained in stoppered plastic tubes, were routinely counted for 10 min. An empty tube was counted to determine the background. The raw counting rate was corrected for background (300 cpm) but not for quenching or coincidence, as these sources of error were considered to be insignificant for the counting of a gamma-emitter in a constant volume in this instrument.

THE SUBSTRATE BLANK

The level and stability of the blank (the proportion of total radioactivity that is soluble in TCA without enzymatic hydrolysis) imposed a constraint on the sensitivity of the assay. The blank was dependent on the concentration of acid used to precipitate the substrate (Figure 2) and the time of incubation of substrate prior to precipitation (Figure 3). The effect of TCA concentration is considered to be due to:

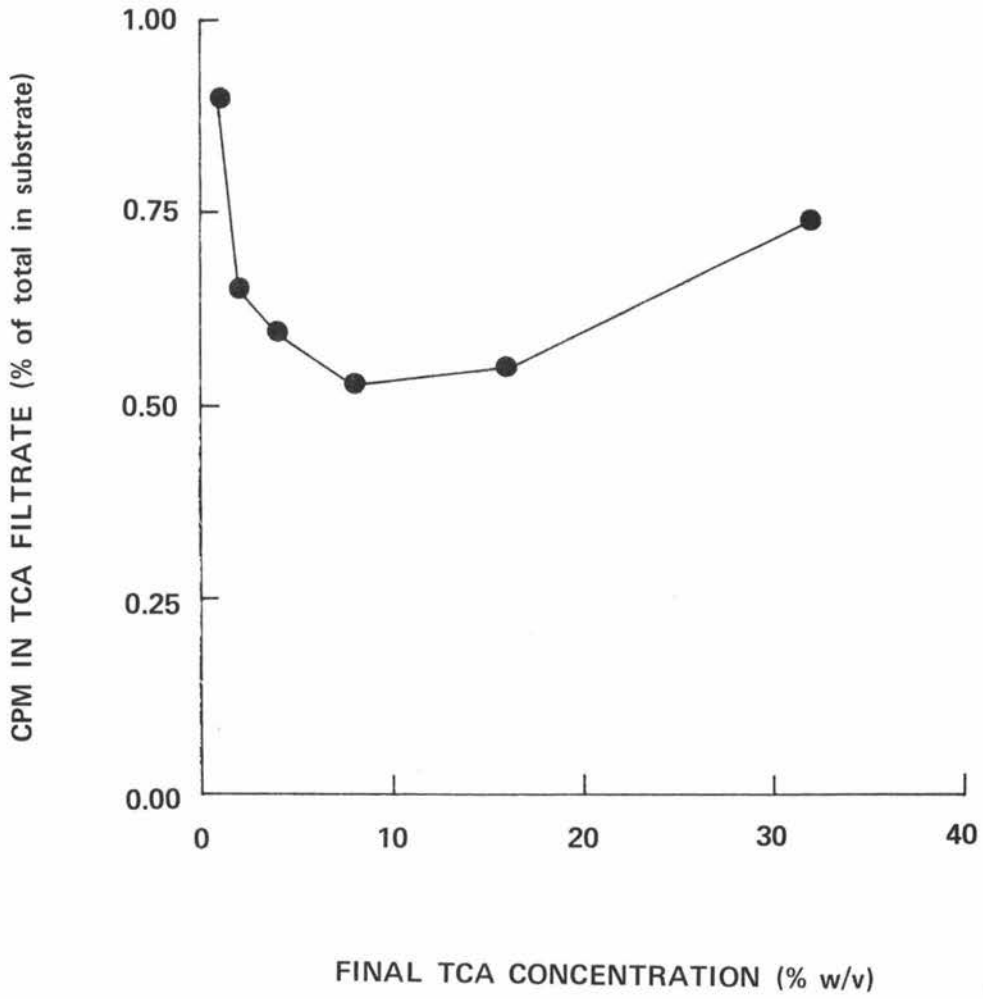


Figure 2. Effect of trichloroacetic acid (TCA) concentration on the substrate blank.
For each point, 1 volume of TCA was added to 1 volume of 0.1M phosphate buffer, pH 7.6, containing iodinated casein (2 mg/ml) and carrier casein (10 mg/ml). The contents were filtered and counted as described in Methods.

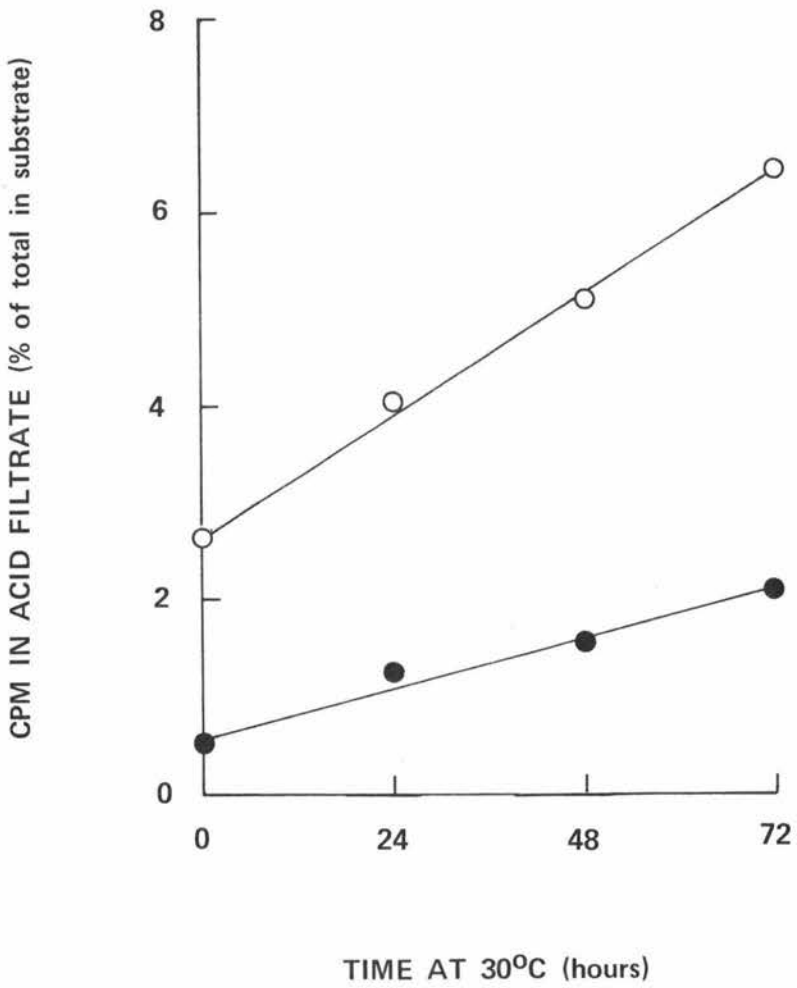


Figure 3. Non enzymatic release of radioactivity from iodinated casein.
Iodinated casein was incubated in 0.1M phosphate buffer, pH 7.6, at 30°C (4 mg casein/ml). At intervals, samples were removed, added to equal volumes of carrier casein (20 mg/ml in 0.1M phosphate buffer, pH 7.6), and treated by the addition of two volumes of either 24% w/v TCA (●) or 10% w/v acetic acid (○). The contents were filtered and counted as described in Methods.

(a) solubilization of casein at concentrations greater than 12% (w/v), and (b) failure to remove large fragments (peptides) at less than 12% (w/v). The optimum concentration, 12%, is in agreement with the data of Bell (1963) which indicated that approximately 10% TCA, final concentration, resulted in the minimum 'non-protein nitrogen' when added to skim milk.

The slight release of TCA soluble material observed when the substrate was incubated at 30°C in buffer (Figure 3) might indicate the presence of a native proteinase, such as the "milk proteinase", which is known to survive the usual casein preparation methods (Dulley, 1971). The different rate of solubilization found with acetate as the protein precipitant (Figure 3) suggested, however, that at least part of the anomaly was due to the nature of the protein-protein precipitant reaction.

STANDARD ASSAY PROCEDURE

The short half-life of the isotope and the anomalous behaviour of the blank necessitated the inclusion of a blank solution in all assays. This was used to measure the total available cpm and the non-enzymatic release of cpm. The non-enzymatic release was consistent, increasing from 0.5% to 0.9% of the total cpm over six hours at 30°C.

Reaction mixtures contained 0.5 ml substrate (2 mg iodinated-casein) and 0.5 ml sample (enzyme for experimental tubes, buffer for blank). After incubation (six hours at 30°C, unless otherwise specified) 0.4 ml samples were removed and added to 1.6 ml lots of carrier-casein (unlabelled, 20 mg/ml in 0.1 M phosphate buffer, Ph 7.6). Two such samples were prepared from the

incubated blank. Trichloroacetic acid (2 ml, 24% w/v) was added to each experimental sample and to one blank (to give the non-enzymatic release of cpm) and distilled water (2 ml) to the other blank (to give total available cpm). All tubes were vortexed for 20 seconds, left at room temperature for 20 min., and the contents filtered through Whatman No. 1 paper; 1 ml from each acid filtrate was counted, the raw counting rates corrected for background and non-enzymatic release, and the corrected count expressed as per cent of the total available cpm.

ASSAY CONDITIONS

The pH, ionic strength, buffer and temperature of assay were varied to suit the proteinase and are detailed in the 'Results' section.

UNITS

One unit of streptococcal proteinase activity was arbitrarily defined as the amount of enzyme which solubilized 1% of the substrate in six hours. Specific activity is expressed as units per mg cell dry weight.[2]

MEASUREMENT OF TRYPSIN AND SUBTILISIN

Trypsin (BDH, from pancreas) and subtilisin (Sigma protease Type VIII, twice re-crystallized) were assayed with the substrate by the standard procedure (Figures 4 and 5). A common feature was the nonlinearity between activity and enzyme concentration at low enzyme levels. It is suggested that this reflects the complex nature of the substrate, which contains various sites for each enzyme.

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2 Specific activities of fractions, washes, etc. were calculated on the basis of cell dry weight equivalent in the samples.

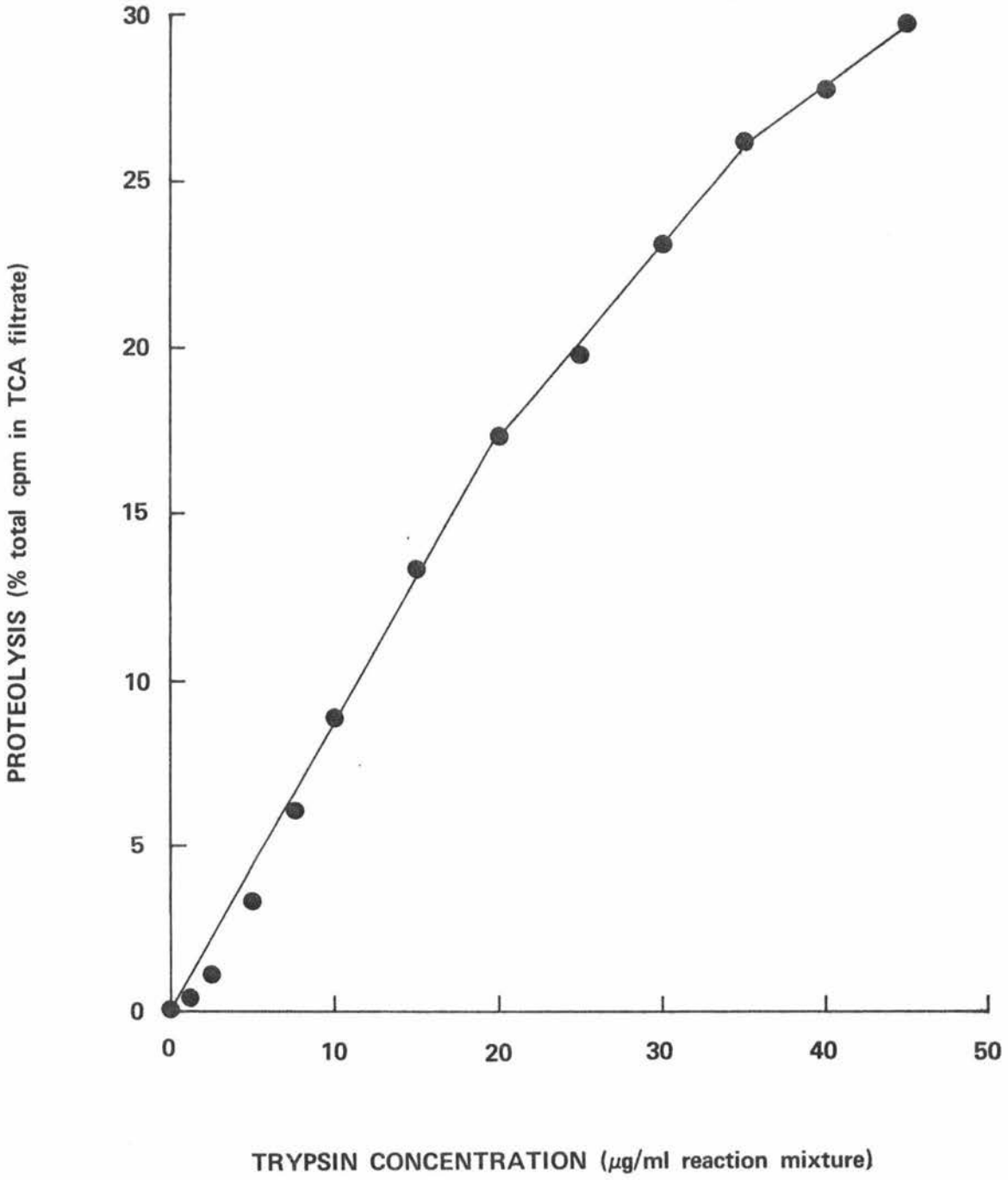

Figure 4. Proteolysis of iodinated casein by trypsin.
Assay procedure : standard.
Assay conditions : 0.1M phosphate buffer, pH 7.6,
40°C, 2 hours incubation.

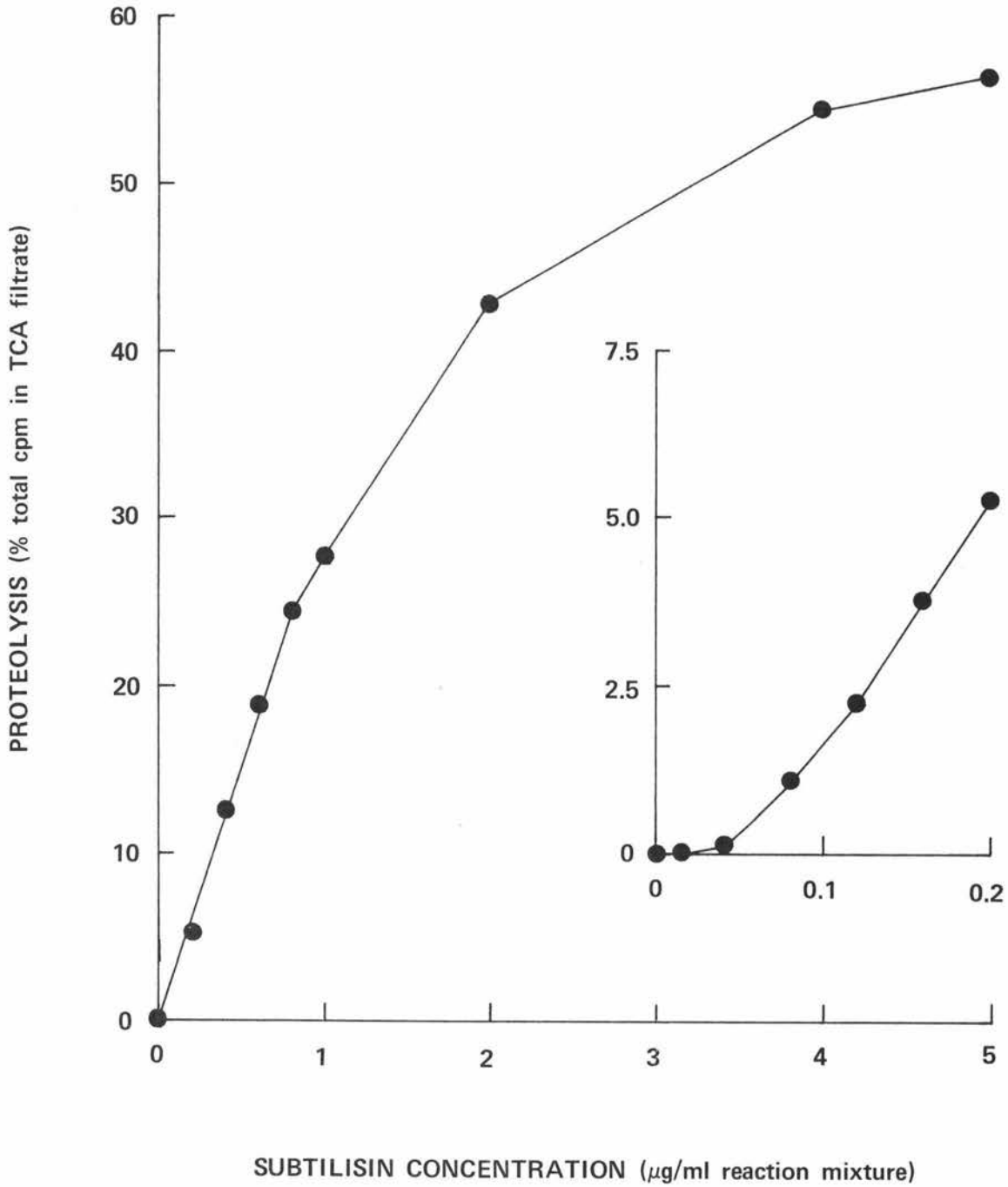

Figure 5. Proteolysis of iodinated casein by subtilisin.
Assay procedure : standard.
Assay conditions : 0.1M phosphate buffer,
pH 7.6, 37°C, 2 hours incubation.

Initial assays of streptococcal proteinase established that, up to 5% hydrolysis, activity was directly proportional to enzyme concentration. All further samples were assayed at a concentration which resulted in less than 5% hydrolysis.

FRACTIONATION PROCEDURES

INTRODUCTION

The procedures given in this section are largely descriptive. Details of individual experiments are given in the 'Results' section.

Cells were harvested, in the mid-logarithmic phase, from T_5 (page 14). All manipulations from this stage were carried out at 0-4°C, except where cell suspensions were incubated with phage lysin. Cell suspensions were stored at -20°C and cell fractions at -75°C until they could be assayed. The stored samples were then thawed by immersion in a 25°C water bath. The progress of cell lysis and of cell conversion to spheroplasts was followed by examination under the phase-contrast microscope.

Two approaches were used to reduce whole cells to their components: ballistic disruption with glass beads (the Mickle method), and conversion of cells to spheroplasts (the spheroplast method).

THE MICKLE METHOD

Cells were washed twice with phosphate buffer, resuspended in either buffer or deionized water (5-8 mg cell dry weight per ml) and disrupted by shaking with glass beads in a Mickle disintegrator (5 ml cell suspension + 3 ml acid-washed Ballotini No. 12, per vial).

At 2-min. intervals during the disruption process the suspensions were chilled on ice for five min. One hundred per cent lysis was achieved within five min. when cells were suspended in water and within seven min. when in buffer. The lysates were filtered through sintered-glass under gentle suction, to remove the beads. The vials and filter apparatus were then washed twice with the appropriate solution and the washings added to the lysate. A summary of this procedure and of the subsequent centrifugation steps is given in Figure 6.

THE SPHEROPLAST METHOD

(i) Preparation of 'cell-wall digest' and 'spheroplast-lysate'. Cells were resuspended (without washing) in spheroplasting medium (0.5 M sucrose, 20 mM Mg^{2+}) to between 2 and 4 mg cell dry weight per ml. Phosphate buffer was added to a final concentration of either 0.1 M (pH 7.0) or 0.2 M (pH 6.4), followed by phage-lysin.[3] The suspension was incubated until 100% conversion to spheroplasts (75 min. at 37°C, 120 min. at 30°C), then chilled. (In some experiments, designed to measure the proteinase released from cells by sucrose only, lysin was omitted from a portion of the cell suspension.) The suspension was centrifuged at 34,800 x g for

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3 Phage m13 lysin (T.D. Thomas, personal communication). High-titre phage lysates of S.lactis ML3 were prepared by the method of Lowrie & Pearce (1971). Phage lysin was purified 30-fold from the lysates by the method of Tsugita, Inouye, Terzaghi & Streisinger (1968). The enzyme was shown to have the same specificity as egg-white lysozyme (Sigma), i.e. a muraminidase, but a much greater specific activity, under the assay conditions. One unit of phage lysin reduces the turbidity (600 nm) of a standard suspension of S.cremoris AM2 by 1.0 in 1 min. at 30°C.
Phage lysin did not hydrolyse the proteinase substrate when assayed at 10 x the highest concentration used in fractionations.

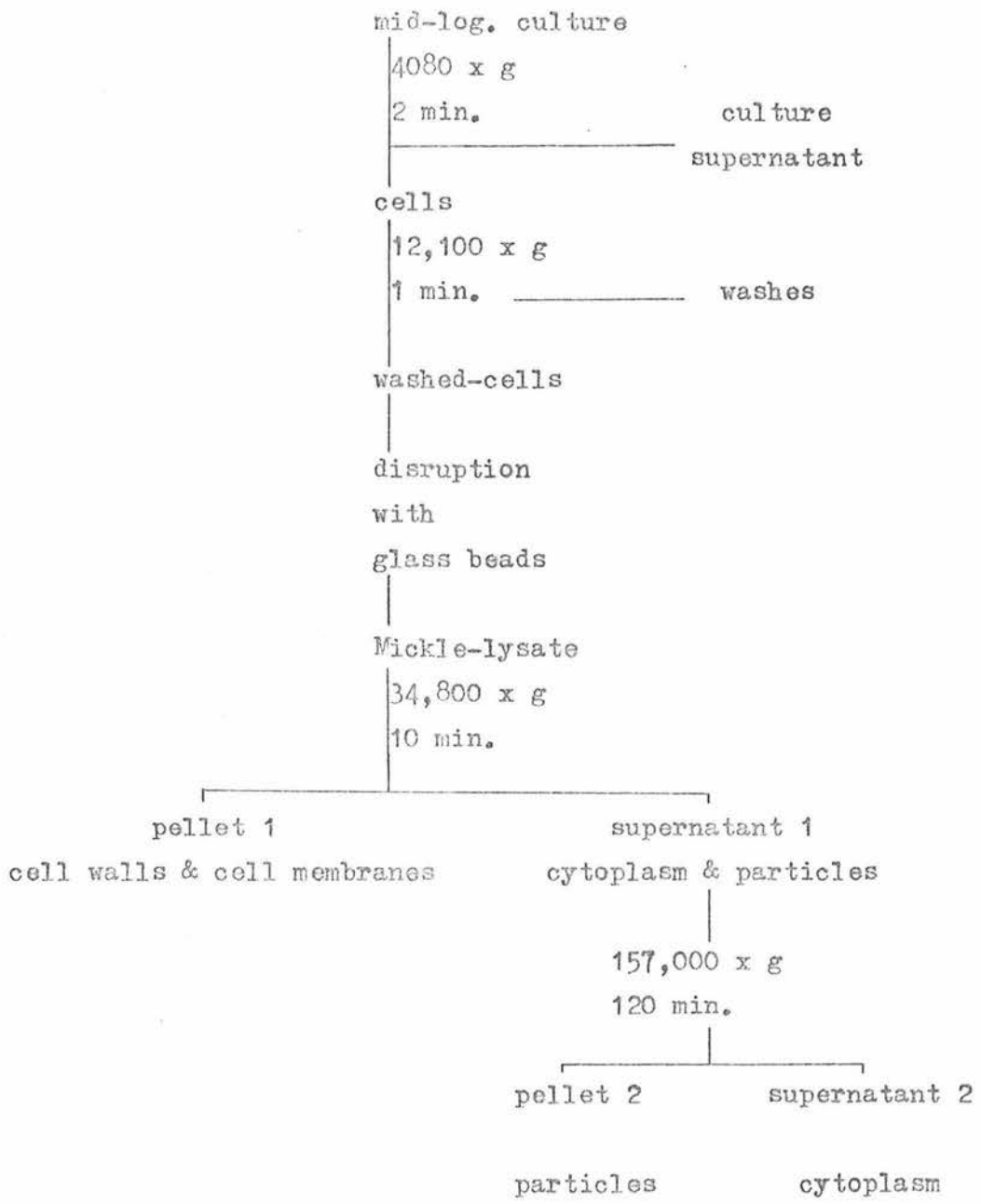


Figure 6. Cell fractionation by the Mickle method.

10 min. to remove the spheroplasts. The 34,800 x g supernatant constituted the 'cell-wall digest'. 'Spheroplast lysates' were prepared by resuspending the pellets in buffer and magnesium^{2+}.

(ii) Preparation of 'lysin-lysates' and 'membranes'. Cells were washed twice with phosphate buffer containing 20 mM Mg^{2+}, resuspended in the same medium and incubated with phage-lysin. Lysis was complete by 75 min. at 37°C and 120 min. at 30°C. A portion of the 'lysin-lysate' was centrifuged at 34,800 x g for 10 min. to pellet the membranes. The 'membranes' were washed three times at the centrifuge with the buffer, Mg^{2+} solution, resuspended in the same, and stored.

R E S U L T S

GROWTH OF S.LACTIS C10

GROWTH IN SKIM MILK. THE SYMPTOMS OF SLOWNESS

S.lactis C10 Slow is defined by its inability to coagulate skim milk (reduce the pH from 6.5 to about 5.1) within 18 hours at 22°C, when inoculated at 1% (v/v) from an 18-hour, 22°C skim milk culture. The overt differences in growth between the variant and parent in milk were examined by inoculating each into the medium, incubating the cultures at 30°C, and sampling them at intervals for pH, lactic acid, and viable count (colony-forming units per ml; cfu/ml) (Figures 7, 8 and 9).

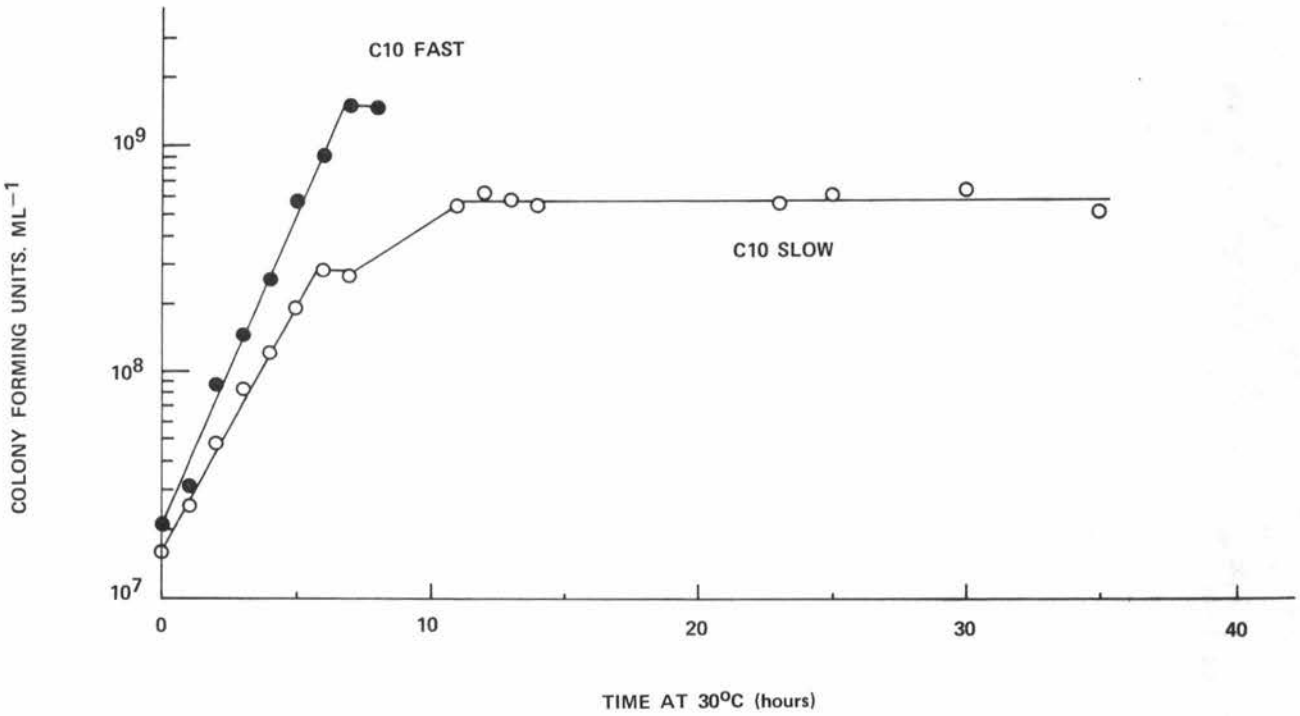
C.10 Fast exhibits the activity of a typical 'fast' Cheddar cheese starter. The population increases exponentially from ca. 2×10^7 to ca. 2×10^9 cfu/ml in seven hours (doubling time 60 min., 6.2 doublings), producing sufficient lactate to clot the medium. In contrast, the growth of C10 Slow is restricted to a maximum population of about 5.5×10^8 cfu/ml, achieved in two stages: (i) an initial period of exponential increase (six hours, 70 min. doubling time, 4.2 doublings), resulting in 2.8×10^8 cfu/ml; (ii) a lag, followed by a single doubling in colony count. At this stage the cells appear to be metabolically normal in that glycolysis continues. The eventual clotting of the milk (28 hours after C10 Fast) reflects a dislocation of acid production from net growth (Figure 9). There was no change in the chain length of either strain during growth in milk.

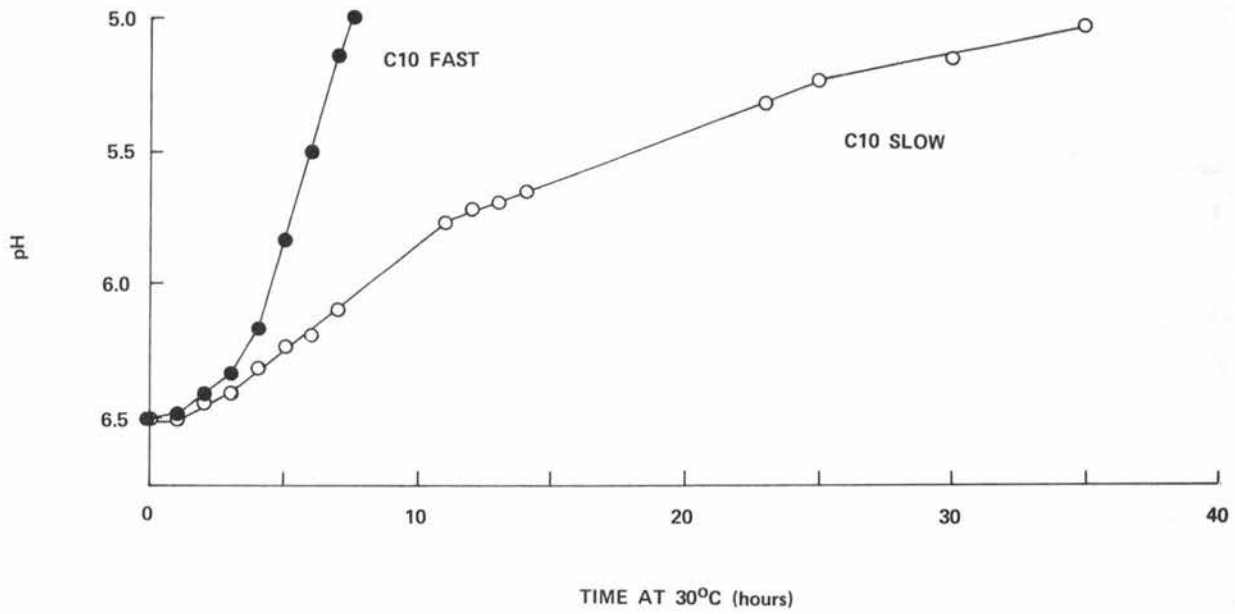
GROWTH IN T_5 BROTH

The development of the parent and variant in broth at 30°C

Figure 7. Growth of S. lactis C10 Fast and S. lactis C10 Slow in skim milk.
200 ml skim milk were inoculated with 2 ml C10 Fast, and 200 ml with 16 ml C10 Slow. Each inoculum was from a skim milk culture (16 hours at 22°C). The cultures were divided into 20 ml portions and incubated at 30°C. At 60 min intervals, samples were chilled, diluted, and plated for colony forming units.

Figure 8. Acid production by S. lactis C10 Fast and S. lactis C10 Slow in skim milk
Skim milk cultures of C10 Fast and C10 Slow were set up as described in Figure 7. At intervals, samples were removed, chilled, and their pH determined.


C10 FAST
C10 SLOW
COLONY FORMING UNITS. ML^{-1}
10^9
10^8
10^7
0
10
20
30
40
TIME AT 30°C (hours)


C10 FAST
C10 SLOW
pH
5.0
5.5
6.0
6.5
0
10
20
30
40
TIME AT 30°C (hours)

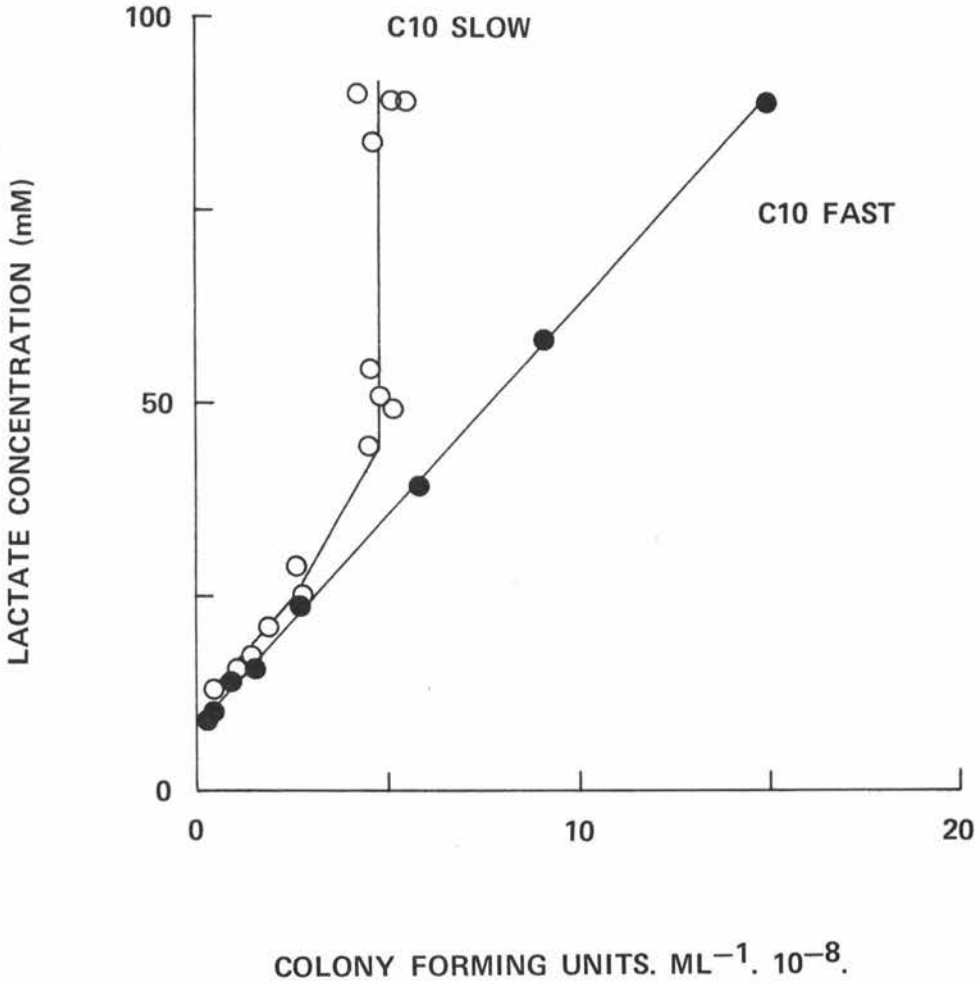


Figure 9. Relationship between growth and acid production in skim milk (30°C).
Skim milk cultures of C10 Fast and C10 Slow were set up as described in Figure 7. At 60 min intervals, portions were removed, chilled, and sampled for lactate and colony forming units.

2. Figures 9 (page 33) and 11 (page 35). Halve the stated lactate values.

served as a control for these growth studies (Figures 10 and 11). In this medium, which contains lactose as the major carbon and energy source and several complex nitrogenous extracts (see 'Methods'), the two strains were indistinguishable. The rapid and extensive growth of C10 Slow (doubling time 38 min., population increase from 1.5×10^7 to 1.2×10^9 cfu/ml) demonstrated that it could utilize lactose as effectively as the parent and was not preferentially inhibited by lactate. Although the relationship between lactate produced and growth was identical for the strains (Figure 11), it was not linear. Furthermore, more lactate was produced per cell in the broth than in milk (cf. Figures 9 and 11).

GROWTH IN MODIFIED SKIM MILK

A series of experiments was set up to test, indirectly, the proteinase-dependency hypothesis. The products of casein hydrolysis, in the form of either "peptides" (enzymatically-hydrolysed casein) or "amino acids" (acid-hydrolysed casein plus L-tryptophane[4]), were added to skim milk before inoculation with either the parent or the variant (Figures 12 and 14). At 1 mg/ml final concentration the effect of the additives on the buffering capacity of the medium was considered to be small, relative to the effect of the metabolic end-product of the cells. For all growth runs in milk C10 Slow was inoculated at four times the concentration (v/v) of C10 Fast, to equalize the initial cell densities.

The addition of "peptides" to milk resulted in a typical

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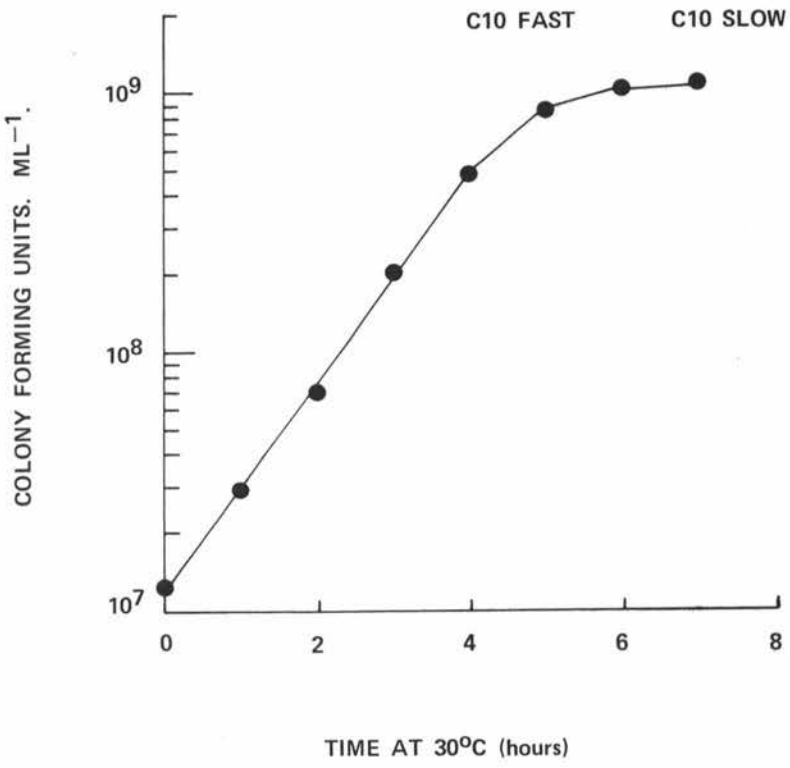
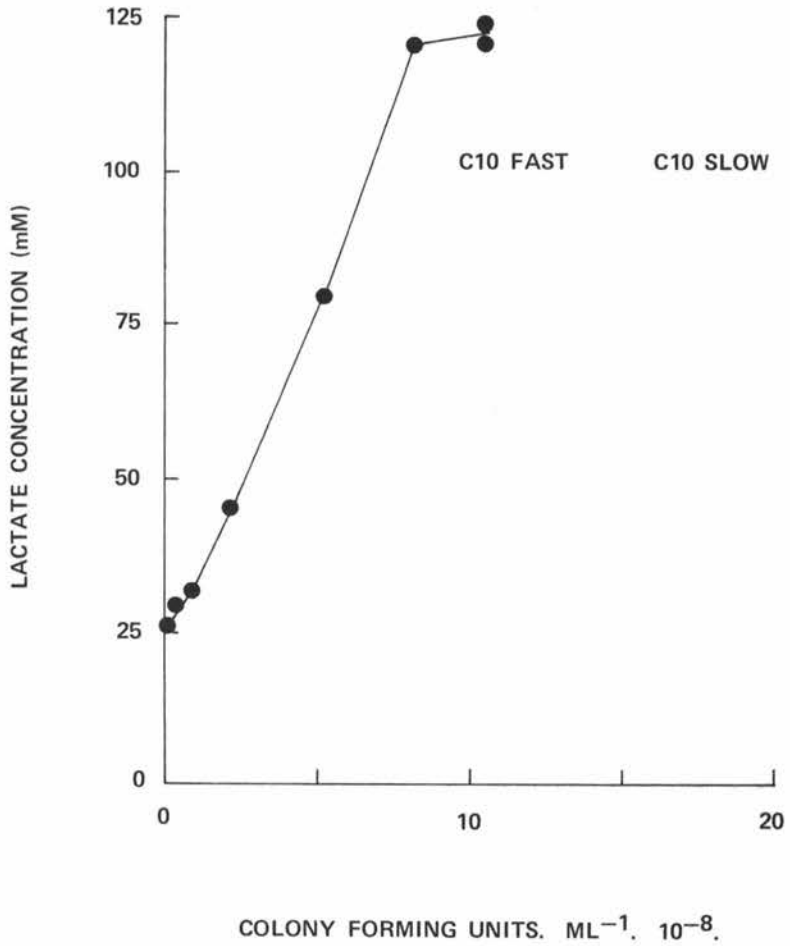
4 L-tryptophane is destroyed by the acid-hydrolysis

Figure 10. Growth of S. lactis C10 Fast and S. lactis C10 Slow in T_5 broth.
200 ml lots of T_5 were inoculated with 2 ml of either C10 Fast or C10 Slow (T_5 cultures, 16 hours at 22°C). The cultures were divided into 20 ml portions and incubated at 30°C. At 60 min intervals, samples were chilled, diluted, and plated for colony forming units.

Figure 11. Relationship between growth and acid production in T_5 broth (30°C).
C10 Fast and C10 Slow cultures were set up as described in Figure 10. At 60 min intervals, portions were removed, chilled, and sampled for lactate and colony forming units.

ERRATUM: Figure 11 (Page 35). Halve the stated lactate values.


C10 FAST
C10 SLOW
COLONY FORMING UNITS. ML^{-1}.
10^9
10^8
10^7
0
2
4
6
8
TIME AT 30°C (hours)


LACTATE CONCENTRATION (mM)
125
100
75
50
25
0
C10 FAST
C10 SLOW
0
10
20
COLONY FORMING UNITS. ML^{-1}. 10^{-8}.

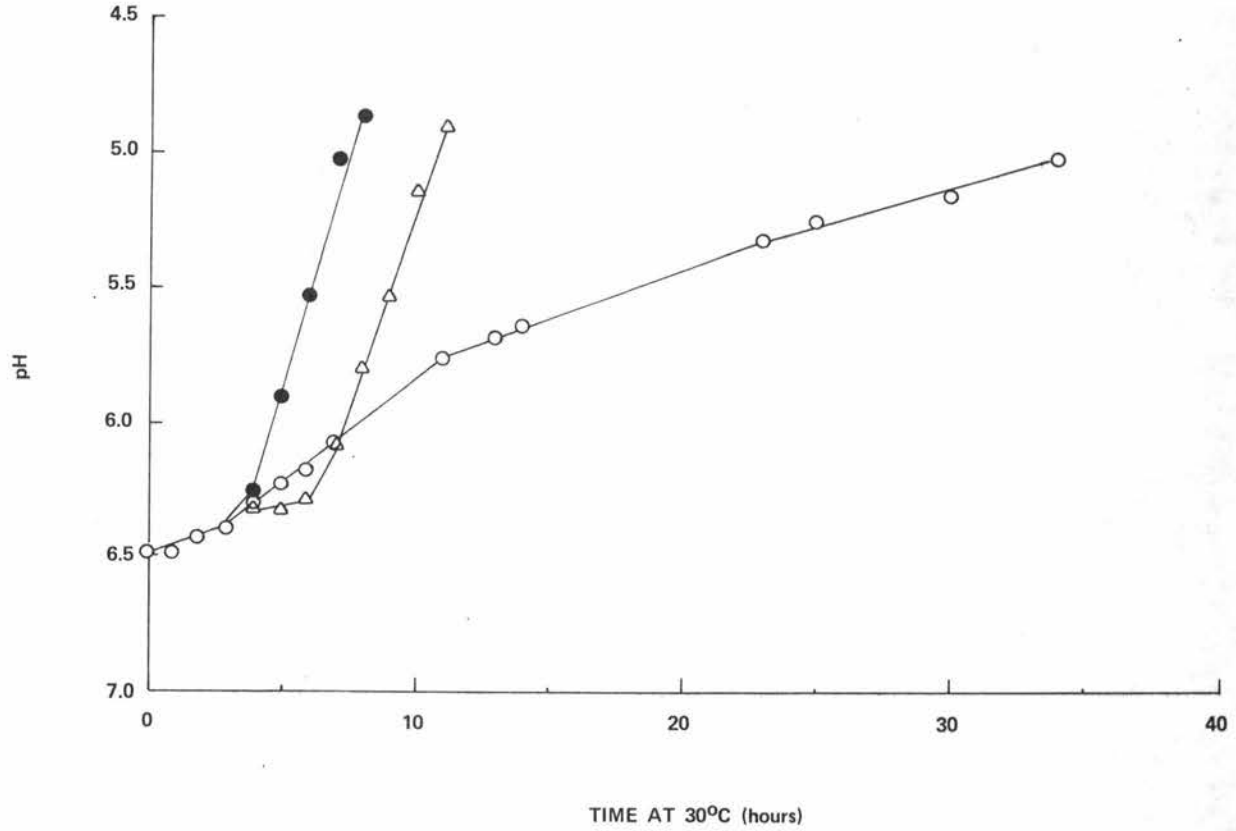
'fast' acid-production curve by the variant (Figure 12). This hydrolysate also stimulated the parent (Figure 14), and both strains reached a population of about 3 x 10^9 cfu/ml, with a doubling time during exponential increase of ca. 54 min. (data not shown). The acid hydrolysate of casein appeared to inhibit the development of both strains to some extent (Figures 12 and 14), both acid production and growth being erratic for six hours. However, the eventual growth of the cultures was both rapid and extensive (doubling time 54 min., to about 3 x 10^9 cfu/ml).

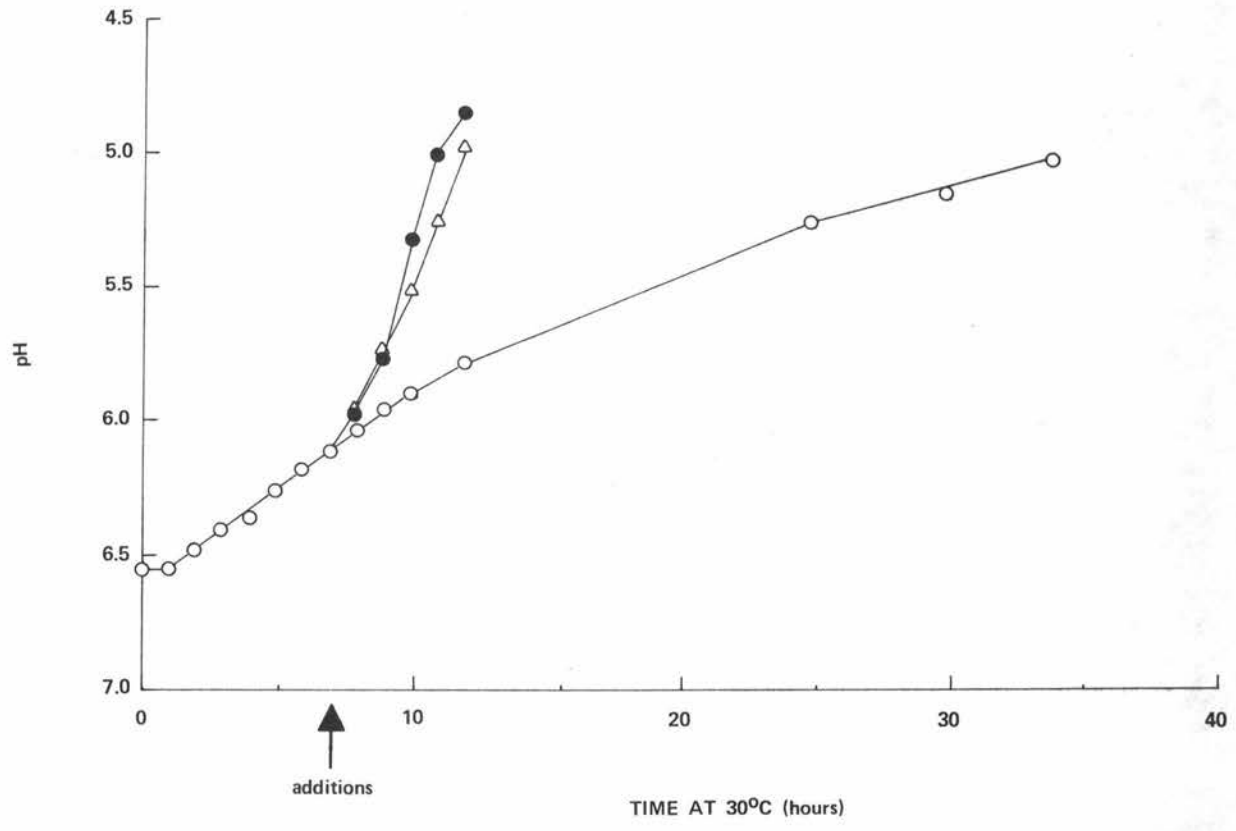
The growth of C10 Slow in unmodified skim milk indicated a two-stage process (Figure 7), the second, short burst of growth being presumably due to a change to an alternative source or form of some nutrient. When the casein hydrolysates were added to the variant in milk after it had reached the end of its initial rapid development, stimulation by both was immediate (Figure 13).

These results provided good evidence that the growth of S. lactis C10 in skim milk is rate-limited and, for C10 Slow, extent-limited, by the supply of protein-degredation products. The apparently preferential stimulation by "peptides" may indicate the preferred form of amino acids for uptake by the cells, but the hydrolysates were too ill-defined to allow firm conclusions on this point.

Figure 12. Effect of casein hydrolysates on S. lactis C10 Slow in skim milk. 1. Hydrolysates added before inoculation. 200 ml batches of skim milk, fortified with either acid hydrolysed casein (1 mg/ml) and L–tryptophane (1μg/ml) or enzyme hydrolysed casein (1 mg/ml), were each inoculated with 8 ml C10 Slow (skim milk culture, 16 hours at 22°C). The cultures were divided into portions and incubated at 30°C. At 60 min intervals, samples were chilled and their pH determined.
(△), skim milk + acid hydrolysate + L–tryptophane; (●), skim milk + enzyme hydrolysate; (○), unmodified skim milk (data from Figure 7).

Figure 13. Effect of casein hydrolysates on S. lactis C10 Slow in skim milk. 2. Hydrolysates added 7 hours after inoculation.
300 ml skim milk were inoculated with 12 ml C10 Slow (skim milk culture, 16 hours at 22°C). The culture was divided into 10 ml lots and incubated at 30°C. After 7 hours, the samples were modified by the addition of either acid hydrolysed casein (to 1mg/ml) and L–tryptophane (to 1μg/ml) (△); enzyme hydrolysed casein (●) to 1 mg/ml; or water (○). At hourly intervals, throughout the incubation, samples were removed, chilled, and their pH determined.


4.5
5.0
5.5
6.0
6.5
7.0
pH
0
10
20
30
40
TIME AT 30°C (hours)


4.5
5.0
5.5
6.0
6.5
7.0
pH
0
10
20
30
40
additions
TIME AT 30°C (hours)

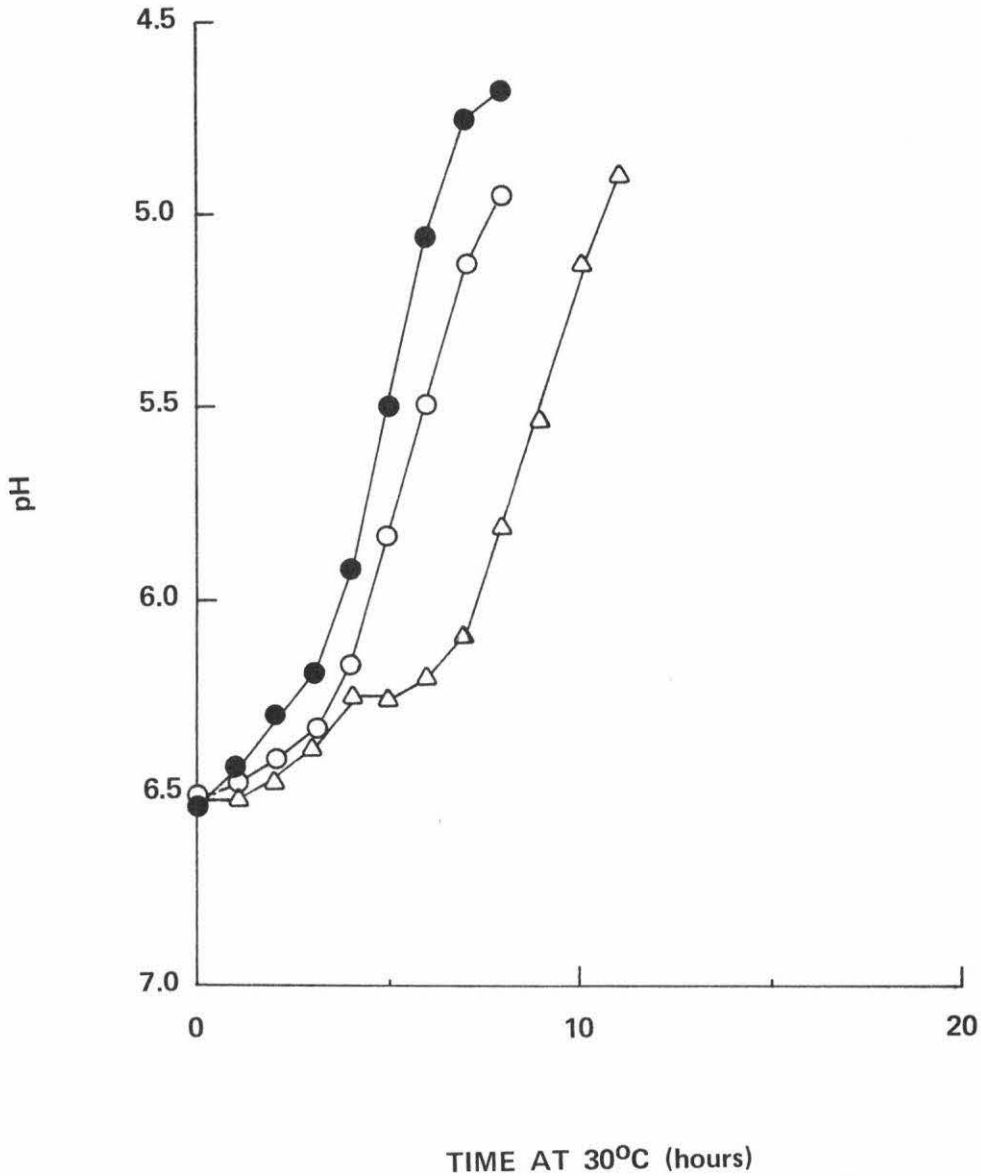


Figure 14. Effect of casein hydrolysates on S. lactis C10 Fast in skim milk.
200 ml batches of skim milk, fortified with either acid hydrolysed casein (1 mg/ml) and L–tryptophane (1μg/ml) or enzyme hydrolysed casein (1 mg/ml), were each inoculated with 2 ml C10 Fast. (skim milk culture, 16 hours at 22°C). The cultures were divided into portions and incubated at 30°C. At 60 min intervals, samples were removed, chilled, and their pH determined.
(△), skim milk + acid hydrolysate + L–tryptophane;
(●), skim milk + enzyme hydrolysate; (○), unmodified skim milk (data from Figure 7).

PRELIMINARY EXPERIMENTS TO LOCALIZE S.LACTIS C10 PROTEINASES

Skim milk was the only medium in which the 'slowness' of the variant was expressed, but presented technical difficulties with harvesting of cells. Attempts to devise an optically clear differential medium were not successful; a casein medium[5], similar to one reported to maintain the growth characteristics of S.lactis 3 Fast and S.lactis 3 Slow (Westhoff, Cowman & Speck, 1971), supported excellent growth of both C10 strains (doubling time 42 min., at 30°C). Assay of washed cells from T_5 broth cultures of C10 Fast and C10 Slow showed that, despite identity of growth, proteolytic activity was different. This medium was, therefore, selected for the preparation of experimental cells, since it permitted efficient harvesting.

The initial experiments to quantify and localize the proteinases synthesized by the two strains in the permissive broth medium were designed to define the most active fractions. It was assumed, on the basis of substrate accessibility, that casein-degrading enzymes would be either extracellular (free in the medium) or surface-bound, and the preliminary fractionations were concentrated in these areas.

PROTEINASE ASSAYS

The proteolytic activity of fractions was determined by the standard procedure, the substrate being diluted in 0.1 M phosphate buffer, pH 7.6, prior to addition to the samples. Each fraction

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5 Composition, per litre: casein, 20 g; lactose, 10 g; yeast extract, 1 g; 0.5 M potassium dihydrogen phosphate, 11.5 ml; 0.5 M disodium hydrogen phosphate, 62.8 ml, autoclaved at 15 psi for 15 min., pH (cool) 6.9.

was assayed at three concentrations, and this established an excellent linear relationship between fraction concentration and per cent cpm appearing in the TCA-filtrate, up to 5%.

ENZYME DISTRIBUTION: MICKLE METHOD

Cells of either C10 Fast or C10 Slow, grown and harvested by the standard procedure (page 14), were washed twice with 0.1 M phosphate buffer, pH 7.0, containing 10 mM Mg^{2+}, resuspended in deionized water (C10 Fast, 7.4 mg cell dry weight per ml; C10 Slow, 8.4 mg cell dry weight per ml), and processed in the Mickle (page 27). A portion of each "Mickle-lysate" was centrifuged to prepare the "cytoplasm", as described in Figure 6. Portions of the original cell pellets were washed twice with buffer, Mg^{2+}, resuspended in the buffer, and stored at -20°C. Proteolytic activities of washed cells, Mickle-lysates, and the cytoplasm fractions are given in Table 3.

ENZYME DISTRIBUTION: SPHEROPLAST METHOD

(i) T_5-grown cells were resuspended directly in spheroplasting medium (0.5 M sucrose, 20 mM Mg^{2+}) and made up to 0.1 M phosphate, pH 7.0, with concentrated buffer. Phage lysin (360 units) was added to each cell suspension. The suspensions (C10 Fast, 5.9 mg cell dry weight per ml; C10 Slow, 6.5 mg cell dry weight per ml) were mixed thoroughly, incubated at 37°C until all cells had been converted to spheroplasts (75 min.), then centrifuged in the cold for 10 min. at 34,800 x g. The supernatants ("cell-wall digests") were centrifuged at 157,000 x g for 120 min. to remove any particles, then stored at -75°C.

(ii) T_5-grown cells were washed twice with 0.1 M phosphate buffer, pH 7.0, containing 10 mM Mg^{2+}, resuspended in the buffer,

Mg^{2+}, and incubated at 37°C with phage lysin (31 units per mg cell dry weight C10 Fast; 28 units per mg cell dry weight C10 Slow). Lysis was complete by 65 min., but the lysates were left at 37°C for an additional 10 min. to equate the treatment with that used to prepare "cell-wall digests". A portion of each "lysin-lysate" was centrifuged at 34,800 x g for 10 min. to pellet "membranes" (also contains undigested cell wall and large cell-wall fragments). The "membranes" were washed three times at the centrifuge with 0.02 M phosphate buffer, pH 7.0, containing 10 mM Mg^{2+}, to remove cytoplasmic materials. In a typical experiment this procedure reduced the E_{260} of the wash from 4.5 to 0.15. This represented a 99% reduction in absorbance, relative to that associated with total lysates ("lysin-lysates") of S.lactis C10, and was interpreted to mean near-complete removal of nucleic acid material. "Membranes" were then resuspended and stored at -75°C. Table 3 gives the proteolytic activities of the fractions.

It was evident that the cell-bound activity of S.lactis C10 was predominantly surface-bound, and further that the slow variant was deficient in this respect. The near equivalence of whole-cell and cell-lysate (Mickle) activities, the low proteinase in "membrane" and "cytoplasm" fractions, and the relatively very active C10 Fast "cell-wall digest" all indicated that the enzymes are associated with the envelope, probably the cell wall. Although the culture supernatants were inactive, the possibility that either strain secretes proteinase into the medium during active growth was not excluded, since such activity would be dilute. The marked loss of activity observed in "lysin-lysates", relative to whole cells, Mickle-lysates, and cell-wall digests, of C10 Fast, was anomalous, but was later partially resolved by

Table 3. Proteinase localization in S.lactis C10 (preliminary data).

	FRACTION	SPECIFIC ACTIVITY[a] absolute C10 Fast	SPECIFIC ACTIVITY[a] absolute C10 Slow	SPECIFIC ACTIVITY[a] relative C10 Fast : C10 Slow	DISTRIBUTION % activity of washed-cells C10 Fast	DISTRIBUTION % activity of washed-cells C10 Slow
b	washed-cells	9.4	3.9	2.4	100	100
b	culture supernatant	0	0	1.0	0	0
b	Mickle-lysate	9.8	3.6	2.7	104	92
b	cytoplasm	1.3	1.0	1.3	14	26
c	washed-cells	9.4	3.9	2.4	100	100
c	cell-wall digest	16.2	0.7	23.1	172	18
c	lysin-lysate	2.4	2.3	1.0	26	59
c	membranes	0	0	1.0	0	0

a units proteinase activity per mg cell dry weight equivalent

b fractions prepared by the "Mickle method"

c fractions prepared by the "spheroplast method"

the finding that the enzymes were unstable at 37°C.

The fractionations described were each repeated several times and it became obvious that progress was hampered by the lack of knowledge of the properties of the enzymes assayed. The C10 Fast cell-wall digest fraction was selected as the source of crude proteinase for characterization since it was both the most active fraction and derived from the cellular compartment likely to be of most relevance to the difference between the two strains.

CHARACTERIZATION OF (CRUDE) S.LACTIS C10 FAST SURFACE-PROTEINASE(S).

The influence of pH, buffer strength, temperature, and well-known activators and inhibitors of proteinases on the activity of the C10 Fast cell-wall digest was examined. Substrate blanks were incorporated into all assays to control for the possible effects of each condition on the stability of the iodinated casein. Preparation of the crude enzyme is described in Figure 15. The dialysis step, designed to remove sucrose, Mg^{2+}, and phosphate from the fraction, reduced the activity by approximately 70%.

Although the enzyme was dilute and the number of experiments limited, it was clear that the activity was very sensitive to changes in pH and temperature and, to a lesser extent, in buffer strength (Figures 15, 16 and 17). The double pH optimum, 6.0 and 6.8, was consistently observed and could be attributed to either the presence of two proteinases or to some peculiarity

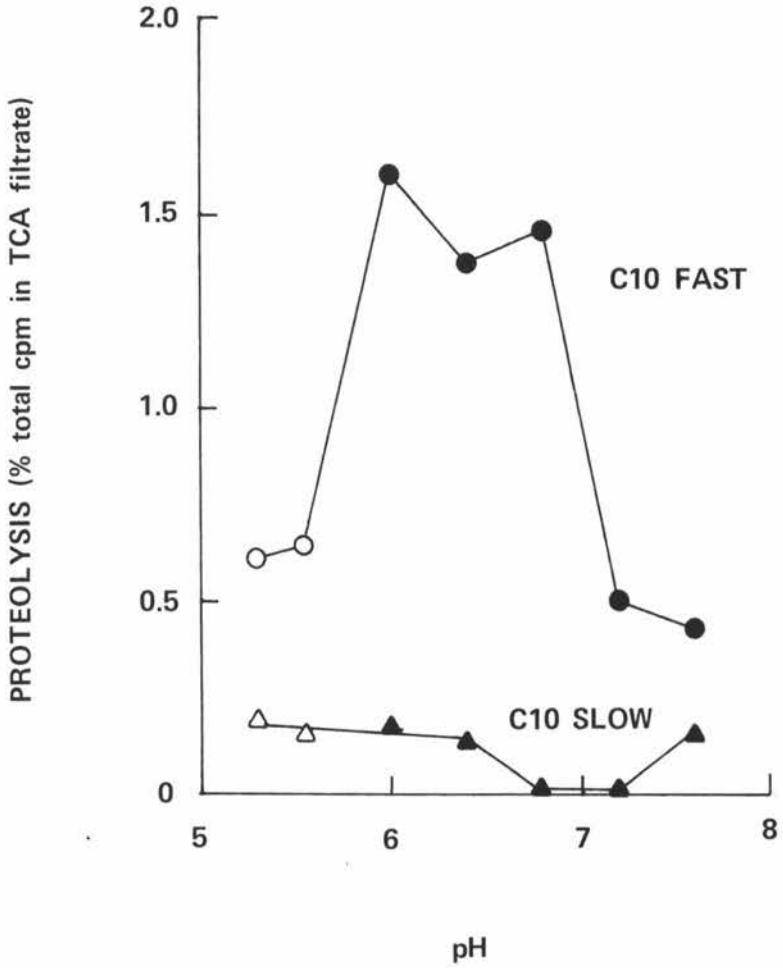


Figure 15. Effect of pH on S. lactis C10 surface proteinase(s).
A mid logarithmic C10 Fast culture (T_5 broth, 30°C) was divided into two portions and centrifuged. One pellet was washed twice with buffer (0.1 M phosphate, pH 7.0, to 20 ml), resuspended in 10 ml buffer, and frozen. The cell dry weight was determined. The second pellet was resuspended in 8.5 ml spheroplasting medium. Phosphate buffer was added to 0.1 M, pH 7.0, final concentration, followed by phage lysin (180 units). The suspension was made up to 10 ml (4.4 mg cell dry weight /ml) with buffer and incubated at 37°C. After 75 min, the suspension was chilled and centrifuged (34,800 g, 10 min), 4 ml of the supernatant (cell wall digest) was dialysed overnight at 4°C against 2 litres of deionised water. The same procedure was used to prepare a dialysed cell wall digest from C10 Slow (5.2 mg cell dry weight/ml incubated cell suspension).
The digests were assayed for proteinase by the standard procedure. Assay conditions : 6 hours at 30°C, in either 0.05M phosphate buffer (●,▲), or 0.05M acetate buffer (○,△).

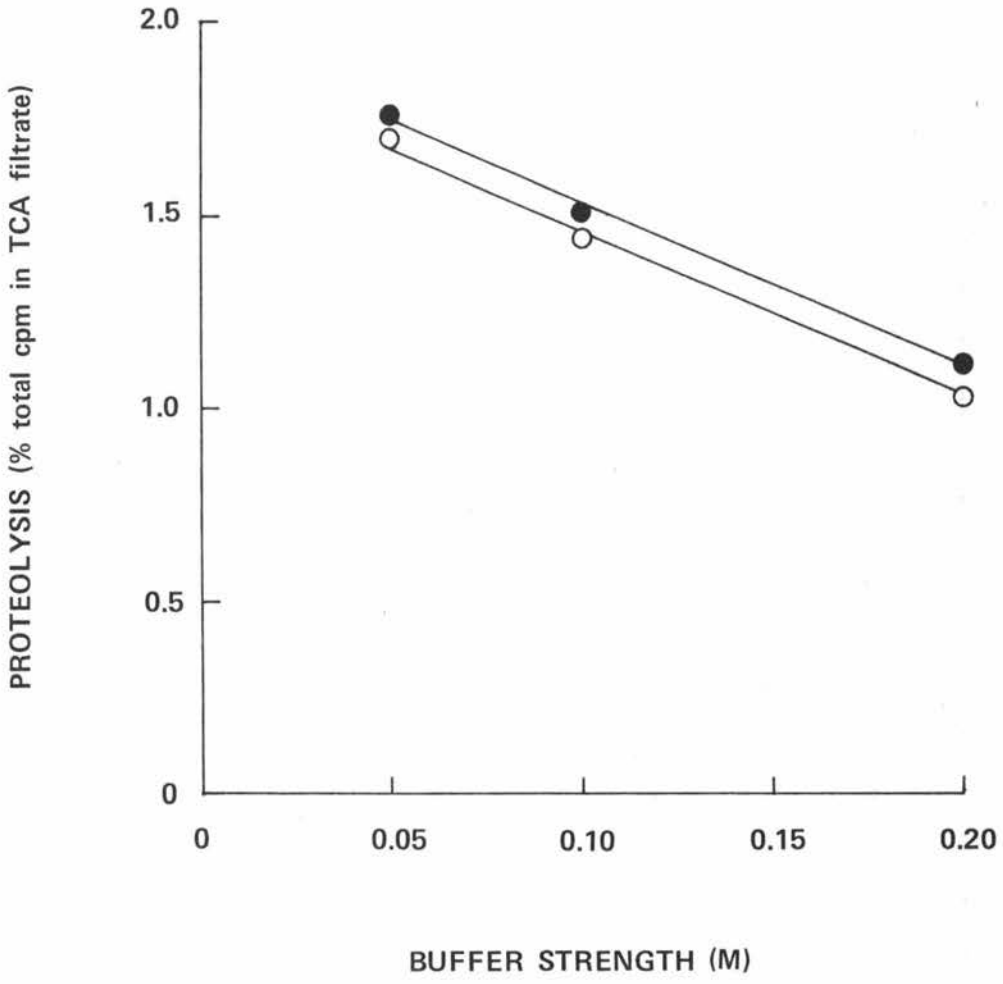


Figure 16. Effect of buffer strength on S. lactis C10 Fast surface proteinase(s).
Dialysed cell wall digest, prepared as described in Figure 15, was assayed for proteinase by the standard procedure, Assay conditions : 6 hours at 30°C, in either phosphate buffer, pH 6.0 (●), or phosphate buffer, ph 6.8 (○).

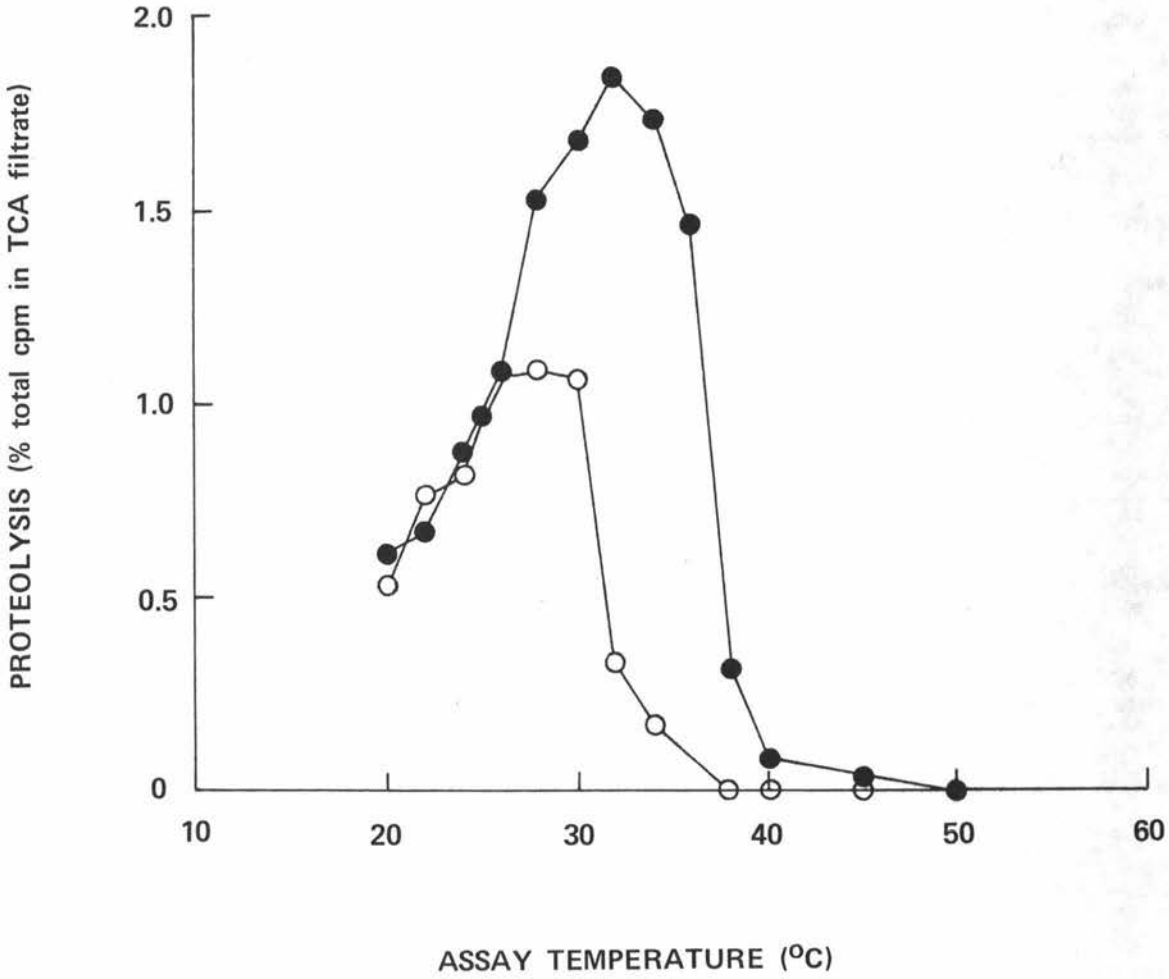


Figure 17. Effect of temperature on S. lactis C10 Fast surface proteinase(s).
Dialysed cell wall digest, prepared as described in Figure 15, was assayed for proteinase by the standard procedure. Assay conditions : 6 hours at the temperature indicated, in either 0.05M phosphate, pH 6.0 (●), or 0.05M phosphate, pH 6.8 (○).

of the complex substrate. The assay pH had a marked influence on the effect of temperature; changing the pH from 6.0 to 6.8 both depressed the maximum proteinase activity and shifted the temperature optimum (Figure 17). The C10 Slow cell-wall digest, prepared and dialysed as for the parent strain fraction, was tested for pH optimum, but its activity was very low under all conditions (Figure 15).

Experiments were carried out to determine the effect of proteinase inhibitors and activators on the C10 Fast fraction (dialysed against water, as described in Figure 15). It was appreciated that the results might be restricted in value by the ill-defined nature of the digest, but the intention was to determine if an activator was necessary for maximum activity. Neither thiol poisons (parachloromercuribenzoic acid, para-hydroxymercuribenzoic acid) nor reducing agents (L-cysteine, sodium thioglycollate) altered the activity when included in the incubated assay mixture at 1.75×10^{-4} M, indicating that the enzyme(s) were not of the well-known sulphydryl group (e.g. papain, ficin, group A streptococcal proteinase) (Liu & Elliot, 1971). Proteolysis was depressed 70-80% by 1 mM EDTA (ethylenediaminetetraacetic acid); this sensitivity, together with the observed large loss of activity upon dialysis against water, suggested that the enzymes required metal ions for maximum activity. This point was not pursued. The properties of thiol poison insensitivity, pH optima near neutrality, and EDTA-sensitivity, are common to several microbial proteinases (the 'metal chelator-sensitive neutral proteinases', Matsubara & Feder, 1971), and the C10 Fast enzymes may belong to this group.

STANDARDIZATION OF CONDITIONS OF FRACTIONATION AND ASSAY

The conditions of assay exerted a major influence on the detected proteinase activity, the effect of any one parameter being modified by alterations in the others (Figures 15, 16 and 17). Overall, maximum proteolysis by the cell-wall digest was obtained in 0.05 M phosphate buffer, at pH 6.0 to 6.8, and from 30-32°C. In order to ensure that the activities of fractions were comparable, it was necessary to define both the environment in which the fractions were prepared and the conditions of subsequent assay. Assay conditions chosen were 0.2 M phosphate buffer, pH 6.4; 30°C, and six hours' incubation. The selected pH was a compromise between the two optima (Figure 15); the temperature of previous assays was retained, since it was close to the determined optimum (Figure 17); and the buffer strength was selected to improve the buffering capacity and to minimize differences in ionic composition between fractions. Although 0.2 M buffer depressed proteolysis 37% relative to 0.05 M (Figure 16) its superior buffering capacity (4-fold at pH 6.4; Long, 1961) was considered an asset. All further fractionations were based on the 0.2 M buffer to allow assay without dialysis. In addition, subsequent fractionations with phage lysin were conducted at 30°C instead of 37°C, since the rapid fall in proteolysis at temperatures above 32°C (Figure 17) suggested irreversible inactivation.

LOCALIZATION OF S.LACTIS C10 PROTEINASES

The localization of proteolytic activity in the two strains

was re-examined, using the standardized conditions.

ENZYME LOCALIZATION: MICKLE METHOD

C10 Fast and C10 Slow were fractionated by the procedure outlined in Figure 6. Each strain was processed twice; a representative experiment is described. Approximately 1200 ml of mid-logarithmic culture (T_5 broth, 30°C) were centrifuged at 4080 x g and the pellet washed twice with 0.2 M phosphate buffer, pH 6.4. A portion of the culture supernatant was filtered through a Millipore membrane (0.22 μ) to remove any cells, and stored for assay. The washed cells were resuspended in 40 ml buffer and divided into two portions. One portion was washed again, re-suspended in buffer, and stored at -20°C ("washed cells", for dry weight and proteinase assay). The second was centrifuged at 12,100 x g, resuspended to 7.6 mg cell dry weight per ml in buffer, and disrupted in the Mickle. Complete lysis required 7 min. shaking with the beads, 2 min. longer than that required for 100% disruption in deionized water. The lysates were pooled and filtered through sintered-glass to remove the beads. The disruption-vials and filter apparatus were rinsed three times with buffer and the washings added to the lysate. A portion of the "Mickle-lysate" was subjected to differential centrifugation (Figure 6) to prepare "cell walls and cell membranes", "particles", and "cytoplasm". The various buffer washes of whole cells were pooled and a sample kept for assay.

All fractions were assayed for proteolytic activity by the standard procedure and under the standardized conditions. The data given in Table 4 represent the averages of results from the replicate experiments with each strain.

Table 4. Proteinase localization in S. lactis C10: enzyme distribution in fractions prepared by the Mickle method[a].

FRACTION	SPECIFIC ACTIVITY[b]			DISTRIBUTION	
	absolute		relative	% activity of washed-cells	
	C10 Fast	C10 Slow	C10 Fast : C10 Slow	C10 Fast	C10 Slow
washed-cells	30.0	5.2	5.8	100	100
buffer washes	0	0	1.0	0	0
Mickle-lysate	37.6	4.4	8.6	125	85
cell walls + cell membranes	18.6	1.3	14.3	62	25
cytoplasm + particles	6.3	2.7	2.3	21	52
cytoplasm	4.2	2.2	1.9	14	42
particles	2.0	1.4	1.4	7	27

a see Figure 6 (page 29)

b units proteinase activity per mg cell dry weight equivalent

Eighty per cent of the C10 Fast Mickle-lysate activity was exhibited by whole cells, indicating that most proteinase is surface-bound. Of the activity recovered in the component fractions (66% of the lysate activity), 75% was associated with the cell walls and cell membranes (the 34,800 x g pellet) and 25% with the cytoplasm and particles. C10 Slow fractions were all less proteolytic than those of the parent strain, the difference being marked in the wall and membrane fractions. Of the proteinase recovered from the C10 Slow total lysate, 32% was associated with walls and membranes, and the remainder with the cytoplasm and particles. In terms of absolute proteinase activity, there was little to distinguish the non-envelope fractions of the two strains; the slightly higher activity of the C10 Fast fractions (1-2 fold) is attributed to solubilization of envelope-bound enzymes by the disruption procedure. Neither culture supernatants nor whole-cell buffer washes contained detectable proteinase.

ENZYME LOCALIZATION: SPHEROPLAST METHOD

Preliminary experiments with phage lysin had indicated that cell-wall digestion removed the large part of the C10 Fast cell-bound proteinases, while the effect on the variant was minimal (Table 3). The following experiments were designed to amplify this statement and to allow a comparison between the two techniques of fractionation.

(i) Time-course of proteinase release.

C10 Fast and C10 Slow were incubated at 30°C in either spheroplasting medium (0.5 M sucrose, 20 mM Mg^{2+}) and buffer, or spheroplasting medium, buffer, and phage lysin. At intervals, samples of the cell suspensions were chilled, centrifuged, and

the supernatants assayed for proteinase (Figure 18). Both the muraminidase and the sugar caused a progressive release of proteinase from the cell-bound form in C10 Fast, with a similar but less easily detectable effect on the variant. Table 5 shows the enzyme activities of the various fractions after 120-min. incubation at 30°C. The proteinase removed by phage lysin alone was calculated by subtracting that released by sucrose from that released by sucrose and lysin. It accounted for 76% of the C10 Fast whole-cell activity and 23% of C10 Slow whole-cell activity. In absolute terms, all of the "cell-wall digests" derived from the parental cells were considerably more proteolytic than the equivalent fractions from the variant.

(ii) Residual proteinase.

The experiments described in (i) were repeated, without attempt to follow the time-course of proteinase release, to establish the levels of enzyme associated with the residual structures.

A 500-ml mid-logarithmic C10 Fast culture (T_5 broth, 30°C) was divided into two portions and centrifuged. One pellet was washed twice with 0.2 M phosphate buffer, pH 6.4, resuspended in buffer, and frozen (washed cells). The second pellet was resuspended directly in 8.5 ml spheroplasting medium and divided in half. Concentrated buffer was added to each portion. Phage lysin (180 units) was added to one portion and an equivalent volume of water to the other. Each was made up to 5.0 ml (2.2 mg cell dry weight per ml) and incubated at 30°C (final composition: 0.2 M phosphate buffer, 0.5 M sucrose, 20 mM Mg^{2+}, phage lysin, cells). After 120 min. the suspensions were chilled, centrifuged at 34,800 x g for 10 min. and the supernatants ("cell-wall digests")

Figure 18. Time course of release of proteinase(s) from osmotically stabilised cells of S. lactis C10 Fast and S. lactis C10 Slow.

A mid logarithmic C10 Fast culture (T_5 broth, 30°C) was divided into two portions and centrifuged. One pellet was washed twice with buffer (0.2M phosphate, pH 6.4, to 40 ml), resuspended in 20 ml buffer, and frozen. The second pellet was resuspended in 19 ml spherophasting medium and divided in half. Phosphate buffer was added to each portion to give 0.2M, pH 6.4, final concentration. Phage lysin (75 units) was added to one portion and water to the other. The suspensions were made up to 10 ml (2.2 mg cell dry weight/ml) with buffer and incubated at 30°C. At intervals, samples were removed, chilled, and centrifuged (34,800 g, 10 min). The supernatants (cell wall digests) were frozen. The same procedure was used to prepare C10 Slow cells and cell wall digests (2.2 mg cell dry weight/ml incubated cell suspensions).

Cells and cell wall digests were assayed for proteinase by the standard procedure, under standard conditions (0.2M phosphate buffer, pH 6.4, 6 hours at 30°C).

(▶), washed cells; (●), sucrose/lysin digest; (○), sucrose digest. C10 FAST

(▶), washed cells; (▲), sucrose/lysin digest; (△), sucrose digest. C10 SLOW.

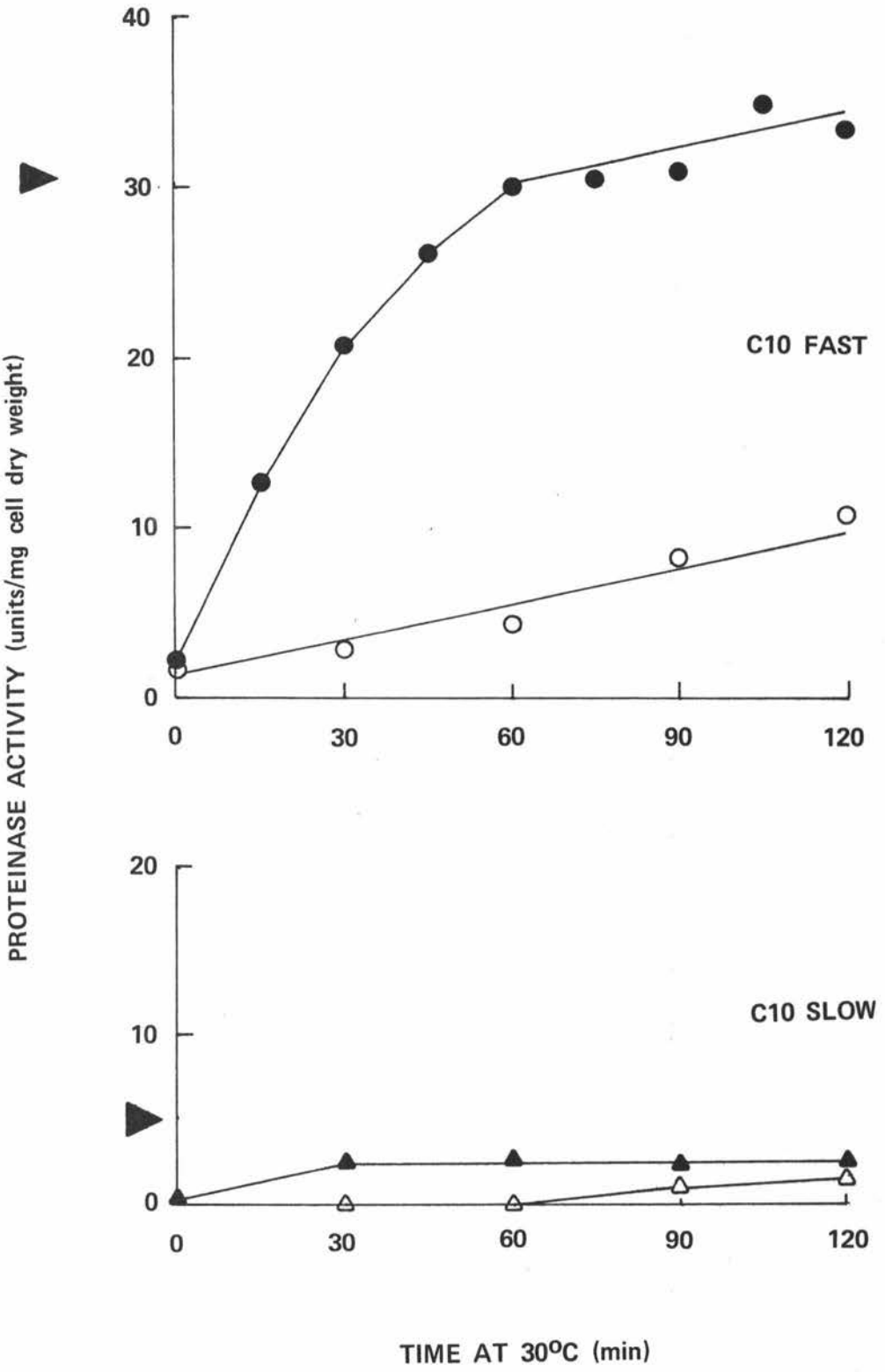

40
30
20
10
0
C10 FAST
0
30
60
90
120
20
10
0
C10 SLOW
0
30
60
90
120
PROTEINASE ACTIVITY (units/mg cell dry weight)
TIME AT 30°C (min)

Table 5. Release of cell-bound proteinase from osmotically-stabilized cells of S.lactis C10 Fast and S.lactis C10 Slow[a].

FRACTION	SPECIFIC ACTIVITY[b]			DISTRIBUTION	
	absolute		relative	% activity of washed-cells	
	C10 Fast	C10 Slow	C10 Fast: C10 Slow	C10 Fast	C10 Slow
washed cells[c]	30.0	5.2	5.8	100	100
lysin/sucrose cell-wall digest[d]	33.6	2.7	12.5	112	52
sucrose cell-wall digest[e]	10.8	1.5	7.2	36	29
lysin cell-wall digest[f]	22.8	1.2	19.0	76	23

a see Figure 18 (page 53) for details of preparation

b units proteinase activity per mg cell dry weight equivalent

c reference activity: T_5-grown cells washed and suspended in buffer

d,e 34,800 x g supernatants of cells incubated for 120 min. at 30°C

f = (d-e)

frozen. The 34,800 x g pellets were each resuspended to 5.0 ml with 0.2 M phosphate buffer. Resuspension of the pelleted cells which had been incubated with lysin caused 100% lysis - the fraction is referred to as the "spheroplast-lysate". Resuspension of the second pellet caused no appreciable lysis (as judged by microscopic examination under phase contrast) and the fraction is referred to as "resuspended cells".

The same procedure was used to prepare C10 Slow fractions (1.7 mg cell dry weight per ml incubated suspensions). All fractions were assayed for proteinase under the standardized conditions; the results are given in Table 6. They substantiate those from the experiments described under (i), although enzyme recoveries were high. Incubation of C10 Fast cells with phage lysin and spheroplasting medium detached 134% of the cell-bound proteinase (washed cells) from the cells, whereas incubation in spheroplasting medium alone caused the release of 32%. The net release, attributed to lysin, was 102%, but this equivalence to the whole-cell activity is probably fortuitous. Activity remaining with residual structures (spheroplast lysates) was 25% of that associated with cells and less than 20% of the "lysin/sucrose cell-wall digest" proteinase.

The specific activity of any given fraction fluctuated considerably from experiment to experiment, as may be seen from examination of Tables 4, 5, and 6. The activities of "cell-wall digests" were especially variable in this respect. Table 7 summarizes and compares the data from the various experiments. Fractions have been equated in terms of the cellular structures and components deduced to be contained in them. "Cell walls and cell membranes", defined as those parts of mechanically-

Table 6. Proteinase localization in S.lactis C10: enzyme distribution in fractions prepared by the spheroplast method.

FRACTION	SPECIFIC ACTIVITY[a]			DISTRIBUTION	
	absolute		relative	% activity of washed-cells	
	C10 Fast	C10 Slow	C10 Fast : C10 Slow	C10 Fast	C10 Slow
washed-cells	35.8	5.2	6.9	100	100
lysin/sucrose cell-wall digest[b]	47.8	2.6	18.4	134	50
spheroplast-lysate[c]	9.0	5.6	1.6	25	108
washed-cells	35.8	5.2	6.9	100	100
sucrose cell-wall digest[d]	11.4	0.4	28.6	32	8
resuspended cells[e]	12.8	6.2	2.1	36	120

a units proteinase activity per mg cell dry weight equivalent

b 34,800 x g supernatant of cells which had been incubated in spheroplasting medium, phosphate buffer, and phage lysin, for 120 min. (30°C)

c 34,800 x g pellet of b, resuspended in phosphate buffer

d 34,800 x g supernatant of cells which had been incubated in spheroplasting medium and phosphate buffer for 120 min. (30°C)

e 34,800 x g pellet of d, resuspended in phosphate buffer

Table 7. Proteinase localization in S.lactis C10: comparison of results from the two fractionation methods.

		MICKLE METHOD			SPHEROPLAST METHOD		
		specific activity	% activity of cells	distribution between particulate and soluble fractions (%)	specific activity	% activity of cells	distribution between particulate and soluble fractions (%)
C10 Fast	whole-cells	30.0	100	-	35.8	100	-
	cell lysate	37.6	125	-	56.8[c]	159	-
	particulate fraction	18.6[a]	62	75	47.8[d]	134	84
	soluble fraction	6.3[b]	21	25	9.0[e]	25	16
C10 Slow	whole-cells	5.2	100	-	5.2	100	-
	cell lysate	4.4	85	-	8.2[c]	158	-
	particulate fraction	1.3[a]	25	32	2.6[d]	50	32
	soluble fraction	2.7[b]	52	68	5.6[e]	108	68

a activity in "cell walls + cell membranes" ┐ data from Table 4
b activity in "cytoplasm + particles" ┘

c activity in "lysin/sucrose cell-wall digest" + "spheroplast lysate" ┐
d activity in "lysin/sucrose cell-wall digest" ├ data from Table 6
e activity in "spheroplast lysate" ┘

disrupted cells which sediment at 34,800 x g, are equated with the "lysin/sucrose cell-wall digests" from cells incubated with lysin. Similarly, "cytoplasm and particle" fractions are considered as equivalent to "spheroplast-lysates". The result is clearly a good agreement between the data provided by each method of localization, in terms of the distribution of proteinase between 'particulate' and 'soluble' fractions. It demonstrates explicitly the fundamental difference between the two strains of S.lactis, in terms of both proteolytic activity and cell-bound enzyme distribution.

INSTABILITY OF S.LACTIS C10 PROTEINASES AT 37°C

It was found during the preliminary experiments that C10 Fast "lysin-lysates", prepared by incubating washed cells in phosphate buffer with phage lysin, contained less activity than both whole cells and "cell-wall digests" (Table 3). The effect of the preparation procedure on C10 Slow was less obvious, although this may have been apparent rather than real, since the slow variant activities were always very low. The following experiments demonstrated that the parental surface-bound enzymes were very unstable at 37°C, the temperature used initially to prepare fractions with lysin.

WHOLE-CELL PROTEINASES

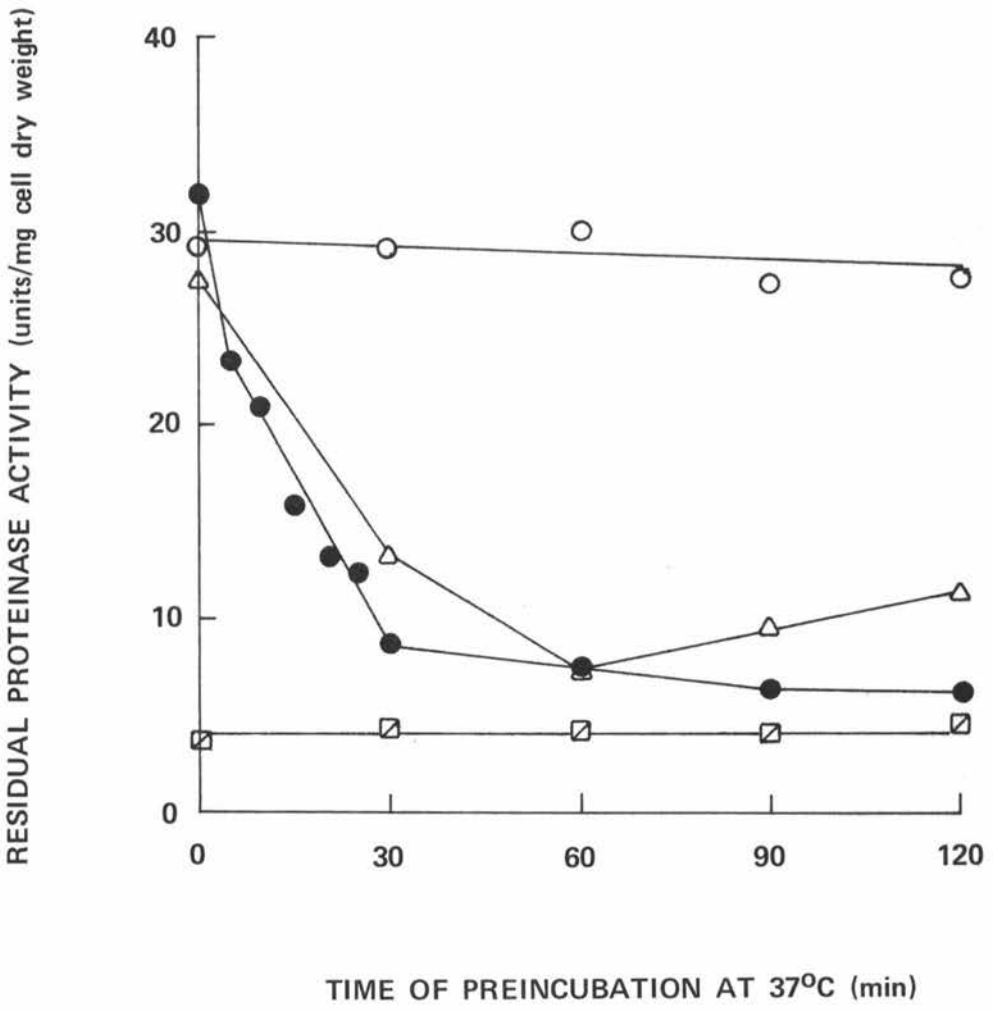
The proteolytic activity of washed C10 Slow cells was unaffected by incubation at 37°C in 0.2 M phosphate buffer (Figure 19). In contrast, C10 Fast cells rapidly lost activity under these conditions. After 60 min., 75% of the proteinase was lost,

Figure 19. Inactivation of S. lactis C10 Fast whole cell proteinase(s) at 37°C.

A mid logarithmic C10 Fast culture (T_5 broth, 30°C) was divided into three portions and centrifuged. The pellets were washed twice with 20 ml of either buffer (0.2M phosphate, pH 6.4), buffer containing magnesium chloride (20 mM), or buffer containing magnesium chloride and sucrose (20 mM Mg, 0.5M sucrose). The washed cells were resuspended to 4.4 mg cell dry weight/ml in the buffer solutions and incubated at 37°C. A C10 Slow cell suspension (4.2 mg cell dry weight/ml) was prepared in a similar way by washing and resuspending in 0.2M phosphate, pH 6.4. It was incubated at 37°C.

At intervals, samples were removed, chilled, and frozen. They were assayed for proteinase under standard conditions.

(○), C10 Fast cells in phosphate, magnesium, sucrose.
(△), C10 Fast cells in phosphate, magnesium.
(●), C10 Fast cells in phosphate.
(▨), C10 Slow cells in phosphate.


RESIDUAL PROTEINASE ACTIVITY (units/mg cell dry weight)
40
30
20
10
0
0
30
60
90
120
TIME OF PREINCUBATION AT 37°C (min)

leaving an activity which was stable and similar quantitatively to that of the C10 Slow cells. The inclusion of sucrose and magnesium, but not magnesium alone, protected the C10 Fast cell-bound enzymes from inactivation (Figure 19).

CELL-WALL DIGEST PROTEINASES

Sucrose and magnesium did not protect the C10 Fast enzymes from the effects of 37°C incubation when included with the cell-free fractions (Figure 20). Both "sucrose/lysin" and "sucrose" cell-wall digests exhibited a progressive loss of activity at this temperature. The inactivation was less rapid than that of whole cells (sucrose/lysin digest, 31%; sucrose digest, 45%; whole cells in buffer, 72%; by 30 min.) but more extensive (98%; 91%; and 78%) by 120 min. As was the case with washed cells, the C10 Slow cell-wall digest activities were unaffected by the treatment.

These results implied that the C10 Fast digest used as the source of enzyme for characterization (Figure 15), already reduced in activity by dialysis, was in addition partially heat-inactivated. They did not completely account for the discrepancy between the activities of cell-wall digests and the "lysin-lysates", both prepared at 37°C (Table 3).

LOCALIZATION OF S.LACTIS H1 PROTEINASES

S.lactis H1 Fast and S.lactis H1 Slow were each fractionated with phage lysin to determine if their cell-bound proteinases were similar, in activity and location, to those of S.lactis C10. The preparation of cells and cell-wall digests is described in

Figure 20. Inactivation of S. lactis C10 Fast surface proteinase(s) at 37°C.

Washed cells, sucrose/lysin cell wall digests, and sucrose cell wall digests were prepared from C10 Fast and C10 Slow as described in Figure 18. (The cell wall digests were separated from the incubated cells after 120 min at 30°C). The digests were thawed (25°C), incubated at 37°C, and sampled at 30 min intervals. The samples were assayed under standard conditions (0.2M phosphate buffer, pH 6.4, 6 hours at 30°C).

(▷), washed cells; (●), sucrose/lysin digest; (○), sucrose digest. C10 Fast.

(▷), washed cells; (▲), sucrose/lysin digest; (△), sucrose digest. C10 Slow.

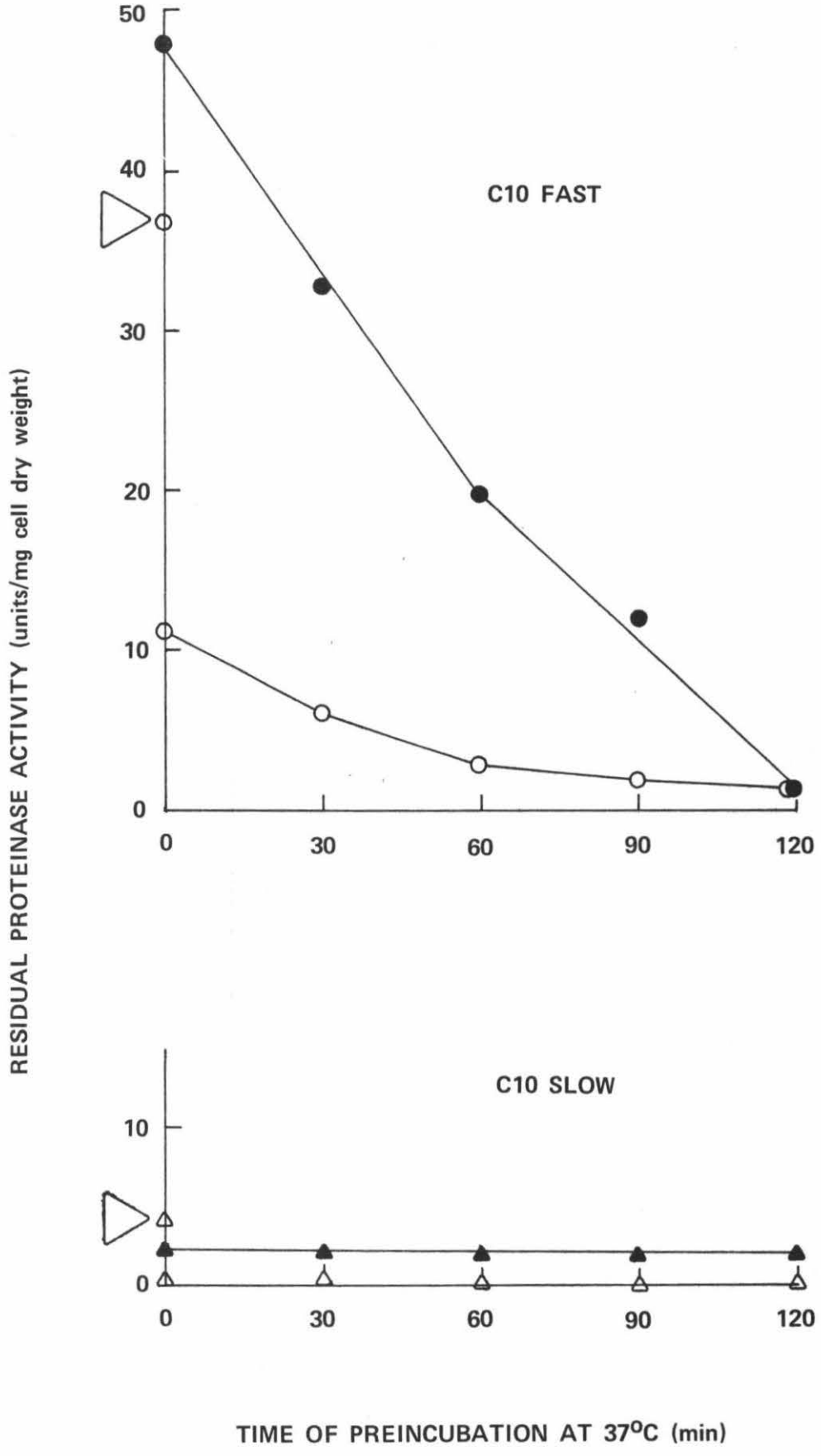

C10 FAST
C10 SLOW
RESIDUAL PROTEINASE ACTIVITY (units/mg cell dry weight)
TIME OF PREINCUBATION AT 37°C (min)
50
40
30
20
10
0
0
30
60
90
120

Figure 21. "Spheroplast-lysates" and "resuspended cells" were prepared, as described for C10, by resuspending (in 0.2 M phosphate buffer, pH 6.4) the pellets from cells incubated in either spheroplasting medium and phage lysin or in spheroplasting medium alone.

The fractions were assayed for proteinase under the conditions determined for S.lactis C10 (0.2 M phosphate buffer, pH 6.4; 30°C; 6 hours' incubation). The results (Table 8) indicated a pattern similar to that established for C10. Incubation of cells with lysin removed proteinase from both the parent and the variant; however, in contrast to C10 Fast, where lysin caused the major part of proteinase-release (Table 5), 73% of the enzymes from H1 Fast were removed by spheroplasting medium alone (Table 8).

The H1 Fast cell-free digests were sensitive to incubation at 37°C (Figure 21) but to a much lesser extent than the equivalent fractions from C10 Fast (cf. Figure 19). By 120 min. at 37°C, the H1 extracts were inactivated by 25-30%, whereas the C10 extracts were reduced in activity by 90-100%. In general, the difference in absolute proteolytic activity between the two strains of S.lactis H1 was less marked than in the case of C10.

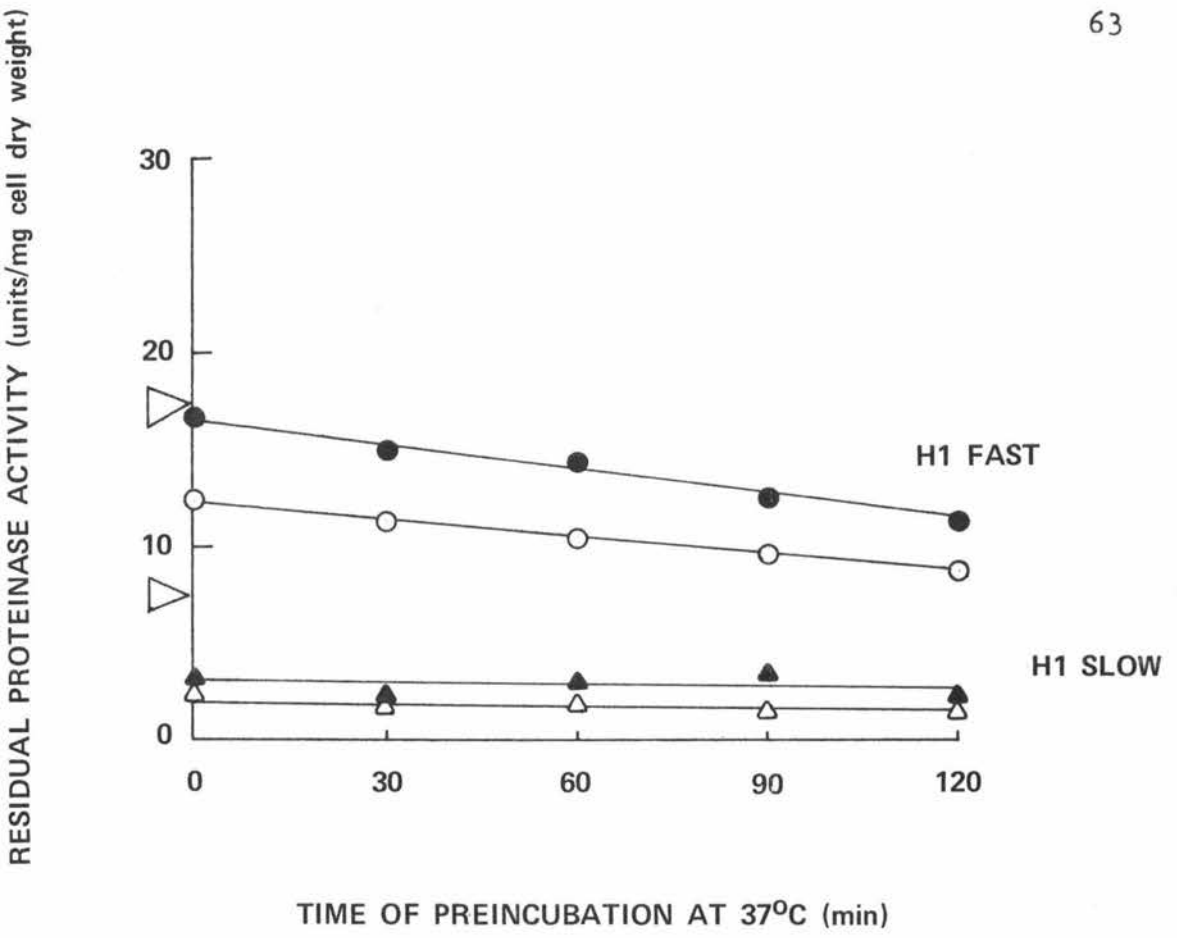


Figure 21. Inactivation of S. lactis H1 Fast surface proteinase(s) at 37°C.
A mid logarithmic H1 Fast culture (T_5 broth, 30°C) was divided into two portions and centrifuged. One pellet was washed twice with buffer (0.2M phosphate, pH 6.4, to 40 ml), resuspended in 20 ml buffer, and frozen. The second pellet was resuspended in 19 ml spheroplasting medium and divided in half. Phosphate buffer was added to each portion to give 0.2M, pH 6.4, final concentration. Phage lysin (75 units) was added to one portion and water to the other. The suspensions were made up to 10 ml (2.2 mg cell dry weight/ml) with buffer and incubated at 37°C. After 120 min, they were chilled, centrifuged (34,800 g, 10 min), and the supernatants (cell wall digests) frozen. The same procedure was used to prepare H1 Slow cells and cell wall digests (2.5 mg cell dry weight/ml incubated cell suspensions).
The four cell wall digests were thawed (25°C), incubated at 37°C, and sampled at 30 min intervals. Samples were assayed for proteinase under standard conditions.
(▷), washed cells; (●) sucrose/lysin digest; (○), sucrose digest. H1 FAST.
(▷), washed cells; (▲), sucrose/lysin digest; (△), sucrose digest. H1 SLOW.

Table 8. Proteinase localization in *S. lactis* H1[a].

FRACTION	SPECIFIC ACTIVITY[b]			DISTRIBUTION	
	absolute		relative	% activity of washed-cells	
	H1 Fast	H1 Slow	H1 Fast : H1 Slow	H1 Fast	H1 Slow
washed-cells	17.0	7.5	2.3	100	100
lysin/sucrose cell-wall digest	16.8	3.6	4.7	99	48
sucrose cell-wall digest	12.4	2.5	5.0	73	33
lysin cell-wall digest[c]	4.4	1.1	4.0	26	15
spheroplast-lysate	7.9	10.7	0.74	46	142
resuspended cells	10.3	11.8	0.88	61	160

a see Figure 18 (page 53) for details of preparation of cells and cell-wall digests; "spheroplast-lysates" were obtained by resuspending the cells from the (sucrose/lysin) suspensions with 0.2 M phosphate buffer, pH 6.4; "resuspended cells" were obtained by resuspending the cells from the (sucrose) suspensions with buffer

b units proteinase activity per mg cell dry weight equivalent

c calculated by subtracting (sucrose cell-wall digest) activity from (sucrose/lysin cell-wall digest) activity

D I S C U S S I O N

PROTEINASE LOCALIZATION

The uptake of amino acids by the bacterial cell is dependent on specific transport systems (Payne & Gilvarg, 1971) and, ultimately, on a supply of transportable units from the medium. Starter enzymes with the ability to degrate proteins and peptides could be secreted into the medium (extracellular), bound to the cell envelope and accessible to the medium (surface-bound), or contained within the cytoplasmic membrane (intracellular). In view of this probable multiplicity of enzymes which can hydrolyse peptide bonds, it is essential that activities be localized; only then can physiological function be rationally deduced.

Proteinases whose function is to supply the cell with amino acids from exogenous protein will be, a-priori, extracellular or surface-bound, since it is unlikely that the substrate can penetrate either the cell wall or the cytoplasmic membrane. It is pertinent, in this regard, that the upper size limit of peptides that are transported into Escherichia coli appears to be that equivalent to six amino acids (Payne & Gilvarg, 1971). Neither strain of S.lactis C10 secreted detectable proteolytic activity into the medium while growing in the nutrient broth. This was, however, expected on the grounds of dilution, and does not exclude the possibility that these organisms produce extracellular enzymes, either in T_5 broth or in milk. Williamson, Tove & Speck (1964) reported that S.lactis E., cultured in a defined medium containing whole-casein, secreted a heat-stable proteinase into the medium. However, the cells were removed from the medium at the end of the logarithmic

phase of growth and, in the absence of data to establish negligible cell-lysis, the description 'extracellular' must be regarded with doubt.

The proteolytic activity associated with cells of C10 Fast (the cell-bound proteinase) has been shown in the present investigation to be predominantly surface-bound. Pollock (1962) has suggested three criteria which would indicate this location for any cell-bound enzyme, as follows: (i) the enzyme can be extracted by procedures specifically designed to destroy the cell wall under conditions where the protoplast is protected from disintegration and the cytoplasmic membrane remains intact; (ii) the enzyme metabolizes a strictly insoluble, highly polymerized, or high molecular weight substrate which is unlikely to be able to penetrate the cell; and (iii) the enzyme is neutralized by a specific antiserum or by any type of large molecule which is believed to be incapable of penetrating the cytoplasmic membrane. Condition (ii) is met and (i) has been established for C10 Fast with some degree of certainty. Treatment of the cells with phage lysin in the presence of hypertonic sucrose, magnesium, and phosphate buffer detached the large part of cell-bound proteinase from the material which sedimented at 34,800 x g (Figure 18 and Table 5). The magnesium (20 mM) was included with incubated cells to maintain membrane integrity (Weibull, 1956; Sheinin, 1959; Pollock, 1961) and to prevent extrusion of mesosomes from the membrane into the surrounding fluid (Reaveley & Rogers, 1969). It has been assumed that this precaution would both prevent significant leakage of intracellular components from the cells during the spheroplasting process and minimize detachment of membrane-bound enzymes.

The detachment of proteinase from C10 Fast cells could not be attributed simply to the effects of the added muraminidase on the streptococcal cell-wall (Figure 18) and, indeed, in the case of H1 Fast only a small part of the release was due specifically to the presence of phage lysin (Table 8). The time-dependent release of C10 Fast enzyme into the spheroplasting medium, in the absence of phage lysin, was apparently due to the sucrose since washing the cells repeatedly with 0.2 M phosphate buffer containing 20 mM Mg^{2+} (at 0-4°C) caused no detectable detachment. The sugar, molecular weight 342, is considered to have free access to the cytoplasmic membrane and its effect on the bound proteinases may involve a plasmolysis of the cytoplasmic membrane away from the wall, resulting in some sort of physical expulsion of proteinase. Kushner & Pollock (1961) found that high sucrose concentrations (0.9 M) were essential for the trypsin-mediated release of penicillinase from Bacillus subtilis. They considered that the physical effects of the sugar on the envelope allowed the protease access to the sites of penicillinase attachment; sucrose alone caused no appreciable release. In contrast, the experiments with C10 Fast suggest that continued incubation of the cells in spheroplasting medium alone would eventually result in complete release of proteinase (Figure 18). If this were true then there is no need to invoke peptidoglycan-degredation by the lysin as having any other function but that of aiding the sucrose-mediated release by weakening the cell wall.

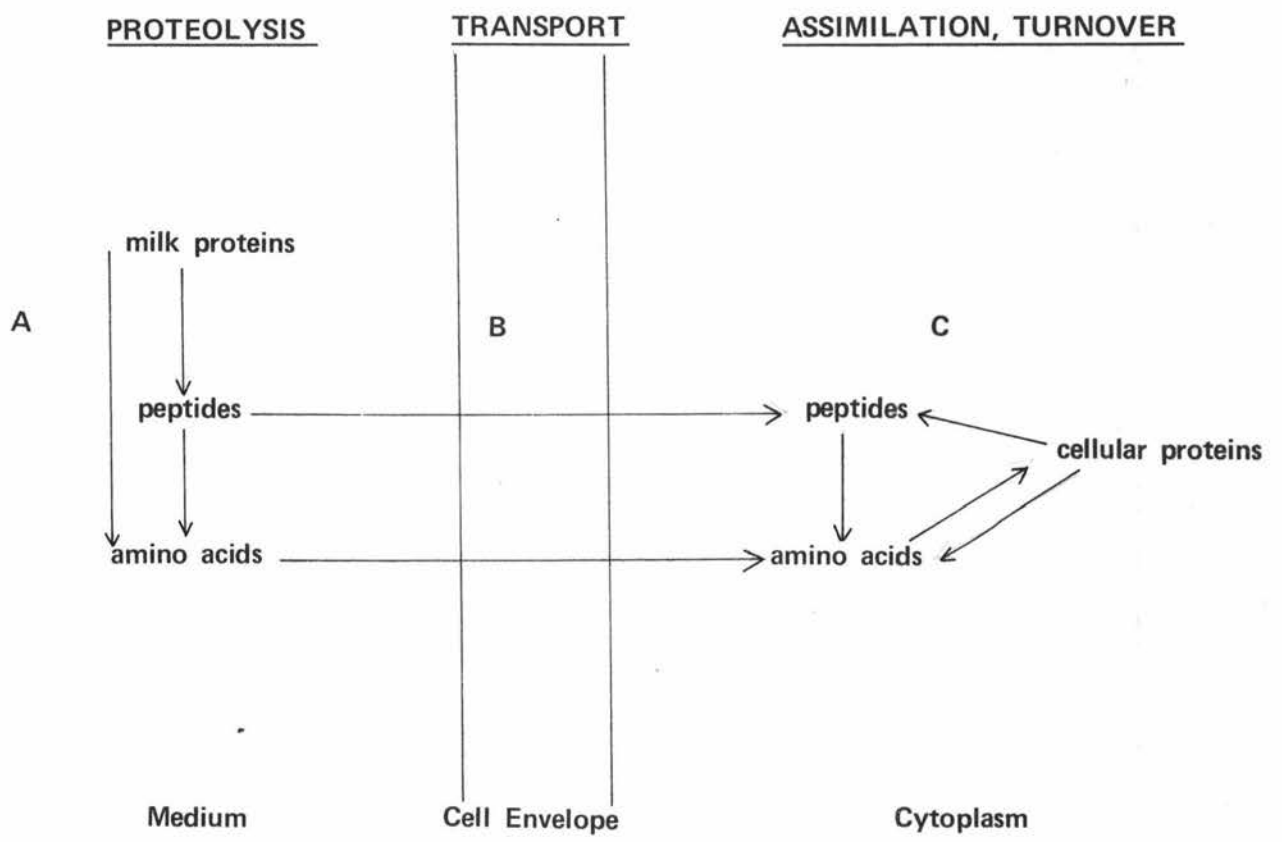
There was no evidence to suggest that the surface-bound C10 Fast proteinases were bound by an ionic linkage, such as has been shown for proteinases of a Micrococcus species and Mucor hiemalis (McDonald, 1962; Wang, 1967), penicillinase in

Staphylococcus aureus (Coles & Gross, 1967), and acid phosphatase in S. aureus (Malveaux & San Clemente, 1967). Neither washing the cells with 0.2 M sodium/potassium phosphate nor the inclusion of 0.3 M sodium chloride in the spheroplasting medium caused release of activity.

Twenty-five per cent of the proteinase recovered from C10 Fast cells and 68% of that from the slow variant was contained in fractions which were not associated with surface-structures (the 'soluble' fractions, Table 7). These activities could be derived in part from the walls or membranes by the manipulations or may indicate a distinct enzyme - the 'intracellular' proteinase (Westhoff & Cowman, 1971). It is, however, unlikely that the bacterial cytoplasm contains enzymes whose function is to degrade casein; it is more likely that the intracellular enzymes catalyse peptide-hydrolysis and the turnover of endogenous nitrogen (Figure 22). There is no doubt that the complexity of the substrate used in the present investigation has precluded any distinction to be made between enzymes which function to hydrolyse protein ('proteinases') and those whose role is peptide-degredation ('peptidases'). This is especially relevant to any interpretation of 'soluble' activities. For instance, E. coli has been shown to contain at least eight distinct peptidases, no less than four of which hydrolyse the lysyl-lysyl bond (Sussman & Gilvarg, 1971). None of the E. coli peptidases was surface-bound. The proteolytic activities associated with the non-envelope fractions of both S. lactis C10 strains are treated with reserve, and are interpreted as indicating, in addition to some contamination from other fractions, the presence of intracellular peptidases.

Both selective treatment of intact cells by the sphero-

Figure 22. The assimilation of exogenous amino acids by S. lactis C10 : an overview.



A Surface bound proteinases/peptidases

B Membrane bound transport enzymes

C Cytoplasmic proteinases/peptidases

plasting procedures and mechanical disruption of washed cells with glass beads resulted in 'particulate' fractions (or their equivalent: cell-wall digests) which contained 75-85% of the recovered C10 Fast proteinase (Table 7); a result which is very similar to that found for Streptococcus faecalis var. liquefasciens cell-bound proteinase (Shugart & Beck, 1966). It is concluded that S.lactis C10 Fast cell-bound proteinase is mainly surface-bound, based on three criteria: (i) the constraint of substrate-accessibility; (ii) the detachment of activity by procedures designed to cause minimal damage to spheroplasts, and (iii) the association of proteinase with 34,800 x *g* pellets from mechanically-disrupted cells. Specifically, the sucrose-mediated release indicates that the enzymes are physically constrained within the normal envelope structure, possibly in the so-called 'periplasmic space' between the cell wall and spheroplast membrane. It may be useful to assess the effect of the 'osmotic shock' technique, used to displace a number of hydrolytic enzymes from gram-negative bacteria (Heppel, 1967), on the binding of proteinase to S.lactis C10.

THE IMPORTANCE OF PROTEOLYSIS TO ACID PRODUCTION

The results of this study show that, (i) S.lactis C10 Slow is stimulated in skim milk by casein hydrolysates to a rate and extent of development (growth and acid-production) which is very similar to that exhibited by S.lactis C10 Fast in unmodified skim milk; and (ii) C10 Slow, when harvested from a medium in which its development is in no way abnormal (T_5 broth), lacks surface-bound proteolytic activity, relative to the parent cells.

There is thus a good agreement between the nutritional and the enzymatic data, a correlation which supports the proteinase-dependency hypothesis, as postulated for this pair of strains. However, the validity of correlating data from measurements of cells grown in one medium (milk) with those from another (T_5 broth) can only be induced, since no attempt was made to assess the effects of the medium on the proteolytic properties of the two strains.

Examination of the literature concerned with the effects of changes in the medium on the regulation of proteinase synthesis does not allow a generally valid mechanism to be deduced. However, several points have been established, (i) amino acids suppress the synthesis of several extracellular-proteinase systems (Neumark & Citri, 1962; Chaloupka & Kreckova, 1966; Hofsten & Tjoder, 1965; May & Elliot, 1968); (ii) carbon sources, including those provided by the intracellular metabolism of accumulated amino acids, suppress extracellular-proteinase synthesis in some species (Bidwell, 1950; Kreckova & Chaloupka, 1969; Levisohn & Aronson, 1967); (iii) in a Micrococcus species, amino acids induced the production of extracellular proteinase (McDonald & Chambers, 1966); these authors considered that this product-induction, together with a repression by utilisable carbohydrates, constitutes a mechanism for regulating the supply of carbon to the cells, i.e. in this case, exogenous amino acids are a source of carbon, not nitrogen; and (iv) extracellular proteinase synthesis does not require the presence of protein (McDonald & Chambers, 1966; Rabin & Zimmerman, 1956).

Westhoff & Cowman (1970) reported that the substitution of "peptides" (enzymatic-hydrolysate of casein) or "amino acids"

(acid-hydrolysate of casein) for whole-casein in a broth medium caused significant change in the relative levels of "membrane" and "intracellular" proteinase associated with sonicated cells of S.lactis 3 Fast which had been grown in the media. They concluded that these effects constituted support for earlier reports (Cowman, Swaisgood & Speck, 1967; Cowman & Speck, 1969) that the "membrane" enzyme functioned only to hydrolyse casein to peptides and the "intracellular" enzyme peptides to amino acids. It is, however, difficult to agree with these authors that the measured proteinase levels reflect the cellular response to the form of exogenous nitrogen, since their basal medium contained yeast extract (1 mg/ml). In addition, the protein hydrolysates were ill-defined.

In summary, there is no evidence of direct relevance to the response of S.lactis-proteinase synthesis to changes in the medium. However, it is argued that in T_5 broth surface-bound proteinase synthesis is under amino-acid repression and that the proteolytic difference found between the two strains of C10 in the broth is an underestimate of the situation in milk. Whether this is true or not, it seems unlikely that transfer of the cells to milk would repress enzyme synthesis, and it is concluded that the proteolytic activity of the two strains will be at least as different in milk as in the permissive medium.

An attempt was made during this study to demonstrate directly the dependence of C10 Fast, in skim milk, on its surface proteinases. Cells, washed and then incubated at 37°C for 120 min. under the conditions known to inactivate

the cell-bound activity (Figure 19), were overtly 'fast' when inoculated into milk and incubated at 30°C. This result may be explained by reference to Figure 7, which shows that the slow variant, known to lack the heat-sensitive enzymes, was capable of rapid growth in milk for six hours. Presumably, the heat-treated C10 Fast cells, initially growing on the amino acid source which is available to C10 Slow, rapidly synthesized, de novo, the surface enzymes and subsequently developed normally. Perhaps the only method which would demonstrate unambiguously the in situ effect of proteinase inactivation would be to include a specific enzyme inhibitor in the medium.

Figure 22 provides an overview of the pathways which are assumed to operate in a multiple-amino acid auxotroph which is growing in an amino acid-limited, protein-rich medium. The stimulation of C10 Slow in such a medium by protein hydrolysates is interpreted to mean that the cells possess fully-operative systems for transporting amino acids and peptides. The enzymatic analyses have provided convincing evidence that the 'slowness' is due to a defective surface-bound proteinase enzyme system, a result that is in direct contrast to that from studies with S.lactis 3 Fast and S.lactis 3 Slow, which indicated that the only significant difference concerned specificity of 'intracellular' proteinase (Westhoff & Cowman, 1971). It is concluded that, at least in the case of S.lactis C10, slow variants are retarded in milk by the supply of amino acids from the medium.

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