PROTEINASES OF LACTIC STREPTOCOCCI

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ABSTRACT

^{14}C-Labelled milk proteins and peptides were required for studies on the nitrogen nutrition and proteolytic enzymes of starter bacteria. Therefore, a cow was injected with a mixture of ^{14}C-labelled amino acids (2mCi) and milked at intervals over 28 h. The first milking (3.75 h) contained the most active components with the caseins, β-lactoglobulin and α-lactalbumin having a specific activity of 2.3×10^6 disintegrations per min (dpm) / g while lactose and triglycerides had specific activities of 2.3×10^5 dpm / g and 3.4×10^4 dpm / g, respectively. Thirteen amino acids isolated from acid hydrolyzed milk protein contained radioactivity. Only 6% of the label injected was recovered in milk protein in the first 28 h.

The nitrogen nutrition of *Streptococcus cremoris* AM_2 and E_8 was studied by adding ^{14}C-labelled milk proteins, peptides and single amino acids separately to unlabelled milk and then determining the incorporation of radioactivity into bacterial protein during growth. A specially prepared low heat skim milk powder having a content of potential nitrogen sources similar to that of fresh milk, was reconstituted for use as the growth medium. At low cell densities, free amino acids and peptides were used as nitrogen sources. As the cell density increased, milk protein became an increasingly important nitrogen source and the cells became dependent on their cell wall-bound proteinase. All caseins tested, β-lactoglobulin and α-lactalbumin were used as nitrogen sources by both strains.

A proteinase assay was evaluated in which ^{14}C-labelled casein (2.3×10^6 dpm / g) was used as the substrate. This assay was used to study the spontaneous release of cell wall-associated proteinases from milk-grown cells of lactic streptococci. Eight strains of *S. cremoris* and two strains of *S. lactis* released proteinase when cells were held in buffer. An exception was *S. cremoris* ML_1 which did not release significant activity. The rate of proteinase release increased with rise in temperature (0 to 34^{o}C) and pH (5.5

to 8.7) although inactivation was apparent at 34°C and pH 8.7. With all strains, release of proteinase was suppressed by the addition of $CaCl_2$ to the buffer, by lowering the temperature to 0°C, or by lowering the pH to 5.5. The rate of proteinase release varied markedly with different strains. A possible mechanism for the release of proteinase from the cell wall is discussed.

Cheddar cheese was made with cultures containing different proportions of proteinase-positive (Prt^+) and proteinase-negative (Prt^-) cells. This allowed the level of starter proteinase to be varied while the total concentration of starter cells in the curd at salting was kept constant. Cheeses with 45 to 75% Prt^- cells developed significantly less bitterness than cheeses containing only Prt^+ cells, thus providing direct evidence that the level of starter proteinase has a role in bitterness development in Cheddar cheese. The involvement of starter peptidases in the removal of bitter peptides is discussed.

PREFACE

The manufacture of Cheddar cheese and lactic casein involve the fermentation of milk by lactic streptococci. Since these fermentation industries are of major importance to New Zealand an understanding of the growth and metabolism of lactic streptococci in milk is desirable. This thesis describes research carried out on a number of topics related to the nitrogen metabolism of lactic streptococci. A novel approach adopted in these investigations was the use of radioactive milk, or milk fractions, prepared biosynthetically in the mammary gland of a cow after ^{14}C-labelled amino acids were injected into the bloodstream. The preparation and analysis of this material is described in Section I. Radioactive components were isolated from this milk and used to evaluate the potential nitrogen sources for growth of lactic streptococci in milk (Section II). Radioactive casein was used as substrate in the assay of cell wall-associated proteinases, enzymes which catalyze the first step in the breakdown of milk protein to the free amino acids required for synthesis of bacterial protein. An evaluation of the proteinase assay and studies on the release of proteinase from the cell wall of intact cells, are contained in Section III. Proteolytic enzyme systems of lactic streptococci are not only involved in supplying essential nutrients for cell growth during cheese manufacture but are also active during subsequent cheese ripening. Section IV deals with the effect of the level of cell wall-associated proteinase on bitterness development in Cheddar cheese.

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Mills, O E (1976). The preparation of ^{14}C-labelled milk proteins. N Z Jl Dairy Sci. Technol. 11, 164 - 168.

Mills, O E & Thomas, T D (1978). Release of cell wall-associated proteinase(s) from lactic streptococci. N Z Jl Dairy Sci. Technol. 13, 209 - 215.

Mills, O E & Thomas, T D (1980). Bitterness development in

Cheddar cheese: Effect of the level of starter proteinase. N Z Jl Dairy Sci. Technol. 15, 131 - 141.

Mills, O E & Thomas, T D (1981). Nitrogen sources for growth of lactic streptococci in milk. N Z Jl Dairy Sci. Technol. 16 (in press).

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CHAPTER 1

INTRODUCTION

The preparation of radioactive bovine milk was undertaken so that the individual proteins could be isolated in ^{14}C-labelled form. It was anticipated that if the proteins had sufficiently high specific activities then they could be used (i) to assess their role in the nitrogen nutrition of lactic streptococci growing in milk, and (ii) as substrates for the assay of proteinases from lactic streptococci.

Milk proteins are synthesized in the mammary gland from amino acids which are absorbed from the blood either as free amino acids or as amino acid precursors (Linzell, 1974). Sufficient amino acids essential for the synthesis of milk protein are absorbed from the blood to account for all the essential amino acids in milk. Some non-essential amino acids are absorbed from the blood with the remainder being synthesized in the mammary gland (Bickerstaffe *et al.*, 1974). Although Barry (1952, 1956, 1958) found that the separate injection of ^{14}C-lysine, ^{14}C-tyrosine, ^{14}C-glutamic acid and ^{14}C-proline into the jugular vein of a goat each resulted in radioactive casein, no data was available to allow estimation of (i) the recovery of injected isotope in bovine milk and (ii) the specific activity of milk protein. In an effort to obtain uniform labelling of the maximum number of amino acids during bovine milk protein biosynthesis, a mixture of ^{14}C-labelled amino acids was injected into the jugular vein of a Friesian cow. The cow, which was in mid-lactation, was then milked at intervals and the milk analyzed to determine the effectiveness of this method in radiolabelling milk protein.

C H A P T E R 2

EXPERIMENTAL

2.1 ^{14}C-Labelled Amino Acids

Algal protein hydrolysate containing amino acids evenly labelled with ^{14}C (specific activity 55 mCi/mg atom of carbon) was obtained from The Radiochemical Centre (Amersham, England). The radioactivity of each amino acid as a percentage of the total is given in Table 1. The hydrolysate, containing a total of 2mCi ^{14}C, was dissolved in NaCl solution (20 ml, 0.9% (w/v)) and adjusted to pH 7.4. The quantity of isotope used was limited by its cost.

2.2 Composition of Scintillation Solutions

The polar scintillation cocktail for aqueous samples contained naphthalene (100 g) and 2, 5 - diphenyloxazole (5 g) made up to 1 l with 1, 4 - dioxan. The non-polar scintillation fluid for organic samples contained 2,5 - diphenyloxazole (5 g/l) in toluene. All chemicals were of scintillation grade.

2.3 Radioactivity Determination

Radioactivity was measured in a Beckman LS-100C Liquid Scintillation System (Beckman Instruments Inc., Irvine, U S A) to an error of ± 5%. The quenching effect of water on the count rate was determined by measuring the count rate and external standard ratio after addition of successive aliquots of water to a known quantity of ^{14}C-glycine in the polar cocktail. This data was then used to convert counts/min of any sample to disintegrations / min (dpm).

2.4 Infusion of ^{14}C-Labelled Amino Acid Mixture and Milk Sampling

A Friesian cow in mid-lactation was milked out prior to infusion of the amino acid mixture. The amino acid mixture

TABLE 1: Distribution of Radioactivity in the Algal Protein Hydrolysate used to Inject into a Cow and the Relative Activities (see text) of Total Amino Acids in Milk from the First Milking

Amino Acid	% of Total Radioactivity of Algal Protein Hydrolysate	Relative Activity in Milk $(\frac{dpm}{A_{570nm}})$	
		Mean	Standard Deviation
aspartic acid	9.0	1.4	0.1
threonine	5.8	50.5	11.0
serine	4.8	20.6	2.1
glutamic acid	11.8	11.0	1.3
proline	5.6	46.5[a]	4.8
glycine	4.6	0	
alanine	9.3	0	
cysteine	0	0	
valine	6.8	21.8	0.5
methionine	0	0	
isoleucine	4.8	21.0	1.6
leucine	11.8	17.2	3.0
tyrosine	3.6	69.2	9.0
phenylalanine	6.7	105.2	18.5
lysine	5.1	53.0	1.4
histidine	4.0	82.5	5.0
arginine	6.3	62.7	12.1
tryptophan	0	b	

a $(\frac{dpm}{A_{440nm}})$

b Destroyed during hydrolysis

(20 ml) was administered in a single dose through a cannula into the jugular vein. The cow was then machine-milked 3.75, 7, 11, 22.5 and 28 h after the injection.

2.5 Treatment of Milk Samples

The milk was centrifuged (2500 x g, 15 min, 20°C) and the fat then removed. The skim milk was frozen in a few seconds by pouring thin layers into trays which were placed in a bath of liquid nitrogen. After freeze-drying the powder was stored at -20°C.

2.6 Composition of Milk

Protein:

Total nitrogen (TN, % (w/v)) and non-protein nitrogen (NPN, % (w/v)) taken as the fraction soluble in 12% (w/v) trichloracetic acid (TCA) were determined by the Kjel - Foss Automatic 16210 (Foss Electric, Hillerød, Denmark) instrument using the Kjeldahl method. Whey protein (WP, % (w/v)) was determined by the amido black method (Roeper & Dolby, 1971). The casein concentration (C, % (w/v)) of reconstituted skim milk was calculated from the formula

$$C = (TN - NPN) \times 6.38 - WP$$

where the factor 6.38 converts nitrogen concentration to protein concentration.

Lactose:

Lactose concentration was determined by the Lane and Eynon method preceded by clarification of the milk with lead acetate and sodium fluoride (McDowall & Dolby, 1935).

Minerals:

The total mineral content of the milk was obtained by charring the powders over a bunsen burner then ashing in a muffle furnace at 550°C for 16 h.

2.7 Determination of Radioactivity in Milk Components

Total casein, total whey protein and a crude lactose fraction were isolated by the procedure outlined in Fig. 1. Total casein (0.05 g) was dissolved in 1 M NaOH (0.5 ml), water (4 ml) was added and the pH adjusted to 7.0 with 1 M HCl, the final volume being made up to 5 ml. Whey protein (0.05 g) was dissolved in 0.001 M NaCl (5 ml). These solutions were counted by mixing aliquots (0.1 ml) of each solution with polar scintillation cocktail (8 ml) and the specific activity (dpm/g) was calculated. The specific activity of the crude lactose fraction was determined by weighing dried material (3 mg) into a counting vial and dissolving in 0.1 M HCl (0.2 ml). This solution was then mixed with polar scintillation cocktail (5 ml). The specific activity of the triglyceride fraction, which comprises 95 to 96% of the total milk fat (Kurtz, 1974), was determined after isolation of this fraction. Separated fat was shaken with hexane and the solvent layer washed several times with distilled water before evaporation. Triglyceride (30 mg) was weighed into a counting vial and dissolved in non-polar scintillation solution (8 ml).

2.8 Sephadex G-75 Chromatography of Whey Protein from the First Milking

A sample of skim milk powder (2 g) from the first milking was dissolved in distilled water (10 ml). The casein was acid-precipitated and removed by centrifugation. The supernatant was adjusted to pH 6 with NaOH and concentrated to 3 ml by immersing a dialysis sack containing the whey in a bed of polyethylene glycol flakes. The sample was then eluted from a 2.5 cm x 46 cm column of Sephadex G-75 with a 0.04 M phosphate buffer (pH 6.0). The absorbance at 280nm (A_{280nm} value) was determined for each fraction and an aliquot (0.1 ml) was mixed with polar cocktail (5 ml) and counted to an error of ± 10%.

2.9 DE52 Cellulose Chromatography of Total Casein from the First Milking

Casein (1 g) was dissolved in 0.01 M imidazole - HCl buffer

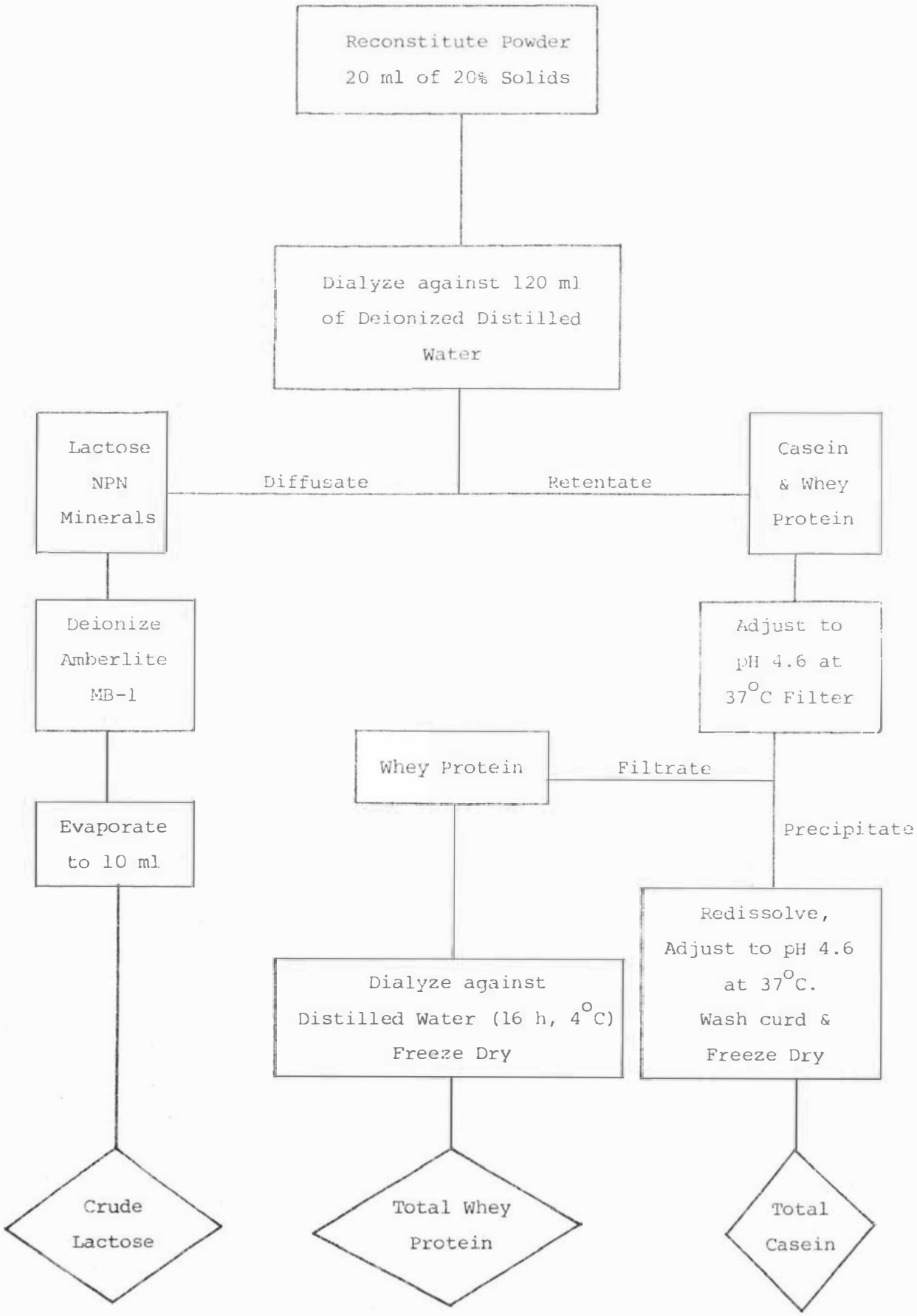

FIGURE 1: Scheme showing the isolation of total casein, total whey protein and crude deionized lactose from radioactive milk powder

(15 ml, pH 7.0) containing 4.5 M urea. The sample was eluted from a 2.5 cm x 45 cm column of Whatman DE52 cellulose with the urea - imidazole - HCl buffer and a 0 to 0.30 M NaCl gradient. The A_{280nm} value was determined for each fraction and an aliquot (0.1 ml) was added to polar cocktail (5 ml) and counted to an error of ± 10%.

2.10 Radioactivity of Individual Amino Acids in Milk Protein

Skim milk powder (0.01 g) from the first milking was digested with 6 M HCl for 24 h at 110°C while under vacuum. The hydrolysate was dried down to remove HCl and redissolved in 0.2 M sodium citrate buffer (1 ml, pH 2.2). This solution was loaded onto the 23 cm column of a Locarte Amino Acid and Peptide Analyzer (The Locarte Company, London, England). After reaction with ninhydrin, the effluent was collected (0.57 ml fractions) after passing through the colorimeter. Three fractions from the top of each peak were selected and the A_{570nm} values (A_{440nm} for proline) were determined in a 2 mm pathlength cell. These values were corrected by dividing by the appropriate colour constant. Aliquots from these fractions were added to polar cocktail (5 ml) and counted to an error of ± 7%. Due to high quenching by the ninhydrin - amino acid complex, it was not possible to count more than 0.1 ml at a time. Relative activity values were determined by dividing the dpm value by the corresponding corrected A_{570nm} (or A_{440nm}) value. A mean relative activity for each amino acid was then calculated.

CHAPTER 3

RESULTS

3.1 Composition of Skim Milk Powders

The concentrations of casein, whey protein, lactose and NPN were determined in reconstituted skim milk then converted to the corresponding concentrations in the skim milk powder (Table 2). There was a decrease in casein and total protein content over the first four milkings while, at the same time, the lactose content increased. The total solids content of the skim milk remained constant at 9.5% (w/v) over the first three milkings (not determined for the fourth or fifth milkings).

The NPN level decreased slightly while a peak in ash content occurred at the fourth milking.

3.2 Specific Activities of Milk Components

The specific activities of the casein, whey protein and crude lactose fraction were highest in milk from the first milking (Table 3). In contrast, the specific activity of the triglyceride fraction peaked at the second milking. Casein had the highest specific activity even taking into account that the average content of carbon by weight in protein, lactose and triglyceride is 50, 40 and 75%, respectively. The specific activity of the crude lactose fraction may be influenced by the presence of small amounts of NPN compounds such as urea and creatinine.

3.3 Sephadex G-75 Chromatography of Whey Proteins from the First Milking

The separation of radioactive whey proteins on a Sephadex G-75 column is shown in Fig. 2. The A_{280nm} peak at fraction 15 contained immunoglobulins, identified by gel electrophoresis, but the high turbidity of the samples indicated

TABLE 2: Composition of Skim Milk Powder (%, w/w)

Milking	Casein	Whey Protein	Non-Protein Nitrogen	Lactose	Ash
1	28.54	6.94	0.34	52.34	7.38
2	26.20	7.40	0.34	54.76	7.63
3	23.87	6.60	0.29	58.40	7.73
4	22.95	6.12	0.29	59.23	8.15
5	23.78	5.54	0.30	58.32	7.68

Footnote:

The relative standard deviations for determination of these milk components by the methods used are 1.0, 5.0, 7.0, 1.0 and 2.0% for casein, whey protein, non-protein nitrogen, lactose and ash, respectively.

TABLE 3: Specific Activities of Milk Components (dpm/g)

Milking	Casein	Whey Protein	Crude Lactose[a]	Triglycerides
1	2,349,000	1,478,000	225,600	33,800
2	979,900	791,100	139,700	35,400
3	313,900	256,100	46,200	25,400
4	126,300	101,800	19,200	11,500
5	44,800	85,300	20,400	6,820

[a] Prepared from protein-free deionized milk serum (Fig. 1)

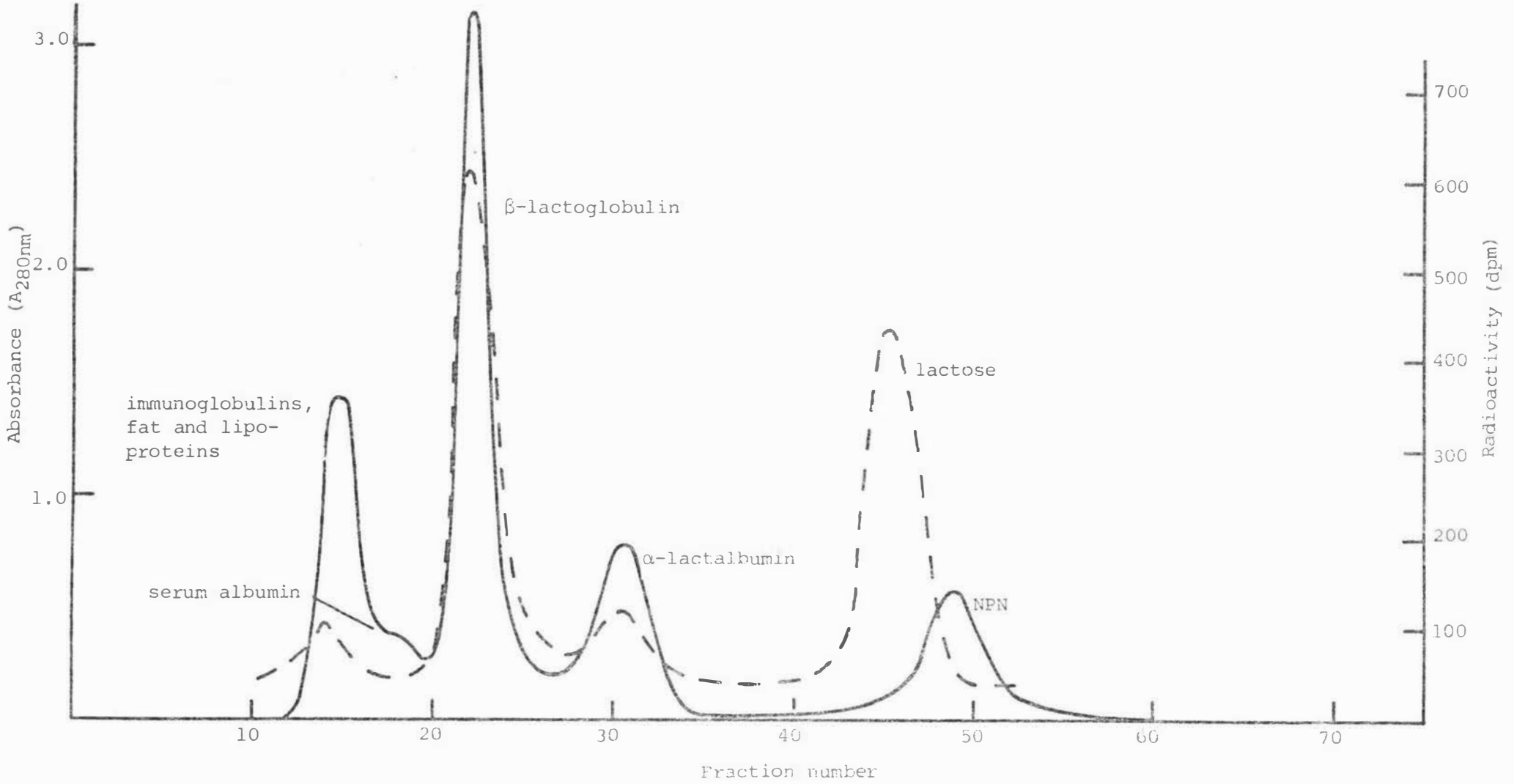

FIGURE 2: Sephadex G-75 chromatographic separation of whey protein (first milking) in 0.04 M phosphate buffer (pH 6.0). The A_{280nm} curve (——) and dpm curve (---) of 0.1 ml samples are given.

that fat and lipoprotein were probably also present. The first ^{14}C activity peak may be due to one of these components which was eluted at the leading edge of the A_{280nm} peak. There was no obvious ^{14}C activity peak for serum albumin. Samples from the centre of the last ^{14}C activity peak (fraction 46) and the last A_{280nm} peak (fraction 49) were analyzed for amino acids (after acid hydrolysis) and for lactose. Both samples contained amino acids in trace amounts while the ^{14}C activity peak sample contained seven times as much lactose as the A_{280nm} peak sample. This ^{14}C activity peak appeared to consist mainly of lactose while the A_{280nm} peak material was comprised of NPN.

The specific activities of α-lactalbumin and β-lactoglobulin were similar to the caseins at about 2.3×10^6 dpm/g but that of serum albumin appeared to be only about 20% of this value. The specific activity of the immunoglobulin fraction could not be determined but is likely to be low since, like serum albumin, immunoglobulins are not synthesized in the mammary gland.

3.4 DE52 Chromatography of Total Casein from the First Milking

The separation of radioactive caseins on a DE52 ion exchange cellulose column is shown in Fig. 3. The individual caseins were identified by electrophoresis. Although the urea in the eluting buffer caused some precipitation of naphthalene in the scintillation cocktail, the efficiency of counting was only slightly impaired. The specific activities were approximately the same for all caseins at about 2.3×10^6 dpm/g.

3.5 Radioactivity in Individual Amino Acids in Milk Protein

Relative activity values $\left(\frac{dpm}{A_{570nm}}\right)$ were determined for all amino acids that contained ^{14}C (Table 1). There was no radioactive cysteine, methionine and tryptophan in the algal protein hydrolysate nor in the milk powder. The glycine and alanine present in the skim milk powder did not contain any ^{14}C.

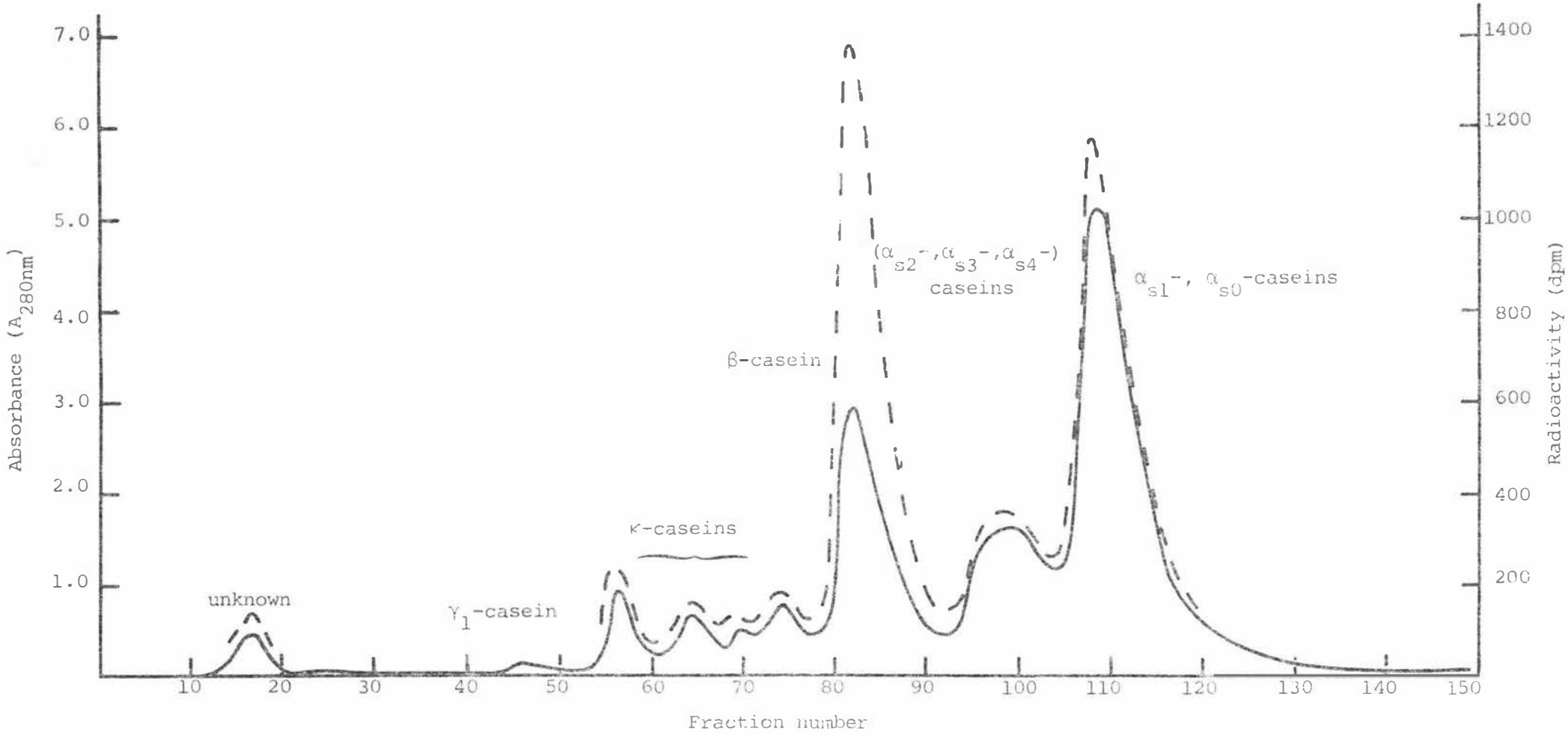

FIGURE 3: DE52 chromotographic separation of casein (first milking) at pH 7.0 in 0.01 M imidazole-HCl-4.5 M urea with a salt gradient of 0 to 0.3 M NaCl. The A_{280nm} curve (——) and dpm curve (---) of 0.1 ml samples are given.

CHAPTER 4

DISCUSSION

Milk composition changed during the 28 h after the injection of the ^{14}C-labelled amino acid mixture (Table 2). These changes could result from any one or a combination of the following factors; (i) the cow's reaction to confinement, (ii) the insertion of the cannula, and (iii) the change in diet. Despite these changes, compositonal data were still within the limits of normal milk. The A_{280nm} curves (Figs 2 and 3) were also typical of normal casein (Creamer, 1974) and whey protein (Davies, 1974).

The injection of a mixture of ^{14}C-labelled amino acids into the jugular vein of a cow was evaluated as a method for producing uniformly labelled milk protein. Of the total ^{14}C-label injected, only 9% was recovered in the first five milkings (18 l milk) and 70% of this recovered isotope was present in milk protein. The specific activity of those milk proteins which are synthesized in the mammary gland was about 2.3×10^6 dpm/g which was considerably less than that obtained by ^{14}C-methylation of casein (40 to 50 $\times 10^6$ dpm/g; Exterkate (1975) see Section III, page 48). The present method, however, resulted in the labelling of at least thirteen of the eighteen amino acids without chemical modification of the molecules. An increase in specific activity of milk protein may have been obtained by milking earlier (at the expense of protein yield) or by infusion of the ^{14}C-labelled amino acids into the mammary artery. The latter procedure may also improve total recovery of ^{14}C but would require extensive surgery.

All milk proteins synthesized in the mammary gland (caseins, β-lactoglobulin and α-lactalbumin) had approximately the same specific activity. The specific activity of total whey protein from the first milking (Table 3) was lower than expected since 80% of whey protein normally consists of β-lactoglobulin and α-lactalbumin (Jenness, 1970). This

suggests that there was a lower proportion of β-lactoglobulin and α-lactalbumin in this milk compared with the subsequent milk. Serum albumin, and the immunoglobulins, have much lower specific activities presumably because they are synthesized in the liver, spleen, lymph nodes and bone marrow (Butler, 1974) and have a much slower rate of turnover.

SECTION II

NITROGEN SOURCES FOR GROWTH OF LACTIC STREPTOCOCCI IN MILK

CHAPTER 5

INTRODUCTION

The requirement for a proteolytic enzyme system, capable of degrading milk protein to the amino acids essential for growth of lactic streptococci, is demonstrated by the limited growth of proteinase-negative (Prt^-) variants in skim milk. These Prt^- organisms, which lack cell wall-associated proteinase (Pearce et al., 1974; Exterkate, 1976a), grow at low cell densities in milk at the same rate as the parent strain (Citti et al., 1965) but growth stops at 10 to 25% of the maximum cell density reached by the parent culture. This growth limitation is, presumably, due to depletion of an essential amino acid initially present either as a free amino acid or in low molecular weight peptides. This explanation is consistent with the observation that addition of casein digest to milk abolishes the growth limitation of Prt^- organisms (Garvie & Mabbitt, 1956; Pearce et al., 1974).

There is a substantial amount of published data on the proteolytic enzyme system of lactic streptococci but much of the earlier work is of limited value in establishing the physiological role of this system for growth of these organisms in milk. Cells were usually grown in media containing high concentrations, relative to milk, of free amino acids and / or peptides which suppress the level of cell wall-associated proteinase (Exterkate, 1979; see also Appendix I). In addition, the rigid criteria required for the cellular localization of enzymes (Pollock, 1962) were not met (Van der Zant & Nelson, 1953, 1954; Williamson et al., 1964; Cowman & Speck, 1967; Cowman et al., 1967; Cowman et al., 1968). These investigations, however, did indicate that lactic streptococci have proteolytic activity with casein as the substrate and the long assay times with large quantities of cells or cell extracts implied that the activity was low.

A more rigorous approach has been adopted in recent investigations and a mechanism for the assimilation of nitrogen by cells has been proposed (see Lawrence & Thomas, 1979). Pro-

teinases capable of hydrolyzing proteins to low molecular weight peptides appear to be located in the cell wall (Thomas et al.,1974; Exterkate, 1975). Large peptides may be further hydrolyzed by peptidases located in the plasma membrane (Exterkate, 1975). Peptides with up to five or six residues can be transported through the cell membrane (Law et al., 1976b; Rice et al., 1978) and hydrolyzed to amino acids by cytoplasmic peptidases (Mou et al 1975; Law, 1977; Schmidt et al., 1977). Sørhaug & Solberg (1973) first showed that sonic extracts of cells contained peptidases with a wide range of specificities but, more importantly, Mou et al.(1975) showed the presence of peptidases having the specificities necessary for the release from casein of all the amino acids essential for growth of lactic streptococci. In addition to these membrane-bound and cytoplasmic peptidases, Law (1977) has reported the release of a dipeptidase into the medium during growth. Recent studies have investigated the transport of amino acids and low molecular weight peptides through the membrane (Law, 1978; Rice et al.,1978).

The potential sources of nitrogen for the growth of lactic streptococci in skim milk are casein, whey protein, peptides and free amino acids. The present study was undertaken to determine the individual contributions of these sources to bacterial protein. Such an assessment requires that the potential nitrogen sources be individually labelled and that their concentrations are known. Therefore, ^{14}C-labelled proteins, peptides and single amino acids were added separately to unlabelled milk and the incorporation of radioactivity into bacterial protein determined during growth. The amount of material added was such that there was only a small change in milk composition. Labelled proteins and peptides were isolated from radioactive milk (see Section I) while single radioactive amino acids were obtained from a commercial source. The choice of radioactive amino acids was limited to those found in milk in free form. Aspartic acid, glutamic acid, alanine and lysine were excluded because they are components of the cell wall peptidoglycan of lactic streptococci (Schleifer & Kandler, 1967) as well as being components of bacterial protein. Threonine was ex-

cluded because it is metabolized to acetaldehyde and glycine by lactic streptococci (Lees & Jago, 1976). Two essential amino acids (isoleucine and valine, Reiter & Oram, 1962) and one non-essential amino acid (glycine) were finally chosen. Because of seasonal variation in the composition of milk and the length of time required for the present investigation, it was not practicable to use fresh milk as the growth medium. The alternative was to reconstitute skim milk from spray dried powder. However, heat treatment during drying causes protein interactions which result in poor chromatographic separation of the individual milk proteins and consequently error in determination of the milk protein composition. Heat treatment can also release low molecular weight nitrogen compounds from protein (Reiter & Møller-Madsen, 1963; Bracquart et al.,1974) thus changing the nutritional characteristics for growth of lactic streptococci. Therefore, a spray dried skim milk powder was specially prepared for this investigation by omitting the usual evaporation stage and thus minimizing heat treatment.

CHAPTER 6

EXPERIMENTAL

6.1 Bacteria and Stock Cultures

Streptococcus cremoris 266, AM_2 and E_8 were obtained from the New Zealand Dairy Research Institute collection. Stock cultures were prepared from single colonies isolated from milk-based agar (MAG) plates (Limsowtin & Terzaghi, 1976). Cultures derived from large colonies which coagulated autoclaved (121°C, 15 min) reconstituted skim milk (RSM) within 24 h at 22°C (0.1% inoculum) were designated proteinase-positive (Prt^+). Cultures derived from small colonies which required more than two days incubation at 22°C to coagulate autoclaved skim milk, but which coagulated within 24 h upon addition of casein hydrolysate (5 mg/ml Trypticase, BBL), were designated Prt^-. The Prt^+ cultures were maintained in autoclaved RSM while this medium was supplemented with 5 mg/ml Trypticase for maintenance of Prt^- cultures. For each experiment, a frozen stock culture was incubated at 30°C for 16 h and then inoculated (0.2 to 8.0%) into a series of tubes containing RSM which had been autoclaved (116°C, 20 min), centrifuged (10,000 x g, 5 min) and buffered with β-glycerophosphate (final concentration 0.073 M and pH 7.1). After 16 h at 22°C the culture nearest pH 5.1 was used to inoculate (1%) the experimental medium.

6.2 Low Heat Skim Milk Powder

Skim milk from a Friesian herd was pasteurized (72°C, 15 s) and dried without prior evaporation in a spray drier (Anhydro A/S, Copenhagen, Denmark). The inlet and exhaust temperatures were 204°C and 93°C, respectively. This powder was used to prepare low heat reconstituted skim milk (LHRSM) medium which was used as the experimental medium for investigations described in this section. The proportions of the individual caseins in total casein were determined by ion-exchange chromatography on Whatman DE52 cellulose (page 6)

while the proportions of the individual whey proteins in the total whey protein were determined by gel filtration chromatography on Sephadex G-75 (page 6).

6.3 LHRSM Medium, Monitoring of Growth and Harvesting of Cells

The LHRSM medium was prepared by reconstituting the skim milk powder in autoclaved distilled water (10 g / 100 ml) at 40 to 45°C. The milk was centrifuged (10,000 x g, 5 min) to reduce the level of contaminant bacteria and to remove particulate material which would otherwise sediment with starter bacteria during harvesting (Thomas & Turner, 1977).

The turbidity at 480 nm (T_{480nm}; Kanasaki et al., 1975) of LHRSM cultures or cell suspensions was used to determine cell density (Thomas & Turner, 1977). The T_{480nm} values (1 cm lightpath) of suspensions of AM_2, E_8 and 266 at a cell density of 1.0 mg (dry weight) bacteria/ml, were 5.59, 5.69 and 5.27, respectively. The experimental cultures were incubated at 30°C until the T_{480nm} value of a ten-fold dilution of the growing culture, reached a maximum of 0.3 (pH ~ 5.0). At the harvest time the culture was adjusted to pH 7.0 with 2 M NaOH and 25% (w/v) sodium citrate solution was added (6 ml / 100 ml culture). After 5 min stirring, the culture was centrifuged (10,000 x g, 5 min) and the cells washed free of milk constituents by twice resuspending in an equal volume of distilled water.

6.4 The Separation of Low Molecular Weight Peptides and Free Amino Acids in the NPN Fraction

The TCA in an aliquot of NPN fraction was extracted by shaking twice with an equal volume of diethyl ether. The resulting solution was evaporated to dryness in a rotary evaporator, redissolved in acetic acid (5 ml, 10% (v/v)) and applied to a 2 cm x 86 cm column containing Sephadex G-15 (fractionation range up to 1500 daltons). Material was eluted from the column with 10% (v/v) acetic acid at a flow rate of 14.3 ml/h. Effluent fractions (2.9 ml) were assayed for amino groups using the fluorescamine reaction (Pearce,

1979). The assay solution consisted of borax buffer (2.3 ml, 4% (w/v), pH 8.0) and sample (0.2 ml) mixed with 4-phenylspiro (furan-2(3H),1-phthalan)-3,3'-dione (fluorescamine) solution (0.5 ml, 0.4 mg/ml acetone). Fluorescence measurements were made at 20°C with an Hitachi MPF-2A fluorescence spectrophotometer (Hitachi Ltd, Tokyo, Japan) set at excitation and emission wavelengths of 395 nm and 480 nm, respectively, and excitation and emission slits of 5 nm and 20 nm, respectively.

6.5 Preparation of ^{14}C-Labelled Peptides

The protein from ^{14}C-labelled milk (100 ml) was precipitated by the addition of TCA (12 g) and separated, after 15 min, by centrifugation (10,000 x g, 5 min). The TCA was extracted from the supernatant and the resulting solution passed down a 1 cm x 7 cm cation exchange column (Bio-Rad AG 50W-X4). Non-cationic radioactive material (mostly lactose) was washed from the column with distilled water before elution of amino acids and peptides with 2M NH_4OH. The effluent was evaporated to dryness, redissolved in acetic acid (10 ml, 10% (v/v)) and the amino acids and peptides separated by Sephadex G-15 chromatography. Samples from column fractions were subjected without prior hydrolysis to amino acid analysis. Using this procedure free amino acids were found to be eluted from fraction 66 onwards (Fig. 4). Fractions 40 to 65 were combined, evaporated to dryness with a rotary evaporator and redissolved in 10 ml water.

6.6 Preparation of ^{14}C-Labelled Total Milk Protein and Isolation of Individual Proteins

Total milk protein free of lactose, amino acids and low molecular weight peptides was prepared from ^{14}C-labelled milk powder (2.35×10^6 dpm/g casein). Milk powder was dissolved (10%, w/v) in sodium citrate (1.5%, w/v) and the pH was adjusted to 8.0. The solution (100 ml) was dialyzed for 24 h against distilled water (2 l) with one change of water. Because the solution gained water during dialysis, it then had to be concentrated to 3.5% (w/v) protein by immersing the dialysis bag in a bed of polyethylene glycol flakes.

The preparation of pure individual ^{14}C-labelled milk proteins

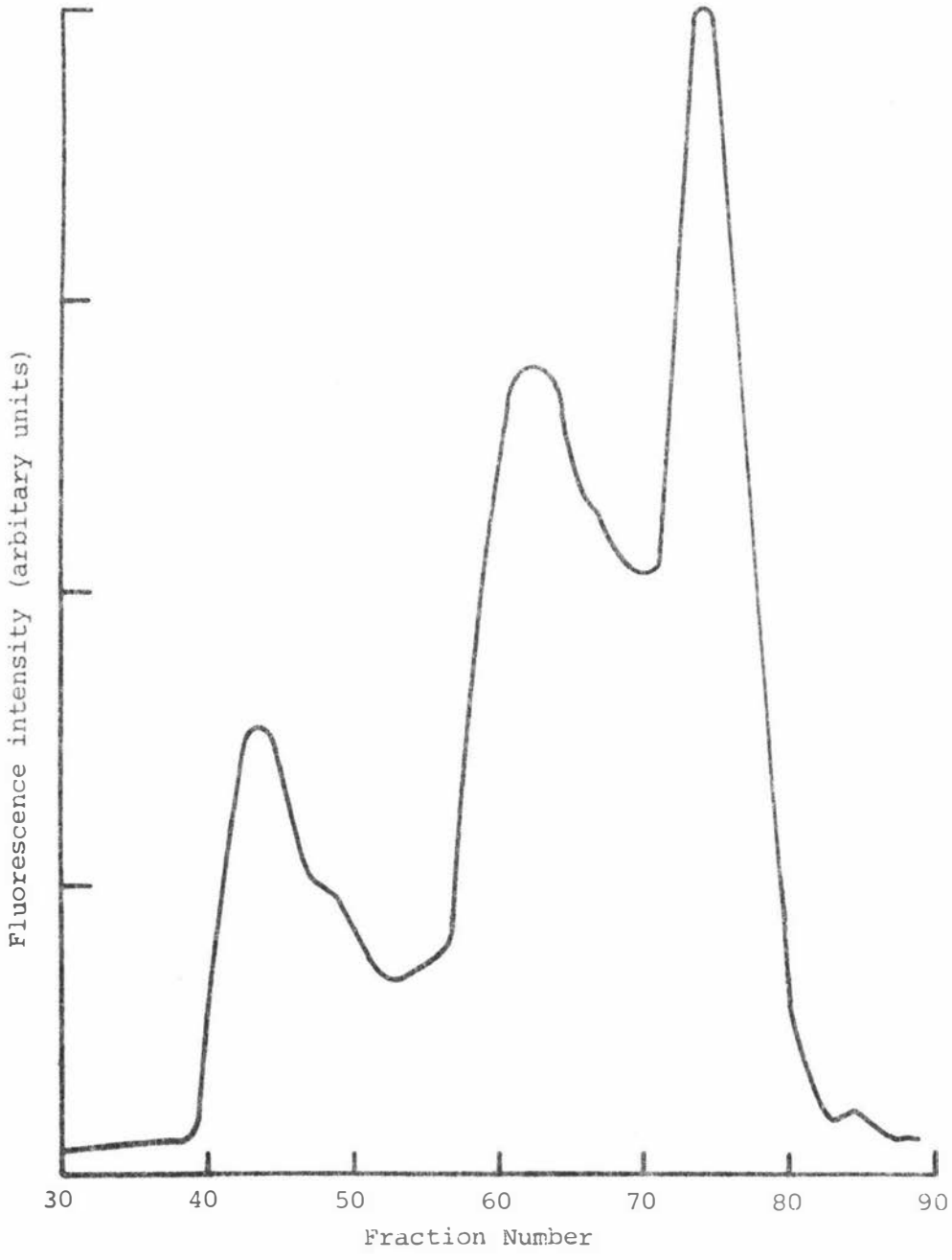

FIGURE 4: Sephadex G-15 chromatographic separation of the amino acids and peptides in the NPN fraction of ^{14}C-labelled milk.

has been described elsewhere (page 6).

6.7 Plate Counts

Culture samples (1 ml) were added to sterile 0.01 M β-glycerophosphate buffer (100 ml, pH 7.0) at 4°C and blended for 1 min at full speed in an Ato Mix Blender (Measuring and Scientific Equipment Ltd, London, England) to reduce the streptococcal chains to uniform size (Martley, 1972). Suitable dilutions were made with the same buffer. Aliquots were plated in M17-agar medium (Terzaghi & Sandine, 1975).

6.8 Free Amino Acids in Milk as a Nitrogen Source for Growth

Uniformly ^{14}C-labelled glycine, valine and isoleucine (The Radiochemical Centre, Amersham, England) were added individually to different aliquots of LHRSM so that the total concentration of each free amino acid (Table 4) was increased by only 0.5%, 2.0% and 6.4%, respectively. The specific activities of the amino acids in LHRSM were 0.05, 0.20 and 0.67 mCi/mmole for glycine, valine and isoleucine, respectively. The LHRSM aliquots were inoculated (1%) with AM_2 or E_8 and incubated at 30°C. Samples were removed at intervals, the cells harvested and washed before protein extraction and measurement of radioactive specific activity. Radioactivity in the culture supernatants was also determined. Cell densities, which were initially too low for turbidimetric measurement, were determined as blended plate counts.

6.9 Milk Protein as a Nitrogen Source for Growth

A solution of ^{14}C-labelled total milk protein was added to LHRSM medium (4% (v/v); final specific activity 89 x 10^3 dpm/g milk protein). The addition of ^{14}C-labelled protein increased the total protein concentration by about 4%. After inoculation (1%) with AM_2 or E_8, the cultures were incubated at 30°C and cells were harvested at intervals. The cell density was determined after washing, cell protein was extracted and the radioactivity counted.

The possibility that milk proteins adsorbed to the cell sur-

face, thereby causing erroneous results in the above experiments, was examined. A culture (100 ml) of strain 266 was grown in RSM at 30°C. Just prior to coagulation (pH ∿ 5.0), chloramphenicol (10.8 mg) was added to stop protein synthesis (Harvey, 1965). After 30 min , a solution (3.7 ml) of ^{14}C-labelled casein was added (specific activity in RSM of 280×10^3 dpm/g, 34 mg casein/ml). After 0, 10, 20 and 40 min a sample (25 ml) was removed and the cells harvested by centrifugation after pH adjustment to 7.0 and dispersion of micelles by addition of sodium citrate (1.5%, w/v). Washed cells were suspended in 1 ml distilled water and the radioactivity counted.

6.10 Individual Milk Proteins as Nitrogen Sources for Growth

Solutions (5 ml) of ^{14}C-labelled pure milk proteins (κ-casein, β-casein, α-lactalbumin and β-lactoglobulin) or α_s-casein fractions were added to the LHRSM cultures (100 ml) of AM_2 and E_8. In the solutions of α_{s1}- and α_{so}-caseins (fractions 107 to 115, Fig. 3) and α_{s2}-, α_{s3}- and α_{s4}-caseins (fractions 92 to 103, Fig. 3), the individual caseins were in the same proportions as found in the original milk. The addition of ^{14}C-labelled milk protein increased the total concentration of that milk protein in LHRSM medium by 5%. The specific activity of each protein in the medium was 118×10^3 dpm/g while the concentration of each protein in LHRSM before the addition of radioactive protein is given in Table 5. The cultures were grown for 5.5 generations and the cells harvested just prior to coagulation. The cell mass was determined after washing, cell protein was extracted and the radioactivity counted.

6.11 Protein Content of Bacterial Cells

Washed cells were resuspended in distilled water (0.2 mg (dry weight) bacteria/ml), mixed with an equal volume of 2M NaOH and heated for 10 min in a boiling water bath (Herbert *et al.*, 1971). The protein content was then determined by the Folin-Ciocalteu method (Lowry *et al.*, 1951) using bovine serum albumin as the reference standard. This value was determined

for each strain and was subsequently used to calculate the amount of bacterial protein in suspensions of known cell density.

6.12 Extraction of Bacterial Protein for Specific Activity Determinations

An equal volume of 10% (w/v) TCA was added to washed cells in distilled water and the suspension heated for 30 min at 90°C (Marchesi & Kennell, 1967). The precipitate was centrifuged (4300 x g, 1 min) and washed twice with 5% (w/v) TCA (2 ml) and once with 1% (v/v) acetic acid (Thomas & Batt, (1969b). The precipitate was finally suspended in distilled water (1 ml), mixed with polar scintillation cocktail (8 ml) (page 3) containing Cab-O-Sil (0.3 g) and counted (page 3).

6.13 Nitrogen Determinations

The nitrogen content of the milk fraction soluble in 0.1 M acetate buffer (pH 4.7) was defined as non-casein nitrogen (NCN). The nitrogen contents of the TN, NCN and NPN fractions were determined as previously described (page 5).

6.14 Amino Acid Analyses

To determine the free amino acid content of LHRSM, a known amount of norleucine was added as an internal standard and the NPN fraction prepared. After TCA extraction, the solution was evaporated to dryness and redissolved in 0.2 M sodium citrate buffer (pH 2.2) before application to the amino acid analyzer (page 8). To determine the total amino acid content of the NPN fraction and bacterial protein, samples were hydrolyzed by dissolving the dry material in 6 M HCl, and heating for 24 h at 110°C under vacuum. The hydrolysate was dried in the rotary evaporator and redissolved in the sodium citrate buffer before application to the amino acid analyzer.

CHAPTER 7

RESULTS

7.1 Free Amino Acid and Peptide Content of LHRSM

A typical amino acid analyzer trace for the NPN fraction of LHRSM is shown in Fig. 5. Cysteine and methionine were not detectable and tryptophan could not be determined as it is eluted with ammonia. The two small peaks that eluted between the buffer change and norleucine are in the region where tyrosine and phenylalanine normally elute but the peaks are unlikely to be due to amino acids because of their irregular shape. Concentrations of amino acids ranged from $\sim$ 0.8 µg/ml for proline and isoleucine to 35.9 µg / ml for glutamic acid (Table 4).

An indication of the concentration of low molecular weight peptides was obtained by acid hydrolyzing the NPN fraction. This led to a three-fold increase in total amino acids and the appearance of methionine (Table 4). A typical analyzer trace is shown in Fig. 6. Other differences with the unhydrolyzed fraction are the change in pattern of the peaks eluted before aspartic acid and the appearance of the large unknown peak between the buffer change and norleucine. This peak would obscure any tyrosine and phenylalanine present.

7.2 Protein Compositon of LHRSM

The percentage compositon of the individual caseins and whey proteins in the total protein fraction of LHRSM was calculated from DE52 and G-75 column elution profiles and the measured values for TN, NCN and NPN. The 280 nm extinction coefficients ($E_{1\,cm}^{1\%}$) used and the resulting compositonal data are given in Table 5. Immunoglobulins and serum albumin were not determined separately as turbid material eluted at the same time, excluding the use of extinction coefficients.

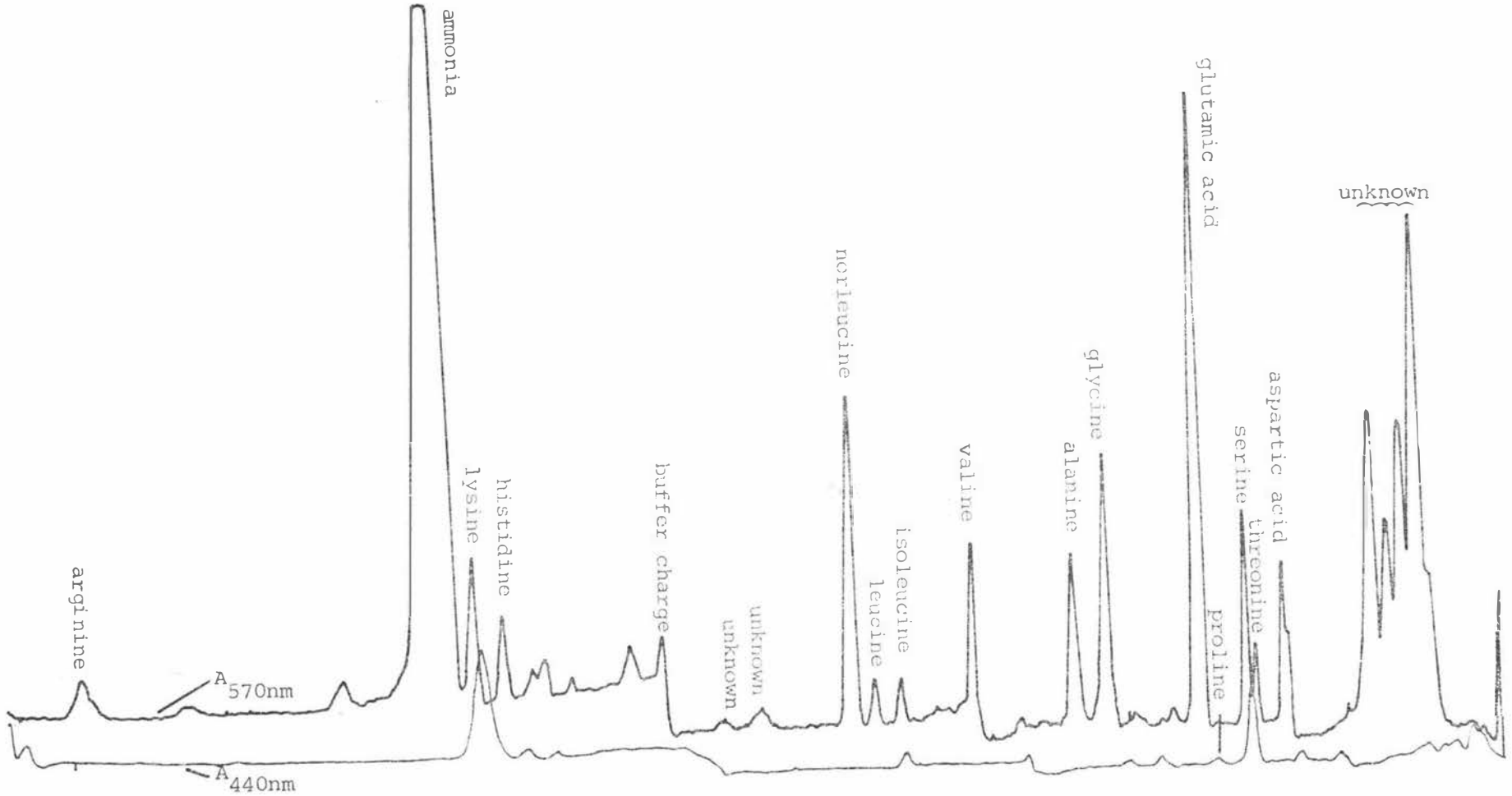

FIGURE 5: Amino acid analyzer chart showing the relative concentration of free amino acids in the NPN fraction of LHRSM. Norleucine was used as an internal standard.

TABLE 4: Free Amino Acids in NPN Fraction of LHRSM Medium and Amino Acid Composition after Acid Hydrolysis of this Fraction

Amino Acids	Free Amino Acids (µg/ml)	After Hydrolysis (µg/ml)
Aspartic Acid	5.1	25.7
Threonine	1.3	10.8
Serine	3.7	18.8
Glutamic Acid	35.9	78.1
Proline	0.8	5.6
Glycine	5.3	19.7
Alanine	3.5	9.6
Cysteine	a	a
Valine	2.6	11.0
Methionine	a	3.7
Isoleucine	0.8	6.4
Leucine	1.2	7.3
Tyrosine	b	b
Phenylalanine	b	b
Histidine	2.8	4.0
Lysine	4.1	18.4
Tryptophan	b	b
Arginine	1.6	3.5
Total	68.7	222.6

a Not detectable

b Not determined (See Results)

Footnote:
Relative standard deviations of the individual amino acids are < 3.0% using the Locarte Analyzer.

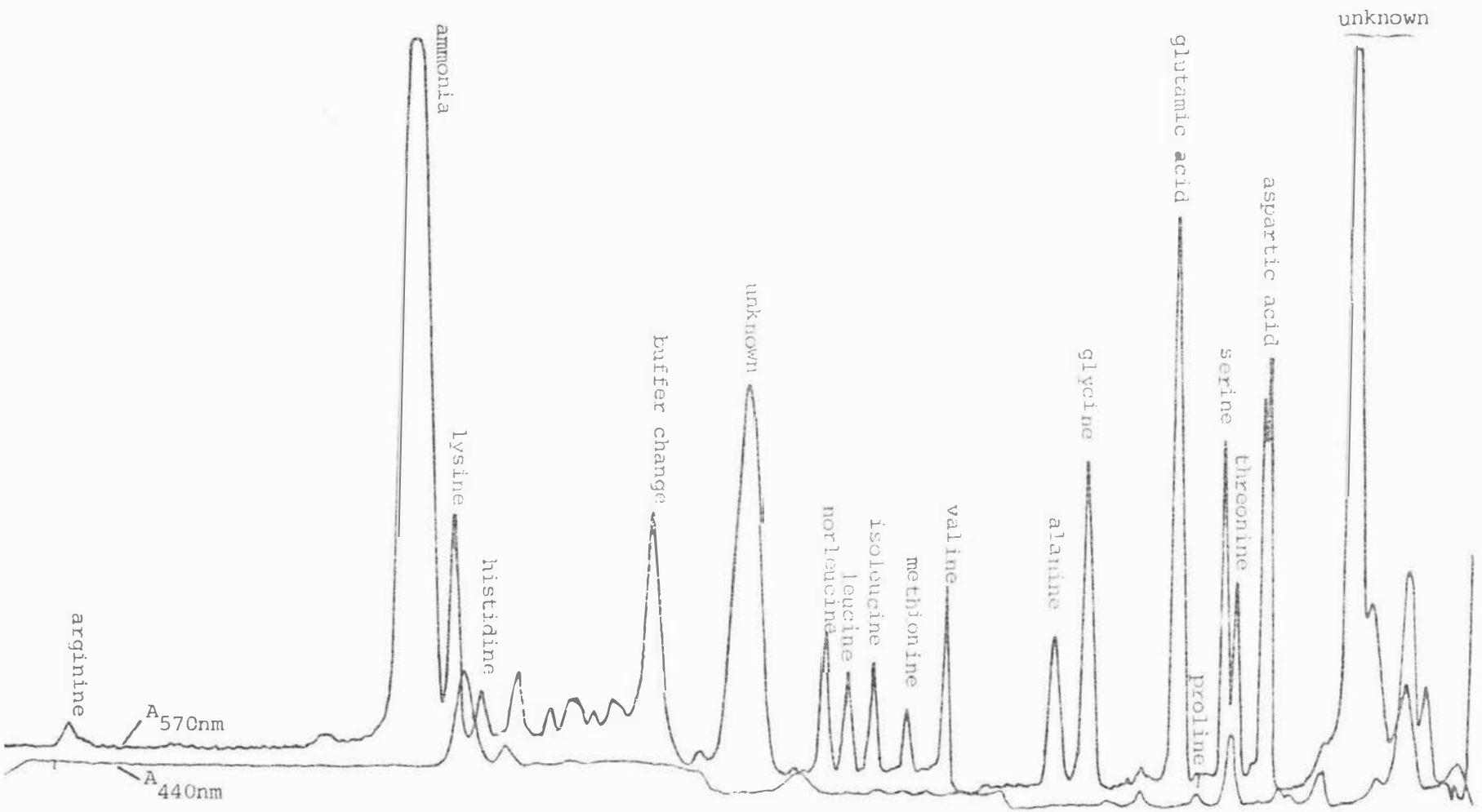

FIGURE 6: Amino acid analyzer chart showing the relative concentration of amino acids in acid hydrolyzed NPN fraction of LHRSM. Norleucine was used as an internal standard.

TABLE 5: Protein Composition of LHRSM

Protein	Percentage of Total Protein[a]	$E_{1\ cm}^{1\%}$ at 280nm	Reference for $E_{1\ cm}^{1\%}$
Caseins[b]			
κ-	11.0	10.0	Waugh et al.(1970)
β-	28.5	4.7	" " " "
α_{s1}-, α_{s0}-	28.2	10.0	" " " "
α_{s2}-, α_{s3}-, α_{s4}-	7.2	10.3	Richardson & Creamer (1975)
γ_1-	3.5	5.5	c
γ_2-	1.2	9.4	c
γ_3-	1.0	9.6	c
β-lactoglobulin	10.8	9.6	Bell & McKenzie (1967)
α-lactalbumin	2.8	20.9	Wetlaufer (1962)
Immunoglobulins + serum albumin	5.8[d]		

a Mean of two determinations

b Caseins defined by Whitney et al.(1976)

c Calculated from the primary structure of β-casein (Mercier et al.,1972)

d Determined by subtracting β-lactoglobulin and α-lactalbumin from total whey protein

7.3 Protein Content of Cells and Amino Acid Composition

Protein accounted for 52% and 57% of the dry matter in LHRSM-grown cells of AM_2 and E_8, respectively.

The amino acid content of bacterial protein from AM_2 and E_8 was determined and used to calculate the concentration of each amino acid contained in bacterial protein in a coagulated milk culture where the cell density is approximately 500 µg (dry weight) bacterial/ml. There was close similarity in the proportion of amino acids with the two strains but the concentrations in the E_8 culture were slightly higher (Table 6) due to the higher percentage of protein in E_8 cells.

7.4 Free Amino Acids in Milk as a Nitrogen Source for Growth

In all cultures the level of radioactive amino acid remaining free in the medium decreased as the culture grew (Fig. 7). The rate of uptake of free isoleucine and valine decreased after one to two generations with both strains while the uptake of glycine occurred at an almost constant rate. In LHRSM containing ^{14}C-labelled amino acids, the specific activity of bacterial protein increased to a maximum during the first few generations (Fig. 8) and then fell sharply except with E_8 grown in the presence of ^{14}C-glycine.

7.5 Low Molecular Weight Peptides in Milk as a Nitrogen Source for Growth

Similar specific activity values for bacterial protein were obtained when the cells had undergone between one and five generations (Table 7) suggesting that the peptides originally present in the milk supplied a constant proportion of the nitrogen used for culture growth.

The Sephadex G-15 elution profiles of the peptides and free amino acids in LHRSM after growth of E_8 were compared to the original milk (Fig. 9). The amount of material eluted in fractions 40 to 50 (> 1500 daltons) and in fractions 55 to 70 (medium to low molecular weight peptides and amino acids) approximately doubled during growth of bacteria.

TABLE 6: Concentration of Amino Acids Contained in Bacterial Protein in Coagulated Milk Cultures of S. cremoris AM_2 and E_8 Containing 500 µg (dry weight) bacteria/ml

Amino Acids	Concentration in Bacterial Protein (µg/ml)	
	AM_2	E_8
Aspartic Acid	29.4	32.0
Threonine	14.9	15.8
Serine	12.0	13.1
Glutamic Acid	40.1	44.8
Proline	8.8	9.8
Glycine	11.9	12.8
Alanine	19.2	21.7
Cysteine	a	a
Valine	14.7	15.7
Methionine	6.5	6.8
Isoleucine	12.5	13.2
Leucine	21.5	22.8
Tyrosine	10.4	11.6
Phenylalanine	15.8	19.1
Histidine	5.9	6.2
Lysine	23.1	24.9
Tryptophan	b	b
Arginine	13.3	14.8
Total	260.0	285.1

[a] Not detectable

[b] Destroyed during hydrolysis

Footnote:

Relative standard deviations of the individual amino acids are < 3.0% using the Locarte Analyzer.

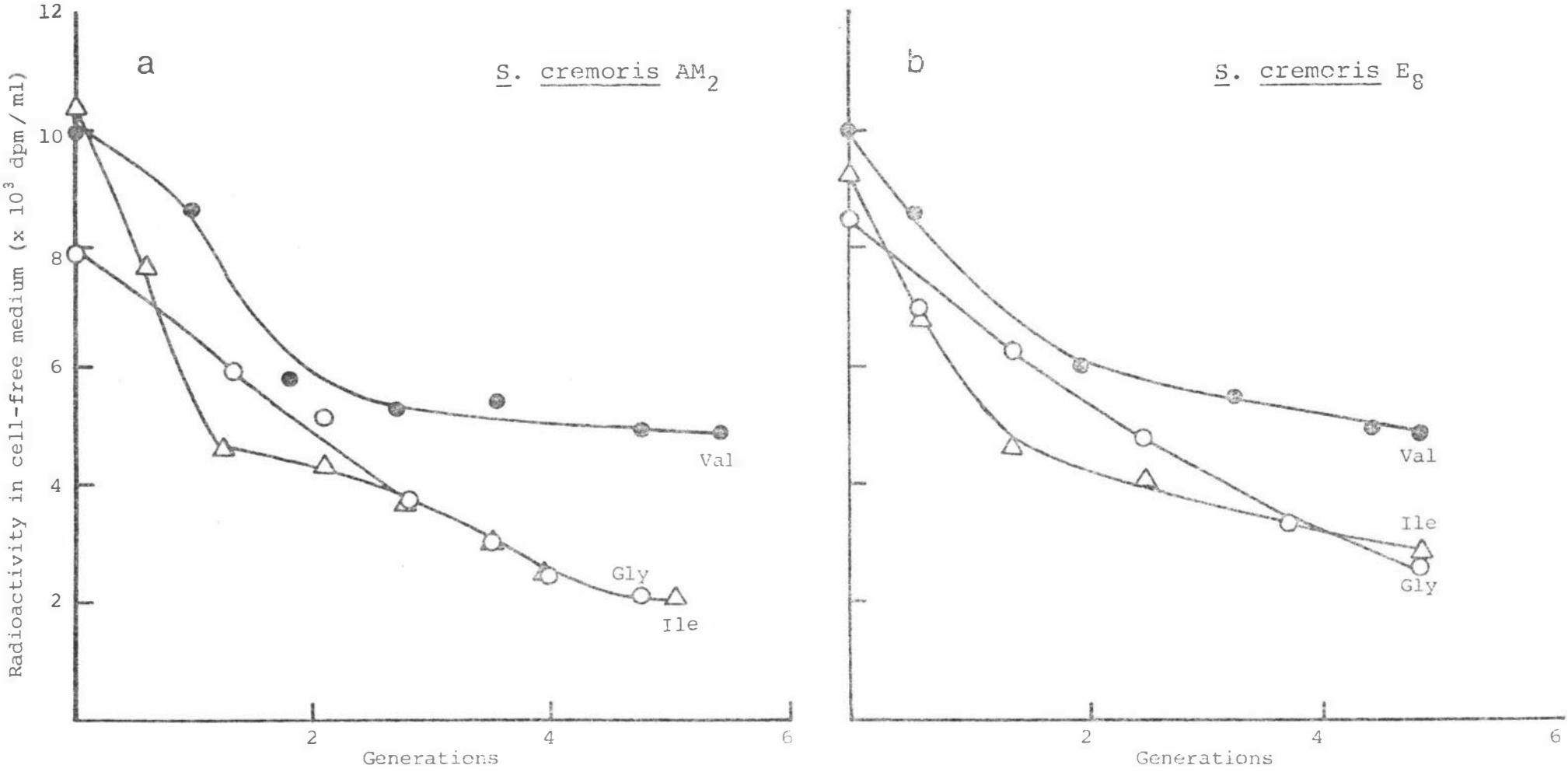

FIGURE 7: Removal of ^{14}C-labelled free amino acids from culture medium during growth of S. cremoris AM_2 and E_8. The media were inoculated (1% of a buffered milk culture-cell density equivalent to ∿ 2% of a normal coagulated milk culture) and incubated at 30°C.

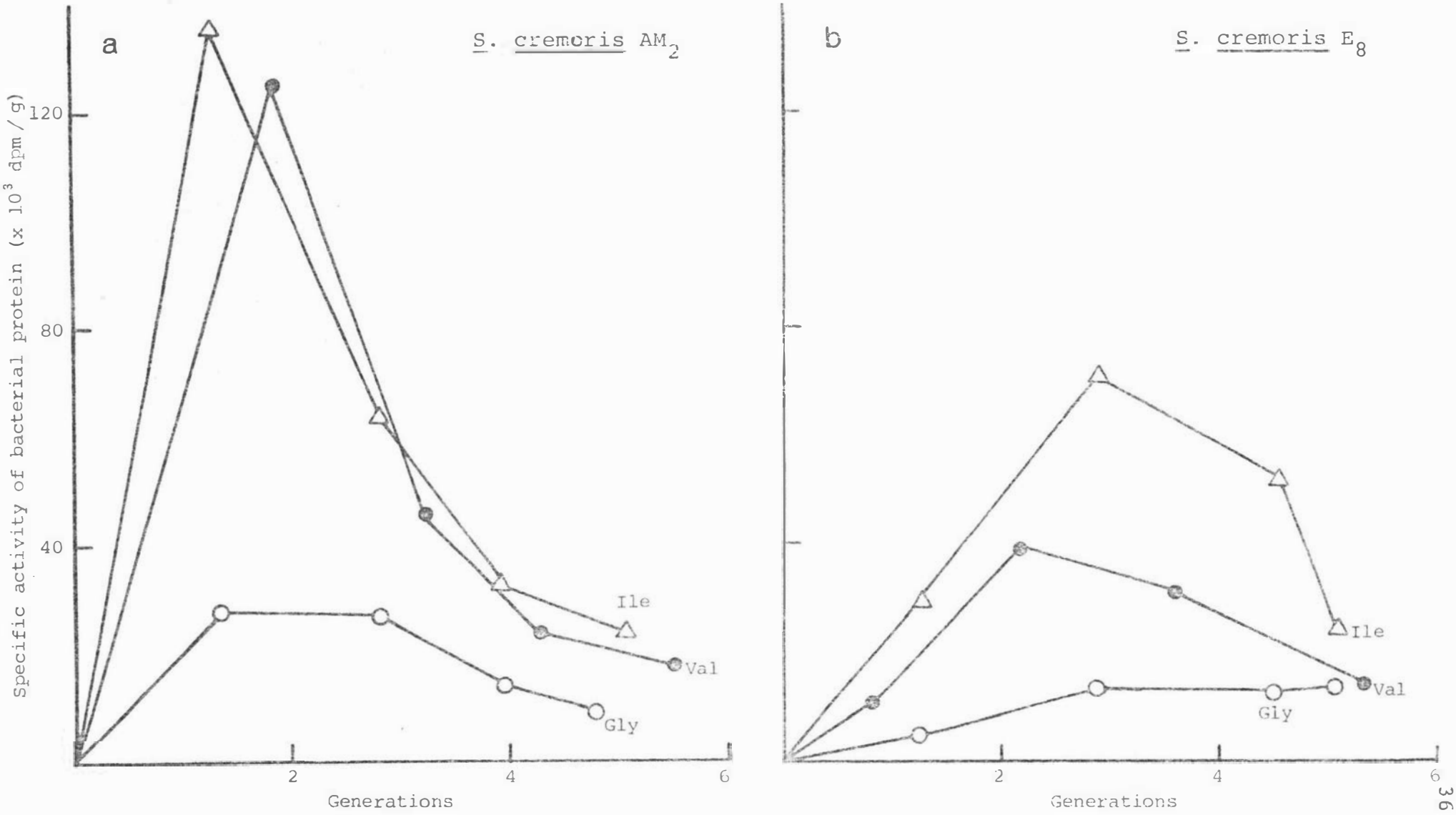

FIGURE 8: Specific activity of bacterial protein with S. cremoris AM_2 and E_8 growing in LHRSM containing ^{14}C-labelled amino acids. For details of inoculation and incubation see Fig. 7.

TABLE 7: Specific Activity of Bacterial Protein from S. cremoris AM_2 and E_8 Cells Growing in LHRSM Containing Added ^{14}C-labelled Peptides

Strain	Generations	Specific Activity of Bacterial Protein ($\times 10^3$ dpm/g)
S. cremoris AM_2	1.1	23
	3.2	18
	4.0	18
S. cremoris E_8	1.2	30
	2.4	32
	4.2	28
	5.3	25

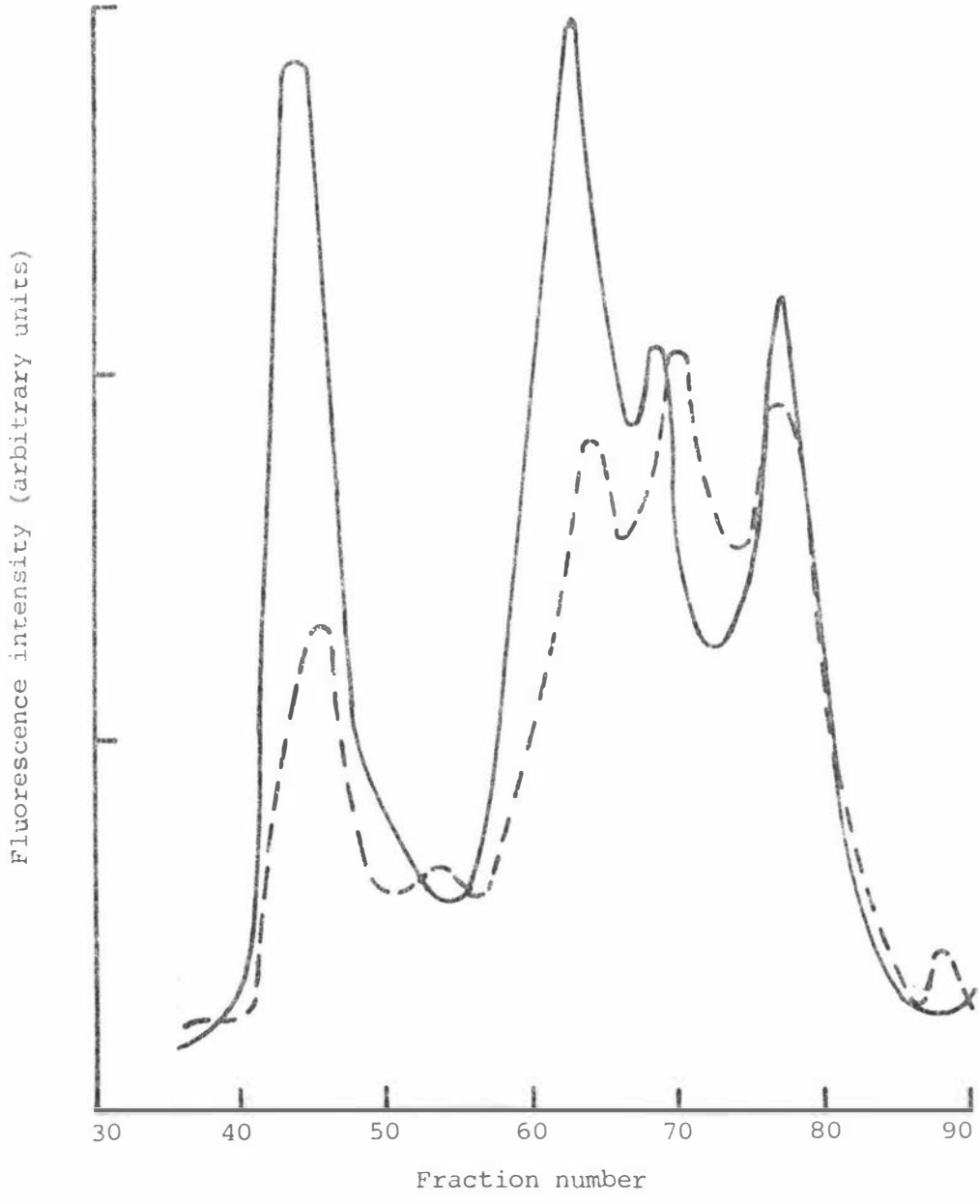

FIGURE 9: Sephadex G-15 chromatographic separation of the amino acids and peptides in the NPN fraction from LHRSM (---) and after growth of S. cremoris E_8 (——). The culture was incubated at 30°C for ∿ 6 generations until pH 5.0 was reached. Samples applied to the column were from the equivalent of 2 ml of medium.

7.6 Milk Protein as a Nitrogen Source for Growth

In the first five generations, the cell protein increased in specific activity with both strains (Fig. 10). Values for repeat experiments were ± 10% of those given in Fig. 10.

Adsorption of milk protein to the cell surface was investigated as a possible source of error in the above experiments. Although there was some adsorption of ^{14}C-labelled casein, this would account for less than 10% of the value obtained for the incorporation of ^{14}C into bacterial protein when cells were grown in the presence of the same amount of ^{14}C-labelled milk protein.

7.7 Individual Milk Proteins as Nitrogen Sources for Growth

The specific activities of bacterial protein from cells of AM_2 and E_8 grown in the presence of individual ^{14}C-labelled milk proteins are given in Table 8. Values for AM_2 in the presence of the α_{s2}-, α_{s3}-, α_{s4}-casein fraction were greater than the corresponding values for E_8. All other values for AM_2 were less than or similar to the corresponding values for E_8.

7.8 Growth of S. Cremoris 266 Prt^+ and Prt^-

S. cremoris 266 Prt^+ grew at an exponential rate (generation time 63 min) until a terminal T_{480nm} value of 3.3 was reached (Fig. 11). The Prt^- variant (proteinase activity negligible against casein) grew initially at a rate similar to the parent strain but growth virtually stopped after about 6 h at a T_{480nm} value which was 12.0% of that attained by the Prt^+ strain (Fig. 11).

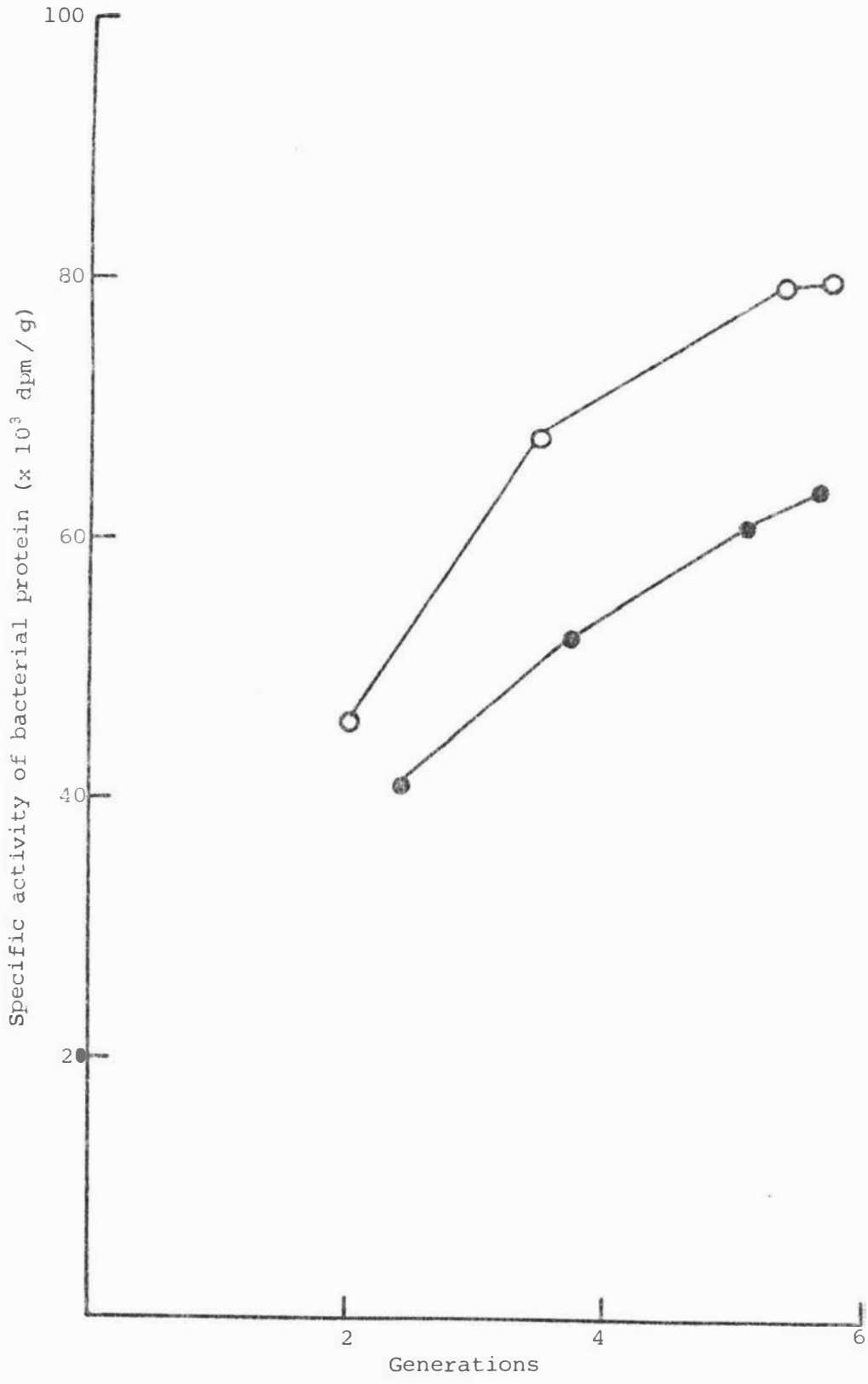

FIGURE 10: Specific activity of bacterial protein with S. cremoris AM_2 (o) and E_8 (●) growing in LHRSM containing added ^{14}C-labelled milk protein. The media were inoculated (1% of a buffered milk culture - cell density equivalent to 2% of a normal coagulated milk culture) and incubated at 30°C.

TABLE 8: Specific Activity of Bacterial Protein from S. cremoris AM_2 and E_8 Cells Grown for 5.5 Generations in LHRSM Containing Pure ^{14}C-Labelled Milk Proteins

Milk Protein	Specific Activity of Bacterial Protein (x 10^3 dpm / g)			
	AM_2		E_8	
	Expt I	II	Expt I	II
κ-casein	14.4	16.1	21.0	18.4
β-casein	15.9	13.8	15.2	11.5
α_{s1}-, α_{s0}-caseins	a	8.0	9.3	10.4
α_{s2}-, α_{s3}-, α_{s4}-caseins	13.9	8.1	4.3	6.9
α-lactalbumin	8.0	6.9	8.8	9.2
β-lactoglobulim	13.0	6.9	13.9	16.1

[a] Not determined

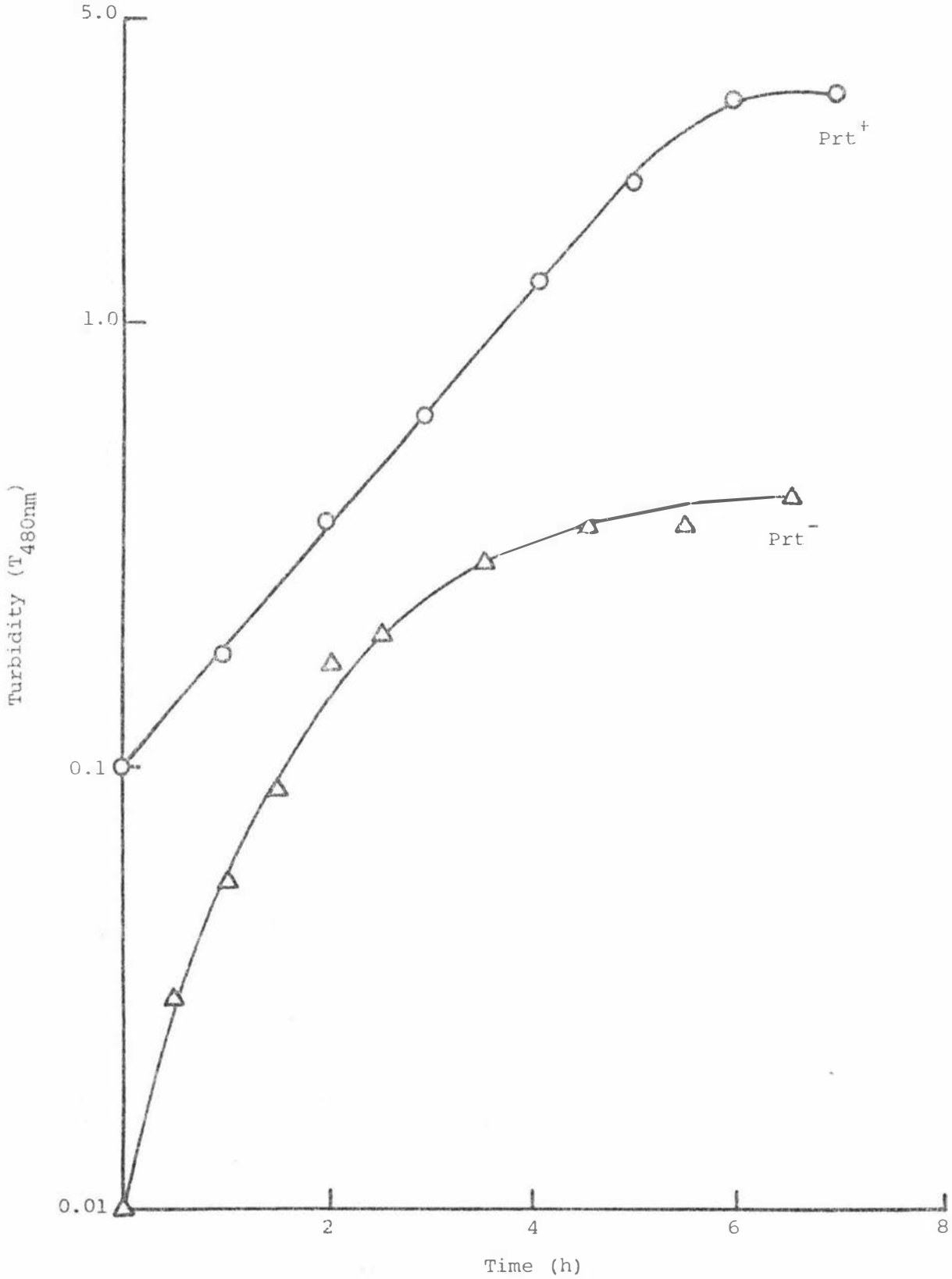

FIGURE 11: Growth of S. cremoris 266 Prt^+ and Prt^- cells in LHRSM

CHAPTER 8

DISCUSSION

Analysis of the low heat skim milk powder using DE52 and G-75 chromatography did not show any evidence of heat-induced protein interaction. The levels of individual free amino acids in the reconstituted milk were similar to those reported by Aston (1975) and Deutsch & Samuelsson (1959) for freshly drawn normal milk. The total free amino acid concentration (68.5 µg / ml) was of the same order as those reported by these workers (30.7 µg / ml and 67.9 µg / ml, respectively). The composition of potential nitrogen sources in the LHRSM medium was, therefore, considered to be similar to that in fresh milk.

With the exception of methionine (and possibly phenylalanine, which could not be determined), all the amino acids essential for growth of lactic streptococci (Reiter & Oram, 1962) were present as free amino acids in LHRSM medium (Table 4). However, acid hydrolysis of the NPN fraction showed that methionine was present in peptides (Table 4). Comparison of data in Tables 4 and 6 shows that most of the essential amino acids were not present in sufficient amounts, either free or in peptides, to account for the synthesis of the bacterial protein required for growth to the cell densities found in coagulated cultures. The limited growth of Prt^- cultures in milk is presumably due to the depletion of an essential amino acid in the low molecular weight nitrogen fraction.

The most accessible potential source of nitrogen for cell growth in milk is the free amino acids. The removal of ^{14}C-labelled amino acids from the medium (Fig. 7) showed that these compounds were taken up by cells growing in milk. In the first few generations the specific activity of cell protein increased but then decreased at higher cell densi-

ties (Fig. 8). This indicates that the free amino acid pool is an important source of nitrogen for growth but only at low cell densities.

The low molecular weight peptide fraction of milk appeared to be a significant potential source of nitrogen as acid hydrolysis of the NPN fraction resulted in a three-fold increase in the total concentration of amino acids (Table 4). The isolation of the NPN fraction containing low molecular weight peptides was based on solubility in 12% (w/v) TCA which is somewhat arbitrary. However, Sephadex G-15 chromatography showed that the bulk of the NPN fraction had a molecular weight of less than 1500 daltons Figs. 4 and 9). The upper size limit for peptide transport through lactic streptococcal membranes is considered to be of five or six residues (Law et al., 1976; Rice et al., 1978) so that some of the peptides in the NPN fraction would require hydrolysis before uptake. The peptide fraction prepared from NPN material in ^{14}C-labelled milk would not contain all the low molecular weight peptides present since large amino acids and small peptides having similar hydrodynamic volumes will tend to overlap during elution and fractions containing detectable free amino acids were excluded from the peptide fraction. The origin of peptides in the NPN fraction is unknown but they can only partly arise from hydrolysis of caseins or whey protein as the specific activity of this fraction was approximately 0.7×10^6 dpm/g compared to 2.3×10^6 dpm/g for milk protein. This low specific activity limited the usefulness of the labelled milk peptides but incorporation of this material into cell protein was observed during the growth of AM_2 and E_8 in LHRSM (Table 7). It was concluded that, in contrast to free amino acids, a constant proportion of bacterial protein was derived from the original pool of peptides in the milk.

The use of some of the milk proteins as nitrogen sources for cell growth may be influenced by the way in which the added milk protein equilibrated with the native milk proteins. In milk the particulate micelles are composed of caseins, immersed in a serum containing the soluble whey proteins, peptides and free amino acids. When ^{14}C-labelled β-casein

was added to milk which was then held at 4°C for 16 h, the added protein largely equilibrated with β-casein in the micelle (Creamer et al., 1977). There is no information concerning the equilibration of other caseins when added to milk.

When ^{14}C-labelled total milk protein was added to LHRSM the specific activity of bacterial protein increased as the culture grew. This indicates that milk protein becomes an increasingly important source of nitrogen for growth as the cell density increases. The specific activity of protein from cells grown in a medium containing ^{14}C-labelled milk protein will depend on the amino acid composition of the milk protein since specific activities of individual amino acids are different (Table 1). Therefore no quantitative comparisons between values in Table 8 for each strain can be made. The only conclusion to be drawn from this data is that all the individual milk proteins tested were used as nitrogen sources for growth. There are a number of factors which may influence the total amount of each milk protein assimilated into cell protein. These include the concentration of each protein in the milk, inherent resistance to proteolysis due to the structure of the protein and the accessibility of each protein to the surface of the bacterial cell where the proteinase system is located. Whey proteins and that fraction of κ-casein that residues on the surface of the micelle are likely to be more accessible than micelle core proteins.

In summary, the source of nitrogen for cell growth in milk appears to depend on the cell density of the culture. At low cell densities, free amino acids and peptides are used by Prt^+ cells and Prt^- cells alike. As the cell density increases an essential amino acid in these low molecular weight materials becomes limiting, and growth of Prt^- cells stops. At cell densities above ∿ 10% of those found in coagulated milk cultures, Prt^+ cells appear to be dependent for growth on their proteinase to supply peptides from milk proteins. This proteinase system is located in the cell wall, possibly near the outside surface (Thomas et al., 1974) so that the proteolysis products are likely to diffuse into the medium. Cell density increase is accompanied by elevation

of the level of cell wall-associated proteinase in the culture and hence by an acceleration in the supply of peptides. Peptides derived from milk protein will thus form an increased proportion of the soluble nitrogen compounds which diffuse through the wall and are transported through the membrane into the cell. The fact that growth of Prt^+ cells of S. cremoris 266 in milk was exponential (Fig. 11) means that proteinase activity must begin to function effectively in supplying proteolysis products before the concentration of the low molecular weight nitrogen initially present becomes rate limiting.

SECTION III

ASPECTS OF CELL WALL-ASSOCIATED PROTEINASES

Part 1

Evaluation of Proteinase Assay with ^{14}C-Labelled Casein Substrate

CHAPTER 9

INTRODUCTION

The activity of proteolytic enzymes is usually determined with casein or haemoglobin as the substrate, especially when the number of proteinases present or their specificities is unknown. Hydrolysis is normally stopped by the addition of TCA and the concentration of soluble products then determined either by measuring A_{280nm} values, or by colorimetry using the Folin-Ciocalteu reaction (Lowry et al., 1951), the biuret reaction (Robinson & Hodgen, 1940) or the ninhydrin reaction (Moore & Stein, 1954). These methods lack sensitivity when used to assay intact cells or cell-free extracts of lactic streptococci, as indicated by incubation times of up to 24 h at 37°C (Westhoff & Cowman, 1970), the use of cells derived from more than 200 ml culture in a single assay (Cowman & Speck, 1967) and sonic extract from 250 mg (wet weight) of cells in a single assay (Van der Zant & Nelson, 1953) to obtain measurable proteolysis. In addition, the traditional methods pose some problems when the proteolytic activity of intact bacterial cells or crude cell extracts are assayed (Lawrence et al., 1976). Extracts from concentrated cell suspensions contain potential substrate for the proteinase system and also usually contain TCA-soluble material which reacts positively in the above assays. In addition, intact cells of S. lactis release free amino acids from endogenous sources when held at 30°C in buffer (Thomas & Batt, 1969a). A method of partially overcoming these problems is to use a protein substrate which is labelled with a radioactive isotope. Although potentially competing substrates and low molecular weight TCA-soluble materials are not eliminated, only products derived from the radioactive protein will be detected. Thomas et al. (1974) covalently bonded ^{125}I to tyrosine residues of casein whereas Exterkate (1975) reductively methylated lysine residues with ^{14}C-formaldehyde. The latter method produces more frequent labelling within the protein molecule, since the various

caseins contain between nine and fourteen lysine residues per casein molecule compared to four to nine tyrosine residues per casein molecule and gives a substrate which has a much longer half-life. Despite these advantages, the substrate is chemically modified and labelling is not random since in all the caseins the frequency of lysine residues is greater towards the amino terminal end of the molecules (Mercier et al., 1972). Preliminary experiments were undertaken to see if chemical modification of casein substrates resulted in different products or rates of proteolysis. The results (see Appendix II) showed that the action of chymosin and trypsin was, in some cases, altered by methylation of α_{s1}- and β-caseins.

In order to clarify the physiological role of proteinase during starter growth in milk, the substrate for proteinase assays should be an effective amino acid source for growth in milk. Potential amino acid sources in milk are free amino acids, peptides and proteins including casein (see Section II). An ideal substrate for assay of the proteinases from lactic streptococci would, therefore, be uniformly labelled casein of high specific activity prepared without chemical modification (see Section I).

This section describes the evaluation of a method using ^{14}C-labelled casein substrate for the assay of proteinases either in solution or bound to intact cells of lactic streptococci.

C H A P T E R 1 0

EXPERIMENTAL

10.1 Bacteria

Preparation of stock cultures and inoculum cultures have been described previously (page 21). The detailed composition of the milk medium was not required as in Section II and since the high bulk density powder for LHRSM was difficult to reconstitute, RSM (page 21) was used instead.

10.2 Experimental RSM Medium, Growth and Harvesting of Cells

The experimental RSM medium was prepared in the same way as LHRSM medium (page 22) except that β-glycerophosphate was added to allow growth to a higher cell density (approx. 10^9 cells/ml) without coagulation (Thomas & Turner, 1977). Concentrated (3 M) disodium β-glycerophosphate (Sigma Chemical Co.) solution was adjusted to pH 7.4 with concentrated HCl, autoclaved, and added to the centrifuged milk (2.5 ml/100 ml experimental RSM), giving a final pH of 7.1.

Experimental RSM was inoculated (3%) with a 16 h / 22°C culture and incubated at 30°C. Growth was monitored turbidimetrically and cells harvested as described previously (page 22).

10.3 Release of Cell Wall-Associated Proteinase

Cell wall-associated proteinase was released from lactic streptococci by holding washed cells (20 mg (dry weight) bacteria/ml) in 0.05 M tris-(hydroxy methyl) amino methane (Tris) buffer (pH 7.8) at 25°C for 2 h. The phenomenon of proteinase release is discussed elsewhere (Section III, Part 2, page 69). Cells were then removed by centrifugation (10,000 x g, 1 min) so that the supernatant contained cell-free proteinase.

10.4 Proteinase Assay With ^{14}C-Labelled Casein

A sample (0.1 ml) of cell-free proteinase solution or cell suspension was added to ^{14}C-labelled casein (0.4 ml, 2.35 x 10^6 dpm/g) solution in buffer. The choice of buffer, casein substrate concentration and incubation temperature depended on the experiment. When intact cells were assayed, $CaCl_2$ was added to a final concentration of 0.02 M to minimize the release of proteinase from the cell wall (page 79). The reaction was stopped by the addition of TCA (0.5 ml, 12% (w/v)). After 15 min the mixture was centrifuged (25,000 x g, 1 min) and the radioactivity in the supernatant (0.5 ml) determined.

A quenching curve for 6% (w/v) TCA was obtained as described for water (page 3). Proteolysis was measured as the radioactive material, soluble in 6% (w/v) TCA, produced during the assay and has units of dpm/ml. Proteinase activity is defined as proteolysis per unit concentration of enzyme and for intact cells has units of dpm/mg (dry weight) bacteria. The proteinase activity of cell-free proteinase was calculated on the basis of the initial bacterial dry weight from which the preparation was derived and has units of dpm/mg (dry weight equivalent) bacteria.

10.5 Proteinase Assays with Non-radioactive Casein

Cell-free proteinase solution (0.1 ml) from *S. cremoris* 266 was added to casein (0.4 ml) in 0.05 M cacodylate buffer (pH 6.5) giving a final casein concentration of 0.5% (w/v). The mixture was maintained at 20°C for 4 h before addition of TCA as in the assay involving ^{14}C-labelled casein. Proteolysis over a range of proteinase concentrations was determined using the Folin-Ciocalteu reaction (Lowry *et al.*, 1951) and also the reaction with fluorescamine at pH 6.0 (Beeby, 1980). In the latter assays, supernatant (0.5 ml) containing 6% (w/v) TCA-soluble material was mixed with 0.2 M phosphate buffer (2.5 ml, pH 7.1) giving a final pH of 6.0. An aliquot (0.5 ml) of fluorescamine in acetone (0.4 mg/ml) was rapidly mixed in, the solution equilibrated to 20°C, and fluorescence intensity determined (page 22).

10.6 Effect of pH on Activity

The activity of cell-free proteinase from 266, E_8, AM_2 and ML_3 was assayed over the pH range 5.1 to 7.8 using ^{14}C-labelled casein (0.5%, w/v) and incubation for 4 h at 20°C. The pH of each assay was determined after the stock casein solution (in 0.05 M cacodylate buffer, pH 6.5) proteinase solution (in 0.05 M Tris buffer, pH 7.8) and assay buffer (0.05 M) were mixed. Buffer salts used in the assay systems were, phthalate, acetate, cacodylate, triethanolamine, Tris, imidazole and 2(N-morpholino)ethane sulfonic acid (Mes).

The proteinase activity of intact cells of 266, E_8, AM_2 and ML_3 was determined over the range pH 4.5 to 8.0. Cells were incubated with 0.5% (w/v) ^{14}C-labelled casein solution for 2 h at 30°C in buffers containing $CaCl_2$ (0.02 M). Except for cacodylate, which induced cell lysis, the same buffer salts were used as for cell-free proteinase. Stock casein solutions for the assay of intact cells contained 0.05 M Mes buffer (pH 6.5).

10.7 Effect of Proteinase Concentration on Proteolysis

Cell-free and cell-bound proteinases were assayed over a range of concentrations. Assay conditions for cell-free proteinase consisted of 0.5% (w/v) casein in 0.05 M cacodylate buffer (pH 6.5) and incubation for 4 h at 20°C while cell-bound proteinase was assayed with 0.5% (w/v) casein in 0.05 M Mes buffer (pH 6.5) with incubation for 2 h at 30°C.

10.8 Assay of Thermolysin, Subtilisin and Chymosin Using ^{14}C-Labelled Casein Substrate

Thermolysin (Protease Type X, Sigma Chemical Co.), subtilisin (Protease Type VIII, Sigma Chemical Co.) and chymosin (page 120) in 0.05 M cacodylate buffer (pH 6.5) were assayed with ^{14}C-labelled casein (0.5%, w/v) at enzyme concentrations up to 0.5 µg/ml, 1.1 µg/ml and 99.6 µg/ml, respectively.

CHAPTER 11

RESULTS

11.1 Effect of pH on Proteinase Activity

The pH had little effect on the activity of cell-free proteinase from strains ML_3 and AM_2 in the range pH 5.2 to 7.8 (Fig. 12). Strain 266 was similar except that activity declined at pH 5.2 while proteinase from strain E_8 showed optimum activity between pH 5.8 and 7.0. Results obtained when different buffers were used at the same pH value indicated that proteinase activity was independent of the buffer salt.

Assays using intact cells of 266, AM_2 and ML_3 (Fig. 13) showed that pH had a similar effect on proteinase activity whether the enzyme(s) was cell-bound or cell-free. However, when intact cells were assayed at lower pH values, AM_2 showed the same level of activity down to pH 4.6 whereas the activity of strains 266 and ML_3 decreased markedly (Fig. 13). The activity peak for intact cells of E_8 was consistently sharper than for both the cell-free proteinase from E_8 and for intact cells of the other strains.

On the basis of the above results, pH 6.5 was chosen as the standard pH for the assay of both cell-free and cell-bound proteinase with all strains.

11.2 Effect of Temperature on Stability of Cell-Free Proteinase

There was no appreciable (< 5%) loss in activity when cell-free proteinase from 266 was held for up to 4 h at 20 or 25°C prior to assay (Table 9). At 30°C, activity was reduced by about 10% when the enzyme solution was held for 2, 3 or 4 h prior to assay. Proteinase from strains E_8, AM_2 and ML_3 showed no appreciable decrease in activity when assayed at 20°C for 4 h after being held at that temperature for 4 h (Table 9).

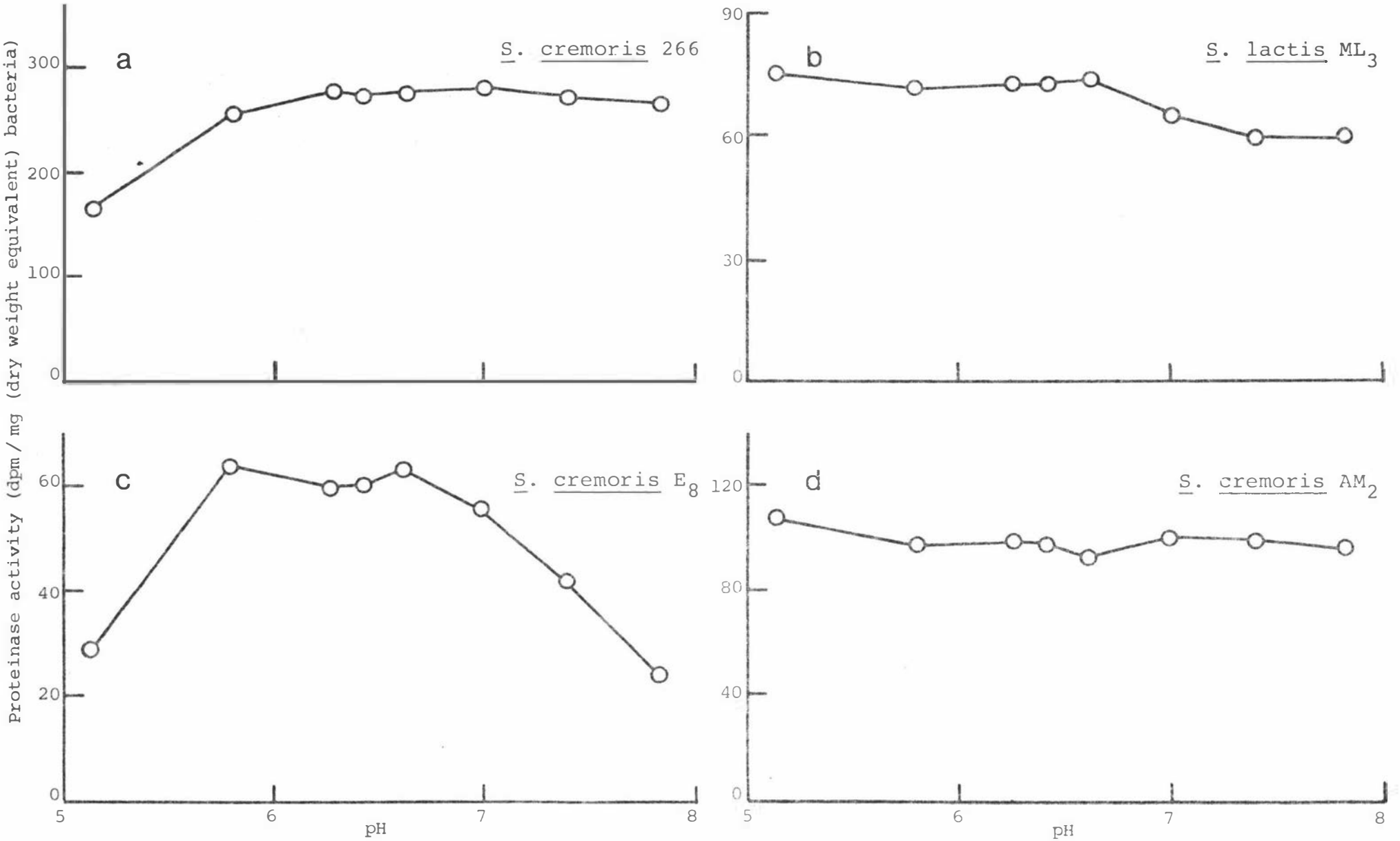

FIGURE 12: Effect of pH on the activity of cell-free proteinase. Proteinase was incubated with ^{14}C-labelled casein (0.5%, w/v) for 4 h at 20°C. See text for details of buffer salts. Note differences in vertical scale.

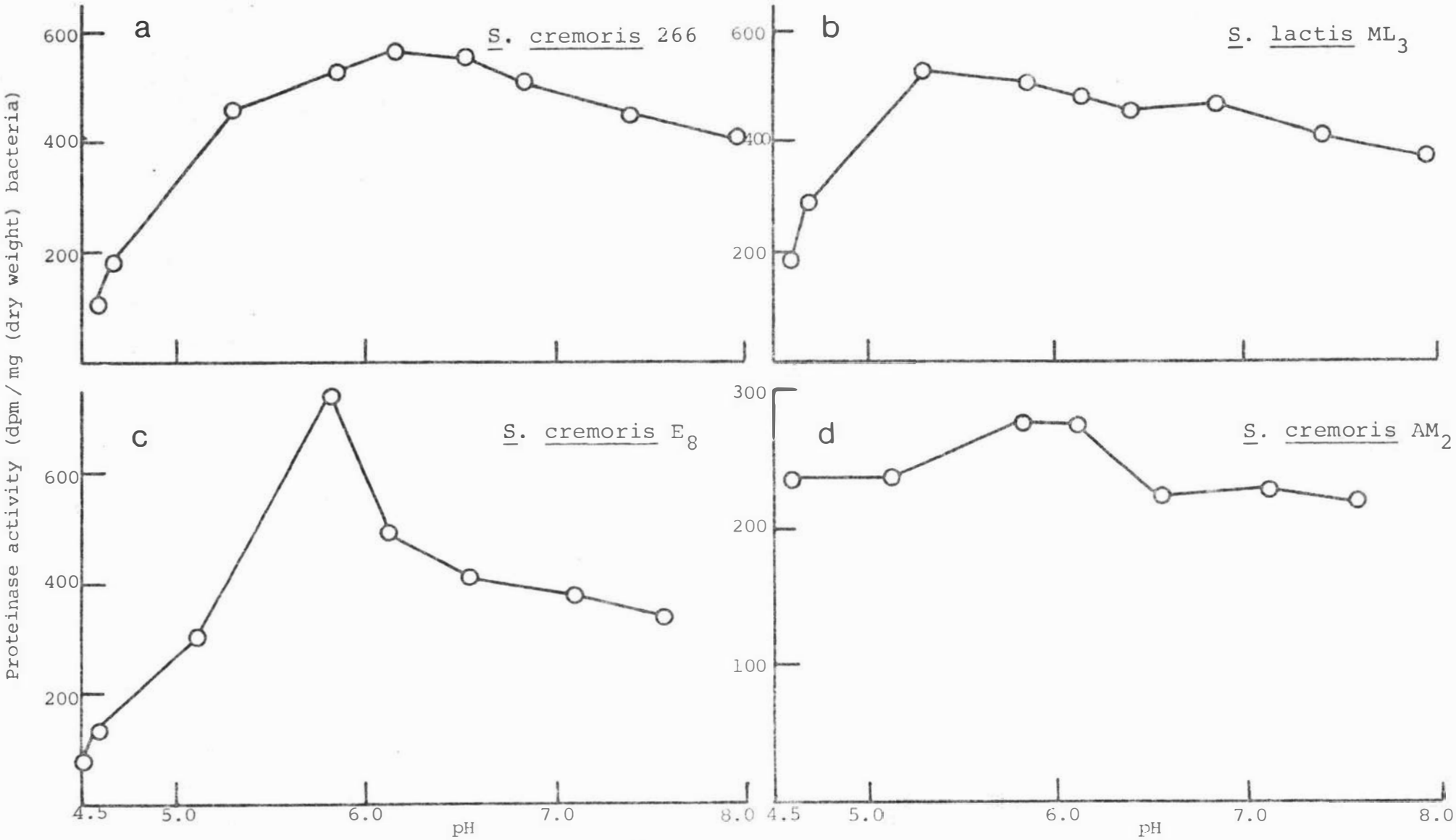

FIGURE 13: Effect of pH on the proteinase activity of intact cells. Cells were incubated with ^{14}C-labelled casein (0.5%, w/v) for 2 h at 30°C. See text for details of buffer salts. Note differences in vertical scale.

TABLE 9: Effect of Temperature on Stability of Cell-Free Proteinase. After being held in 0.05 M Cacodylate Buffer (pH 6.5) under the Conditions Specified, Proteinase (4 mg (dry weight equivalent) bacteria / ml) was Assayed with ^{14}C-labelled Casein (0.05%, w/v) in the Same Buffer

Strain		Temp before assay °C	Time Before assay h	Proteinase Activity dpm/mg (dry weight equivalent) bacteria
S. cremoris	266	control		311
	"	20	4	300
	"	25	2	293
	"	25	3	310
	"	25	4	296
	"	30	1	297
	"	30	2	277
	"	30	3	279
	"	30	4	273
	E_8	control		87
	"	20	4	86
	AM_2	control		180
	"	20	4	186
S. lactis	ML_3	control		140
		20	4	137

Footnote: The relative standard deviation for proteinase assays using ^{14}C-labelled casein and a single batch of cells was < 4%. This variation is applicable to Tables 9 to 14, Figs 12 to 17 and Figs 19 to 25. The relative standard deviation between batches of cells ranged from 9% for ML_3 to 29% for E_8.

Routine assays of cell-free proteinase activity were, therefore, incubated for 4 h at 20°C.

11.3 Effect of Temperature on Activity and Stability of Cell-Bound Proteinase

Proteinase activity of intact cells of 266 and E_8 increased with time at incubation temperatures 25, 30 and 35°C (Table 10). With the same incubation time, proteinase activity increased with increasing temperature (Table 10). The stability of cell-bound proteinase was examined by holding cells in assay buffer, under the same conditions as specified in Table 10, before assay. In general, holding intact cells of 266 and E_8 at 20°C to 35°C before assay resulted in slightly increased proteinase activity, especially with E_8. Only with strain 266 held at 35°C was proteinase activity decreased (Table 11). The possibility of proteinase release during assay was examined by holding cells in Mes buffer (pH 6.5) containing $CaCl_2$ (0.02 M) but no casein. Suspensions were held under the conditions specified in Table 11, centrifuged (10,000 x g, 1 min) and the supernatant assayed for proteinase activity (4 h at 20°C). No detectable proteinase was released from E_8 under any of these conditions. Proteinase was released from 266 although, after 3 h at 30°C, the activity in solution was equivalent to 4% of the activity of intact cells. At 35°C, proteinase released after 2 h was equivalent to 15% of the proteinase activity of intact cells.

Routine assays with intact cells were subsequently incubated for 2 h at 30°C.

11.4 Effect of Substrate Concentration on Proteolysis

Casein substrate concentration had a marked effect on proteolysis after 0.5 h (Fig. 14). After this time, the rate with 0.1% (w/v) casein slowed markedly while with 0.25% (w/v) and 0.5% (w/v) casein the decrease in rate was delayed (Fig. 14). With 1% (w/v) casein, an almost linear rate was observed up to 4 h. The amount of substrate solubilized after 4 h was 20, 14, 18 and 4% for casein concentrations of 0.1, 0.25, 0.5 and 1.0% (w/v), respectively.

TABLE 10: Effect of Assay Temperature on the Proteinase Activity of Intact Cells of S. cremoris 266 and E_8. Intact Cells (4 mg (dry weight) bacteria/ml) were Incubated with ^{14}C-labelled Casein (0.5%, w/v) in 0.05 M Mes Buffer (pH 6.5) containing $CaCl_2$ (0.02 M) under the Conditions Specified

Temp (^{o}C)	Time (h)	Proteinase Activity (dpm/mg (dry weight) bacteria)	
		S. cremoris 266	S. cremoris E_8
20	4	415	838
25	2	371	687
25	3	472	822
30	1	284	558
30	2	425	758
30	3	560	a
35	1	298	578
35	2	466	780

[a] Not determined

TABLE 11: Effect of Temperature on the Stability of Cell-Bound Proteinase of S. cremoris 266 and E_8. Intact cells (4 mg (dry weight) bacteria/ml) were Incubated with ^{14}C-labelled casein (0.5%, w/v) in 0.05 M Mes Buffer (pH 6.5) containing $CaCl_2$ (0.02 M) for 4 h at 20°C after being held in this Buffer under the Conditions Specified

Temperature Before Assay (°C)	Time Before Assay (h)	Proteinase Activity (dpm/mg (dry weight) bacteria)	
		S. cremoris 266	S. cremoris E_8
control		415	838
20	4	419	876
25	2	429	947
25	3	437	922
30	1	430	876
30	2	426	939
30	3	433	a
35	1	426	925
35	2	382	913

[a] Not determined

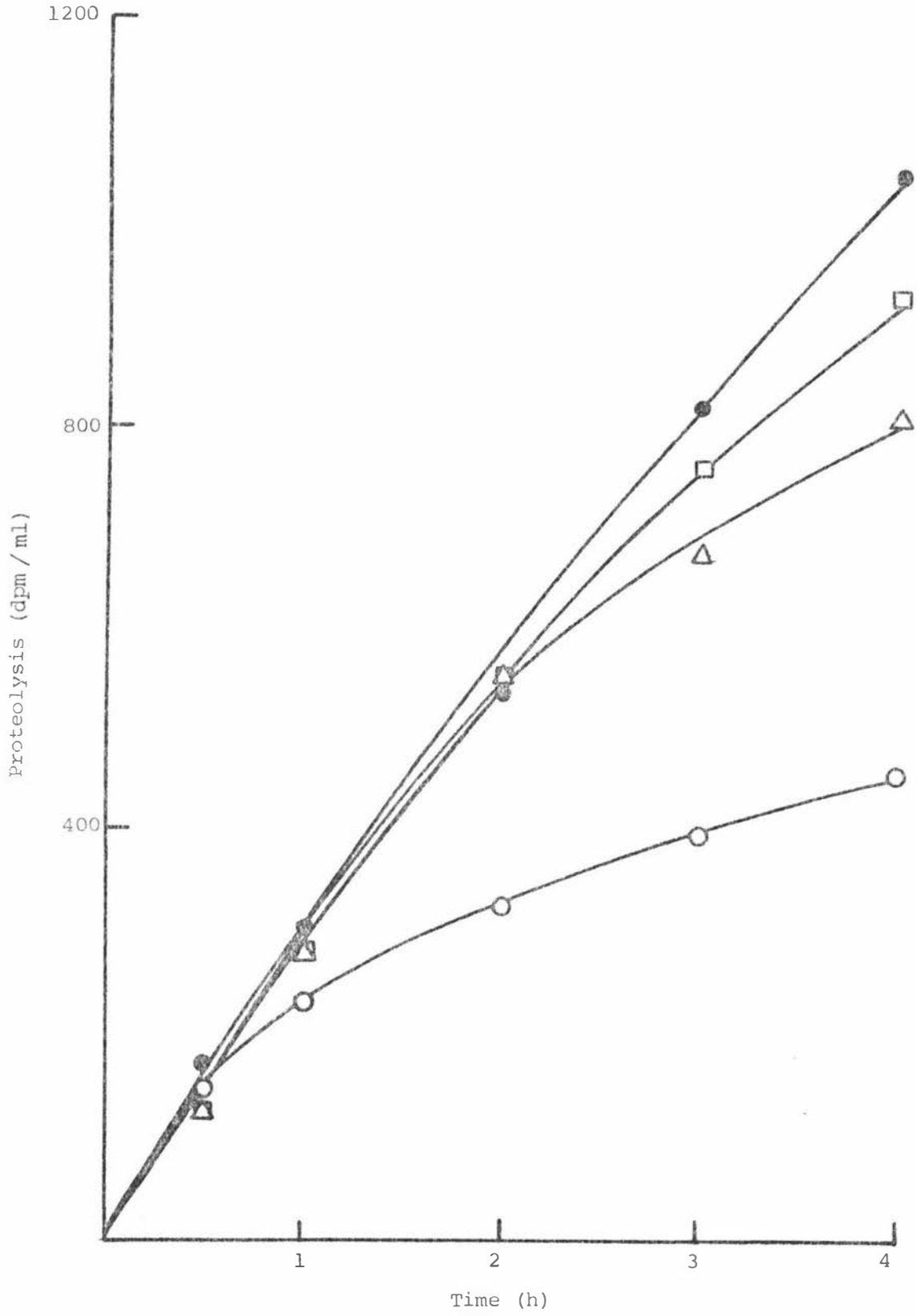

FIGURE 14: Effect of substrate concentration on activity of cell-free proteinase from S. cremoris 266. Proteinase was incubated with ^{14}C-labelled casein in 0.05 M cacodylate buffer (pH 6.5) for 4 h at 20°C. Enzyme concentration was constant. Casein concentrations used were 0.1% (o), 0.25% (Δ), 0.5% (□) and 1.0% (●), w/v.

11.5 Effect of Proteinase Concentration on Proteolysis

There was an almost linear increase in proteolysis with concentration of the cell-free enzyme (Fig. 15). At the highest concentrations of enzyme 4.7, 5.4, 6.2 and 9.2% of the substrate was solubilized during the assay period (4 h) with enzymes from strains E_8, ML_3, AM_2 and 266, respectively.

More extensive proteolysis was obtained during the assay of cell-bound proteinase (Fig. 16) compared with cell-free proteinase (Fig. 15). This difference is largely due to the release of only part of the cell-bound proteinase during preparation of the cell-free enzyme (page 87). With the highest concentrations of cell-bound enzyme 31.5, 24.2, 18.3 and 22.1% of the substrate was solubilized with intact cells of E_8, ML_3, AM_2 and 266, respectively. At low enzyme concentrations (up to about 1.5 mg (dry weight) bacteria / ml for AM_2, 266 and ML_3 and 0.5 mg (dry weight) bacteria / ml for E_8) proteolysis (up to about 1000 dpm / ml) was almost proportional to enzyme concentration. At higher enzyme concentrations the relationship was non-linear.

11.6 Comparison of ^{14}C-Labelled Casein Assay with Folin-Ciocalteu and Fluorescamine Assays

The results of proteinase assay by the three methods for determining TCA-soluble material were normalized for comparative purposes so that the absorbance, fluorescence and radioactivity values obtained from the greatest concentration of proteinase became 100. The curves for all three methods were non-linear and coincident (Fig. 17).

11.7 Assay of Thermolysin, Subtilisin and Chymosin Using ^{14}C-Labelled Casein

There was a linear relationship between the concentration of thermolysin and subtilisin and the amount of material released which was soluble in 6% (w/v) TCA (Fig. 18). This linearity was observed with enzyme concentrations which produced, in the 4 h assay period, up to 45% and 17% substrate

solubilization with thermolysin and subtilisin, respectively. In contrast, increasing chymosin concentrations did not give a linear increase in the amount of casein solubilized during the 4 h assay period.

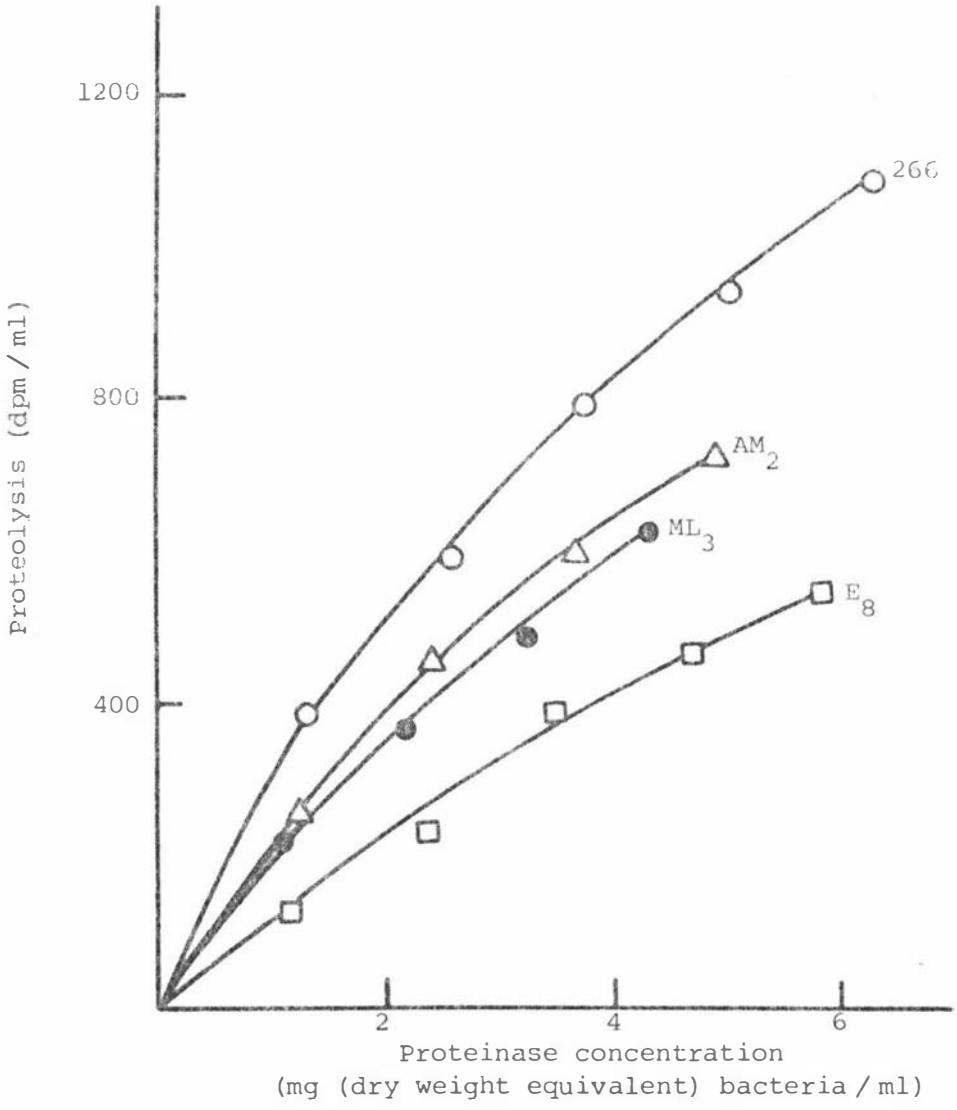

FIGURE 15: Cell-free proteinase concentration versus proteolysis. Standard assay conditions were used (see text).

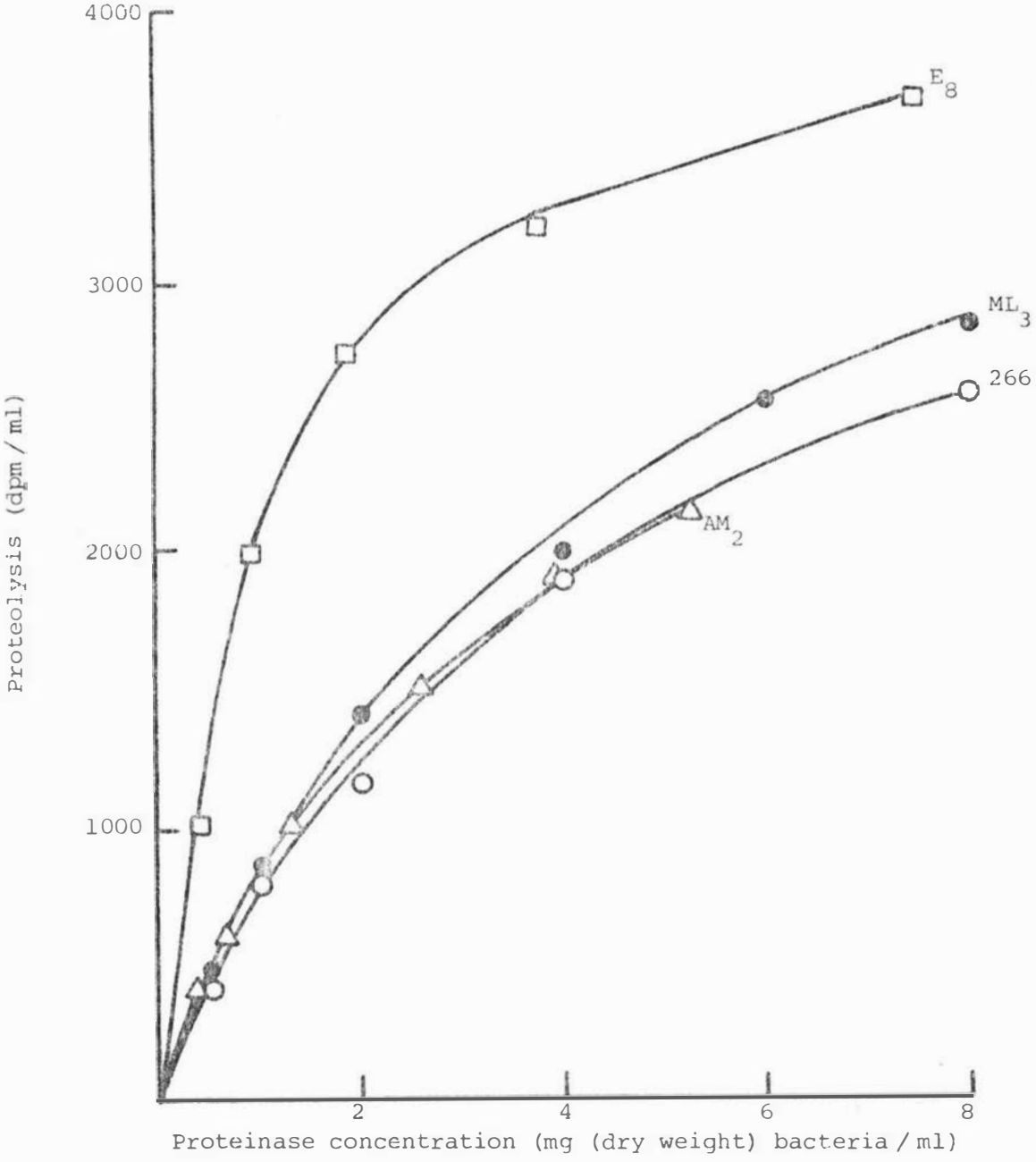

FIGURE 16: Cell-bound proteinase concentration versus proteolysis. Standard assay conditions were used (see text).

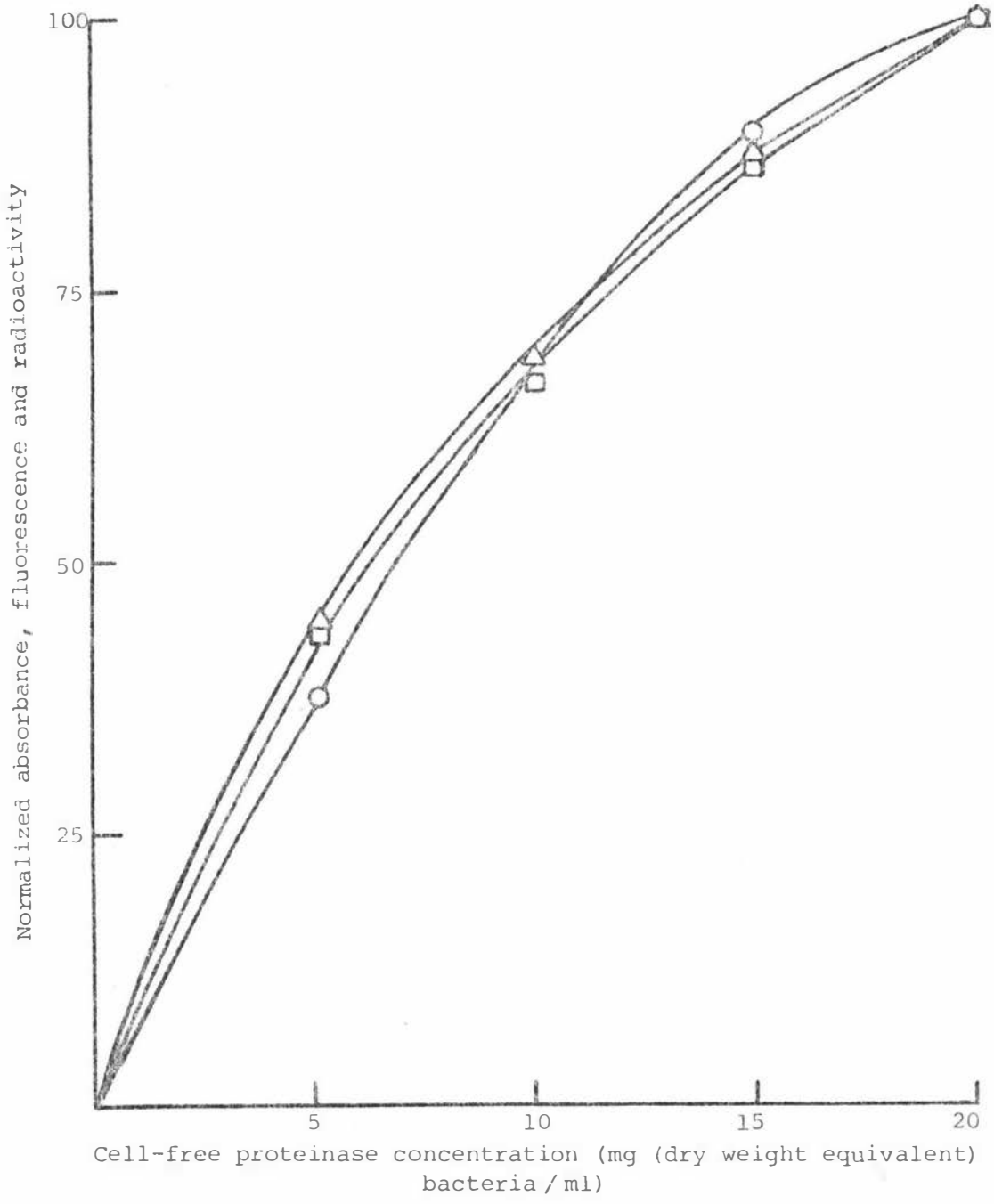

FIGURE 17: Comparison of methods for determining TCA-soluble material in assays of cell-free proteinase from S. cremoris 266. Proteinase was incubated with 0.5% (w/v) casein in 0.05 M cacodylate buffer (pH 6.5) for 4 h at 20°C and the material soluble in 6% TCA was estimated by radioactivity determination (o), Folin - Ciocalteu reaction (Δ) and fluorescamine reaction (□).

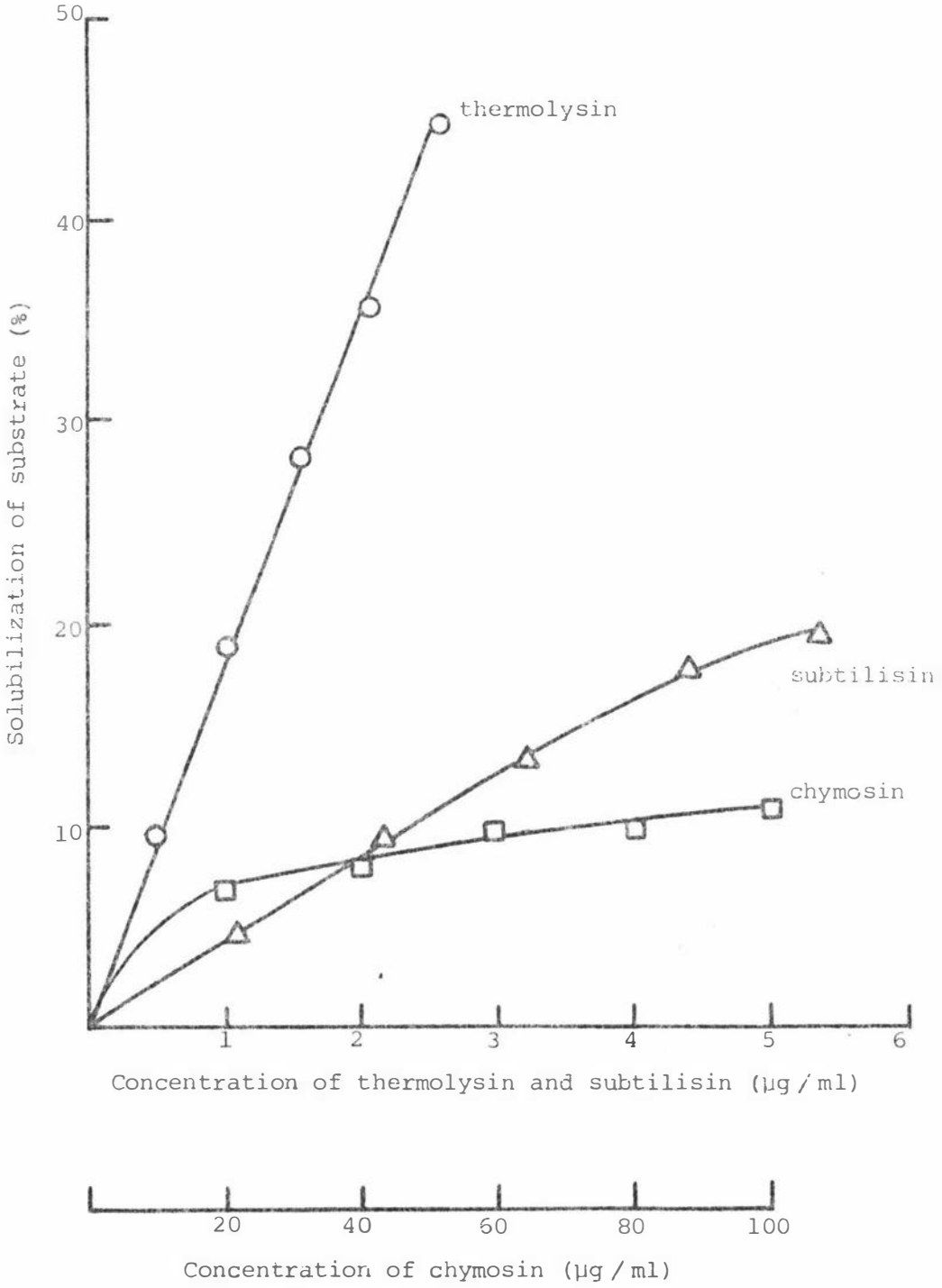

FIGURE 18: Activity of thermolysin, subtilisin and chymosin using ^{14}C-labelled casein as substrate. The enzymes were incubated with 0.5% (w/v) casein in 0.05 M cacodylate buffer (pH 6.5) for 4 h at 20°C.

CHAPTER 12

DISCUSSION

The effect of pH on proteinase activity is difficult to interpret since the protein substrate may be modified as well as the enzyme. Casein is heterogeneous and pH adjustment will cause changes in ionization resulting, not only in conformational changes of the individual caseins, but also in the degree of association of the various caseins. Another complicating factor is that there may be more than one proteinase associated with the cell wall of each strain. Broad pH versus activity curves with most strains are consistent with this possibility. Differences between the pH versus activity profiles of cell-free and cell-bound proteinase of the same strain may result from only partial release of cell wall-associated proteinase. The cell-free proteinase preparation, therefore, may not be representative of proteinase in the intact cell. This could be especially relevant to E_8 where the activity of cell-free proteinase at pH 5.8 (Fig. 12) was equivalent to only 8% of the activity of intact cells at the same pH (Fig. 13) and the pH versus activity profiles were different for the cell-free and cell-bound enzymes.

Non-linear curves were obtained for enzyme concentration (cell-free and cell-bound) versus proteolysis for less than 10% (1175 dpm / ml) solubilization of the substrate (Figs 15 & 16). A non-linear relationship with such low levels of substrate solubilization is characteristic of other proteinases acting on protein substrates (Dixon & Webb, 1964). Factors which may be involved in this relationship include change in substrate during the assay coupled with narrow enzyme specificity, different affinities for different peptide bonds, and the fact that the only proteolysis products detected were those which were soluble in 6% TCA. Hydrolysis of a peptide bond resulting in two 6% TCA-insoluble peptides would be undetected. The likelihood of this event will increase as the proteinase becomes more specific. With intact cells, substrate accessibility to the cell wall-bound

enzyme could also affect results. The curves in Fig. 14 are consistent with proteinase of narrow specificity since deviation from the initial velocity was more pronounced at low substrate concentrations and yet only limited (< 20%) substrate solubilization took place in 4 h. An indication of broad or narrow enzyme specificity can be obtained from the relationship between enzyme concentration and proteolysis. Thermolysin and subtilisin which are both enzymes with broad specificity (Matsubara, 1966; Markland & Smith, 1971), have linear relationships under conditions where extensive substrate solubilization took place (Fig. 18). In contrast, chymosin, which has a narrow specificity (Pelissier et al., 1974), gave a non-linear relationship (Fig. 18). Chymosin action on β-casein resulted in three major oligopeptides (Creamer et al., 1971). The action of cell-free proteinase from strain 266 on β-casein was also limited resulting in four major oligopeptides (Appendix III). As a consequence of the non-linear relationship between proteolysis and enzyme concentration, (cell-free and cell-bound) standard assay conditions were adopted for the subsequent assay of proteinase from all strains. These conditions were those described in Chapter 10.7. Standard enzyme concentrations used were 4 mg (dry weight) bacteria / ml or the cell-free enzyme equivalent. Although a higher substrate concentration may have resulted in a linear relationship between proteolysis and enzyme concentration, for up to the same maximum levels of proteolysis in Fig 15 & 16, the limited supply of substrate had to be considered.

The sensitivity of the three methods used for measuring 6% TCA-soluble material was almost the same. Assay sensitivity of the radioactive casein method is dependent upon the specific activity of the casein substrate which was disappointingly low (see page 48) and insufficient for use in more detailed studies involving fractionation and characterization of cell wall-associated proteinases.

SECTION III

ASPECTS OF CELL WALL-ASSOCIATED PROTEINASES

Part 2

Release of Cell Wall-Associated Proteinase(s) from Lactic Streptococci

CHAPTER 13

INTRODUCTION

Exterkate (1976b) studied the effect of pH between 5.4 and 7.0 and temperature on the proteinase activity with casein substrate, of intact cells of fourteen S. cremoris strains and classified these strains into five groups. Although enzyme fractionation was not undertaken, it was concluded that some strains had as many as three different proteinases, two of which were acid proteinases and the other a neutral proteinase. Other strains appeared to have one or two proteinases, while strain ML_1 had not detectable proteolytic activity with casein as the substrate. Møller-Madsen & Hansen (1976) determined the pH versus activity profiles of nineteen strains of lactic streptococci using intact cells and casein substrate and concluded that acid, neutral and alkaline proteinases were present. Some strains lacked acid or neutral proteinases but all strains possessed an alkaline proteinase.

The proteolytic activity of intact cells of lactic streptococci is due to proteinase(s) located in the cell wall (Thomas et al, 1974; Exterkate, 1975). Evidence presented so far (Exterkate, 1976b; Møller-Madsen & Hansen, 1976) concerning the number and types of cell wall-associated proteinases is indirect and isolation of the individual enzymes followed by their characterization is required. In the present study, experiments were undertaken to isolate all the cell wall-bound proteinases from milk-grown bacteria by enzymatic solubilization of the cell walls either with phage-associated lysin or with egg white lysozyme, in hypertonic media. In this way it was hoped that contamination by peptidase / proteinase enzymes located either in the cytoplasm or the membrane could be avoided. Proteinase was released in the presence of the lytic enzymes but, surprisingly, cells which were simply suspended in buffer released proteinase spontaneously at a similar rate. It seemed likely that cell wall solubilization by the lytic enzymes was not extensive,

despite the formation of spheroplasts. In the present study, physiochemical factors affecting the release of proteinase from intact cells grown in milk were investigated. Milk was chosen as the growth medium since proteinase synthesis was repressed in other media containing high concentrations of free amino acids and low molecular weight peptides (Exterkate, 1976b, 1979; Appendix I). A survey was also carried out to determine whether the release of the cell wall-associated proteinase was a general phenomenon among lactic streptococci.

CHAPTER 14

EXPERIMENTAL

14.1 Bacteria and Stock Cultures

The preparation of stock cultures and inoculum cultures for experimental RSM was described previously (page 50).

14.2 Experimental RSM, Growth and Harvesting of Cells

Experimental RSM was prepared by the addition of disodium β-glycerophosphate to reconstituted low-heat skim milk (page 21) and inoculated with 3% of a 16 h / 22°C RSM culture. The T_{480nm} value was used to monitor growth and to determine the cell density of bacterial suspensions (page 22). Experimental RSM cultures were incubated at 32°C until the T_{480nm} value reached 3.6 (pH 5.8 - 6.0). The culture was centrifuged (10,000 x g, 1 min) and the cells washed free of milk protein by twice resuspending in distilled water at 0 to 4°C. There was no significant release of proteinase activity during washing.

14.3 Proteinase Assay

Cell-free proteinase was assayed at 20°C for 4 h with 0.5% (w/v) ^{14}C-labelled casein in 0.05 M cacodylate buffer (pH 6.5). Intact cells were assayed in the same way except that 0.05 M Mes buffer was used instead of cacodylate which induced cell lysis.

14.4 Release of Proteinase from Intact Cells Incubated with Lytic Enzymes in a Hypertonic Medium

Milk-grown cells of strains 266, E_8, P_2 and ML_3 were washed and resuspended in 0.1 M phosphate buffer (pH 7.0) containing 1.2 M sucrose and 0.02 M $MgCl_2$. Solutions of phage-associated lysin (a muramidase derived from S. lactis ML_3 phage lysates, gift from T D Thomas), egg white lysozyme (Sigma Chemical Co.) or

buffer were added to each cell suspension. These suspensions were held at 32°C and sampled at intervals. After centrifugation (35,000 x g, 15 min), supernatant samples were assayed for proteinase activity.

14.5 Spontaneous Release of Proteinase from Intact Cells

General method:

Washed cells were resuspended at approximately 20 mg (dry weight) bacteria/ml in the appropriate buffer at 0°C and then equilibrated at the required temperature. The pH of buffers was adjusted at the working temperature. Samples (1 ml) of the cell suspension were taken at intervals and centrifuged (27,000 x g, 5 min). Supernatants samples were then assayed for proteinase activity.

Effect of pH and buffer composition:

The rate of release of proteinase at 25°C was determined for cells of 266 resuspended in 0.05 M acetate buffer (pH 5.5), 0.05 M triethanolamine buffer (pH 6.7 and 7.5) and 0.05 M Tris buffer (pH 7.8, 8.2 and 8.7). The effect of buffer concentration at pH 7.8 and 25°C was determined by resuspending cells in 0.05, 0.1, 0.2 and 0.5 M Tris.

Effect of temperature:

The rate of release of proteinase from cells of 266 in 0.05 M Tris buffer (pH 7.8) was determined at 0, 25, 28, 31 and 34°C.

Effect of Ca^{2+} and Mg^{2+}:

Cells of 266 were suspended in 0.05 M Tris buffer (pH 7.8) containing $CaCl_2$ or $MgCl_2$ at concentrations up to 0.02 M. Proteinase activity released after 2h at 25°C was then determined.

Effect of NaCl:

The rate of release of proteinase at 25°C, from cells of 266 suspended in 0.05 M Tris buffer (pH 7.8) containing 0, 0.1, 0.2 and 0.5 M NaCl, was determined.

Effect of sucrose:

The rate of release of proteinase at 25°C, from cells of 266 suspended in 0.05 M Tris buffer (pH 7.8) containing 0, 0.17, 0.31 and 0.68 M sucrose, was determined.

14.6 Stability of Proteinase

The stability of proteinase under the conditions which gave optimum release was investigated. Washed cells of 266 were resuspended in 0.05 M Tris (pH 7.8) and maintained at 25°C for 120 min. The suspension was centrifuged, and an aliquot of the supernatant immediately assayed. The remainder was then held for a further 120 min at 25°C before assay.

14.7 Estimation of Lysis

The release of lactate dehydrogenase (LDH) from cells incubated in buffer was used to estimate cell lysis. This intracellular enzyme was assayed in supernatants by the method of Thomas (1975) while the total LDH activity was determined after complete lysis, as judged by phase contrast microscopy, of cells treated with phage-associated lysin in hypotonic media (0.1 M phosphate, pH 7.0).

14.8 Cell Wall Preparation

Milk-grown 266 cells were washed and resuspended at 19 mg (dry weight) bacterial/ml in 0.01 M Mes buffer (pH 6.5) containing 0.02 M $CaCl_2$. Cells were disrupted by shaking with glass beads (Thomas *et al.*, 1974) and after centrifugation (35,000 x *g*, 10 min.) the pelleted cell wall material was washed first in Mes buffer and then in distilled water. The cells were maintained at 0 to 4°C throughout these procedures. The walls were finally resuspended in 0.05 M Tris buffer (pH 7.8) and held at 25°C. Release of proteinase was determined as described for intact cells.

CHAPTER 15

RESULTS

15.1 Release of Proteinase from Intact Cells in Hypertonic Media

Proteinase was released from intact cells of 266, AM_2, E_8 and ML_3 in the presence of lysozyme and phage-associated lysin although spontaneous release from control cells also occurred (Figs 19 & 20). With strains 266, ML_3 (Fig. 19) and P_2 (Fig. 20a) proteinase release was similar in each system and decreased with time. With strain E_8, phage-associated lysin markedly increased proteinase release so that after 120 min about twice as much cell-free proteinase was present (Fig. 20b). With all strains, the presence of lytic enzymes resulted in spheroplast formation, as judged by phase contrast microscopy, in about 30 min.

15.2 Spontaneous Release of Proteinase from Intact Cells of S. cremoris 266

Effect of pH and buffer composition:

The initial rate of release of proteinase increased progressively as the buffer pH increased from 5.5 to 8.2 (Fig. 21). The activity released after 120 min was markedly dependent on pH with a maximum occurring at pH 7.8. At pH 8.7 a time-dependent inactivation of proteinase was evident. The trends observed in Fig. 21 are independent of buffer ion composition since similar results were obtained when 0.05 M phosphate buffers were used over the pH range 5.9 to 8.0. However, phosphate was unsuitable for studies on the effect of Ca^{2+} on proteinase release and Tris buffer was selected for general use.

The rate of release of proteinase at pH 7.8 decreased as Tris concentration increased from 0.05 M to 0.1 M, this rate then remained constant to 0.5 M Tris. A Tris buffer concentration of 0.05 M was, therefore, used in subsequent

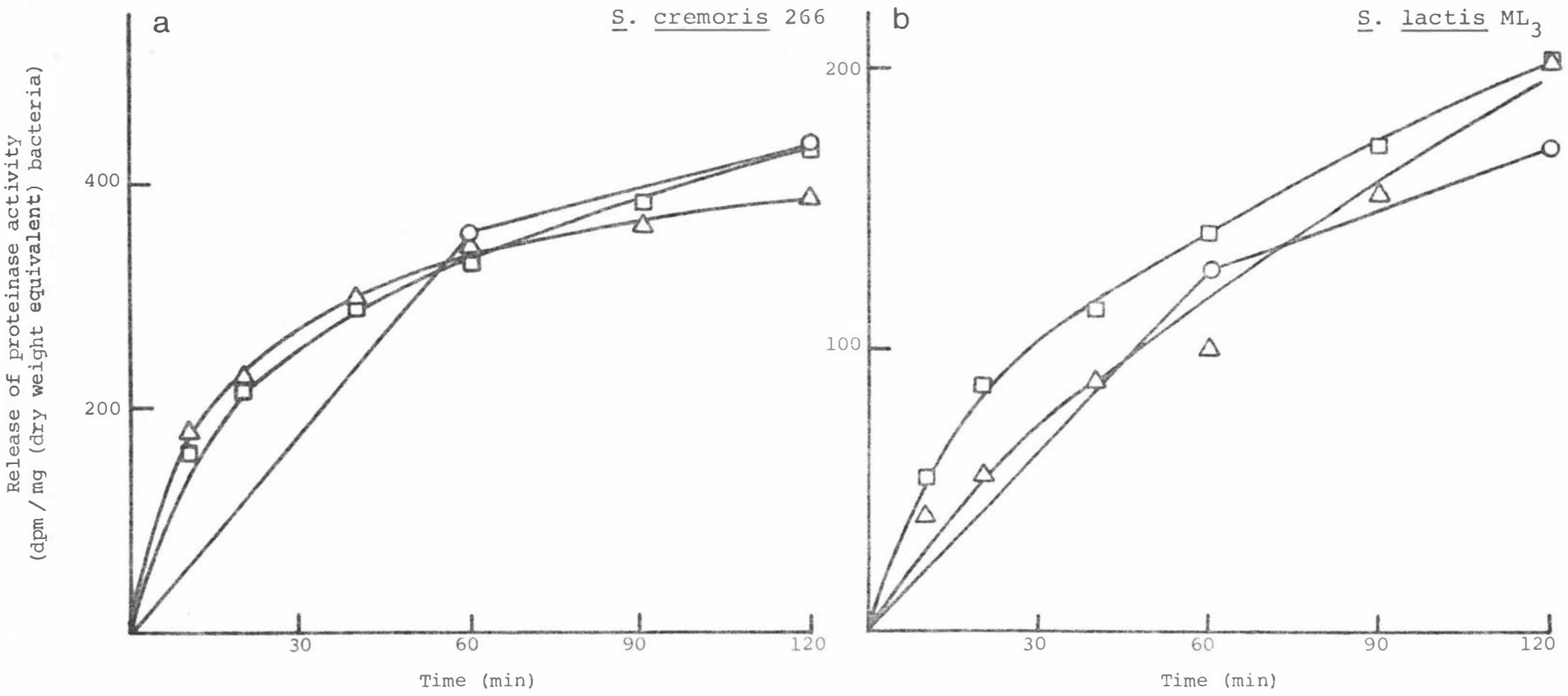

FIGURE 19: Release of proteinase from intact cells of a) S. cremoris 266 and b) S. lactis ML_3 in the presence of added lytic enzymes; control (o), lysozyme (□), phage-associated lysin (Δ). Cells were suspended at 32°C in 0.1 M phosphate buffer (pH 7.0) containing 1.2 M sucrose and 0.02 M $MgCl_2$.

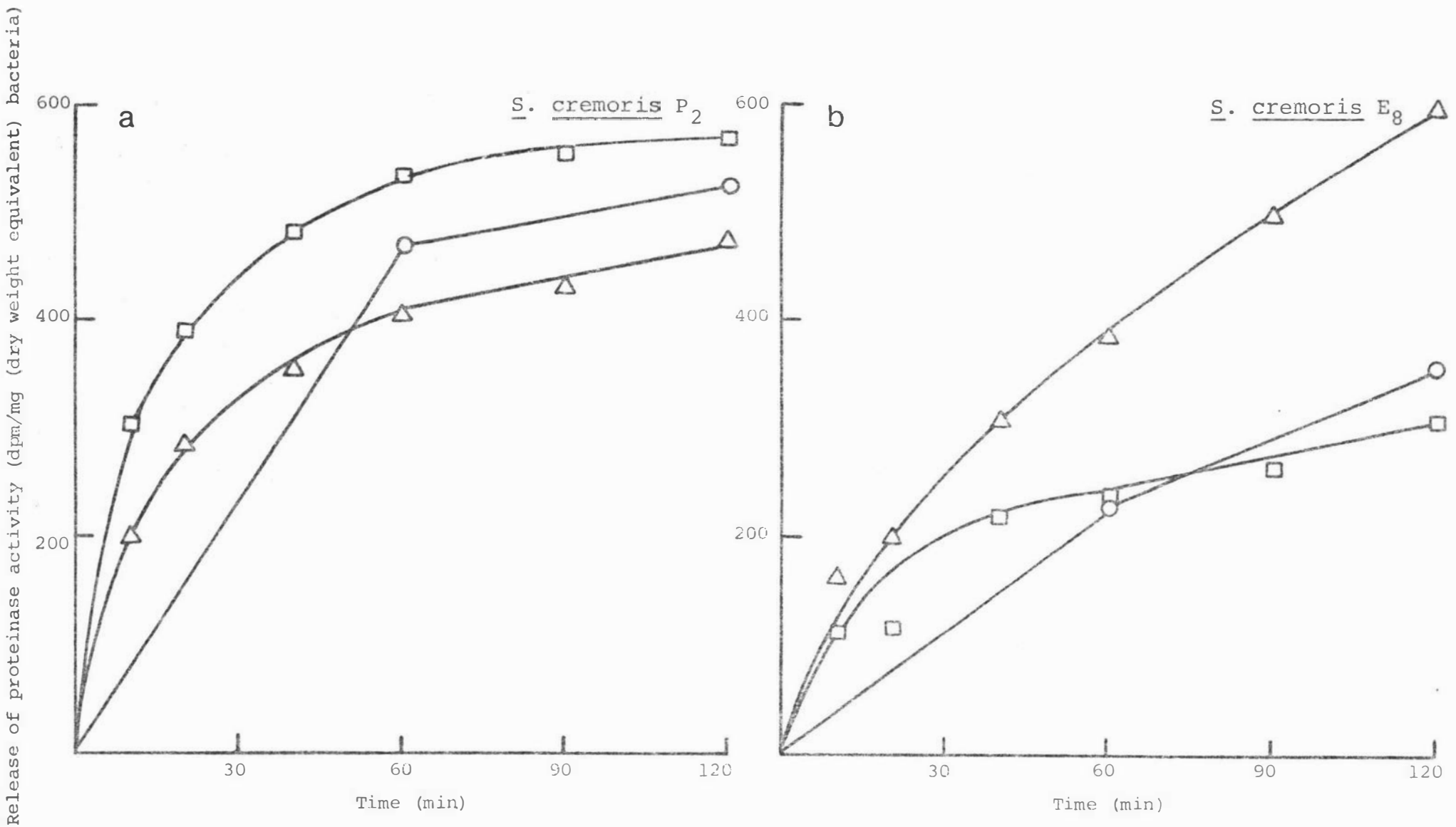

FIGURE 20: Release of proteinase from intact cells of a) S. cremoris P_2 and b) S. cremoris E_8. For conditions of release and symbols refer to Fig. 19.

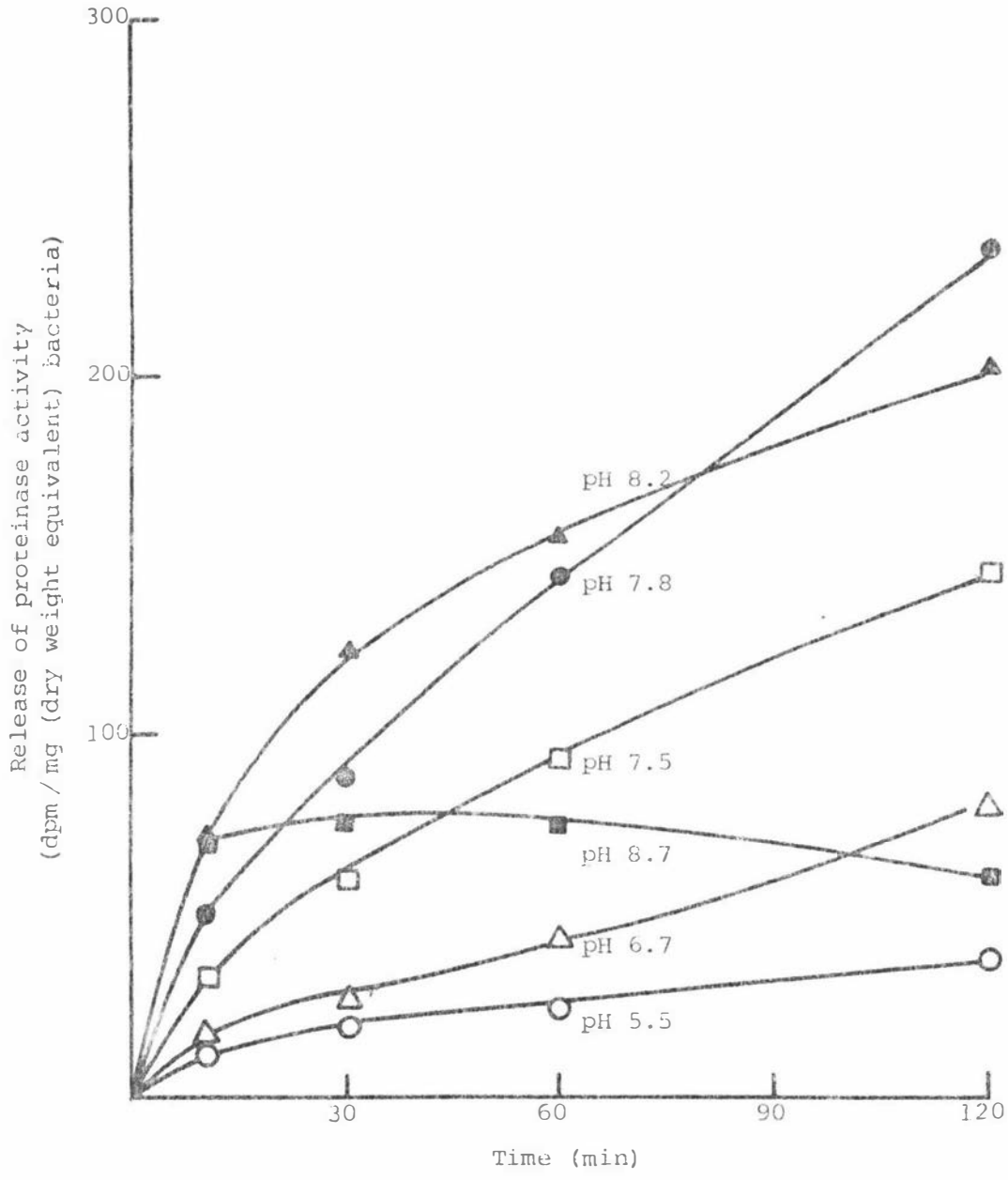

FIGURE 21: Effect of pH on the release of proteinase from S. cremoris 266. Cells were suspended at 20.0 mg (dry weight) / ml in 0.05 M acetate buffer (pH 5.5), 0.05 M triethanolamine buffers (pH 6.7 and pH 7.5) and 0.05 M Tris buffers (pH 7.8, 8.2 and 8.7) and incubated at $25^{o}C$ for the times indicated. Samples were taken at intervals, centrifuged and proteinase activity in the supernatants was determined as described under Experimental.

experiments.

Effect of temperature:

The initial rate of proteinase release from intact cells of 266 in 0.05 M Tris buffer (pH 7.8) increased with increasing temperature (Fig. 22). At $0^{o}C$ release occurred very slowly. At $34^{o}C$ an apparent maximum was obtained after about 45 min followed by a decrease in activity, presumably due to thermal inactivation.

Effect of Ca^{2+} and Mg^{2+}:

Calcium ions (0.02 M) decreased the release of proteinase activity by 90% and Mg^{2+} (0.02 M) by 40% (Fig. 23). A reduction of 50% was achieved in the presence of only 0.002 M $CaCl_2$. In these experiments, Ca^{2+} and Mg^{2+} were present in the assay system due to carry over in the proteinase solution. When the final concentration of $CaCl_2$ or $MgCl_2$ in the assay buffer was 0.004 M (0.1 ml proteinase solution containing 0.02 M $CaCl_2$ ($MgCl_2$) + 0.04 ml casein solution), Ca^{2+} enhanced activity of the proteinase by 10% and Mg^{2+} by 3%.

Effect of NaCl:

While the rate of release of proteinase decreased in the presence of NaCl (Fig. 24), no trend could be observed as the concentration was increased from 0.1 to 0.5 M NaCl. Sodium chloride which was carried through from the release buffer into the assay mixture had little effect on the proteinase activity. When the release buffer contained 0.5 M NaCl, the activity of proteinase in the assay was reduced by 5%.

Effect of sucrose:

The rate of release over the first 40 min decreased with increasing sucrose concentration. However, after 120 min the amount of proteinase released in the presence of 0.17 and 0.31 M sucrose was greater than that for the control (Fig. 25). The presence of sucrose, carried through from the release buffer into the assay system, made little difference to the activity of the proteinase. When the release buffer contained 0.68 M sucrose, the activity of

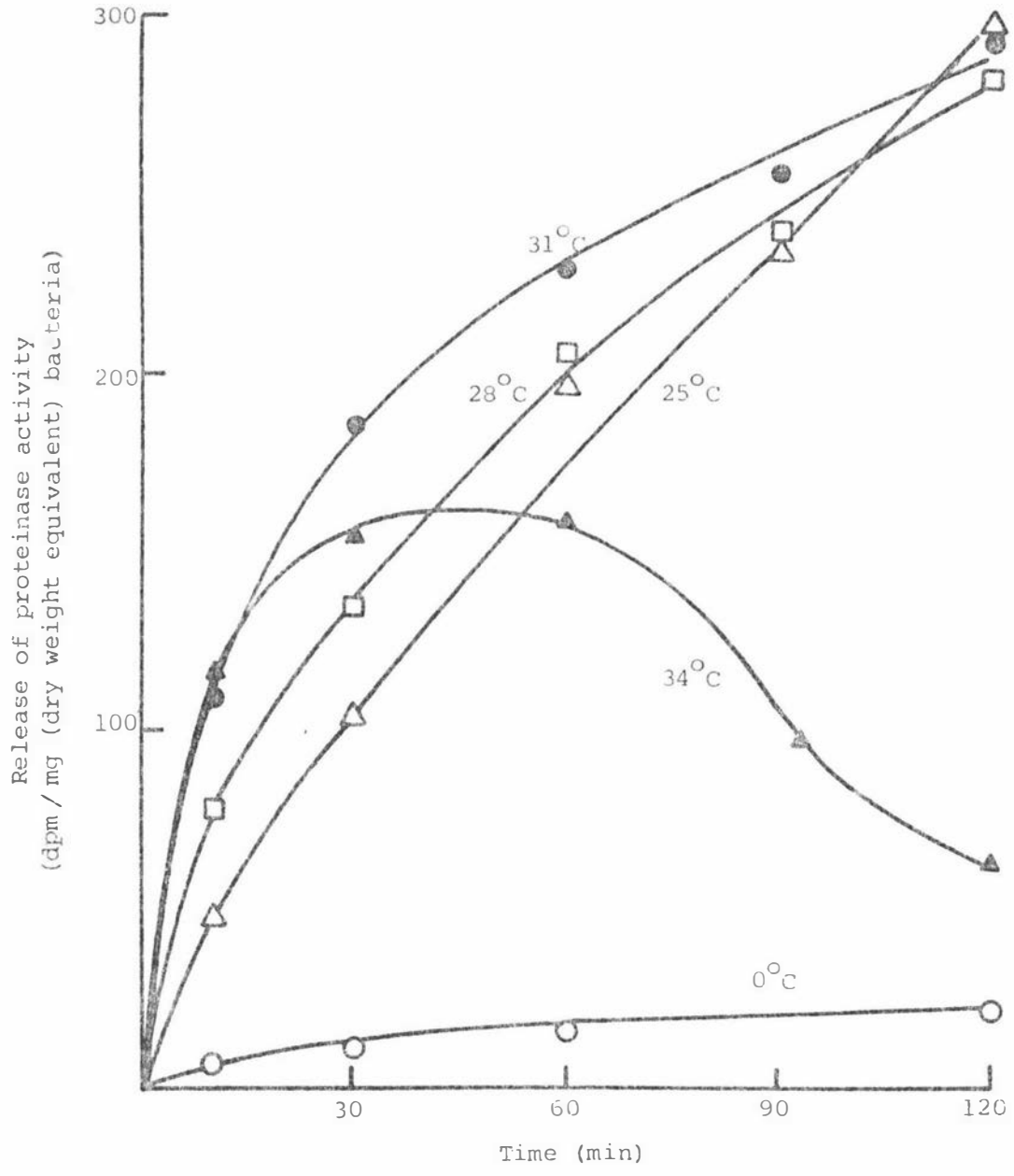

FIGURE 22: Effect of temperature on the release of proteinase from S. cremoris 266. Cells were suspended at 23.9 mg (dry weight) / ml in 0.05 M Tris buffer (pH 7.8) at 0°C, 25°C, 28°C, 31°C and 34°C. Samples were taken at intervals, centrifuged and proteinase activity in the supernatants was determined as described under Experimental.

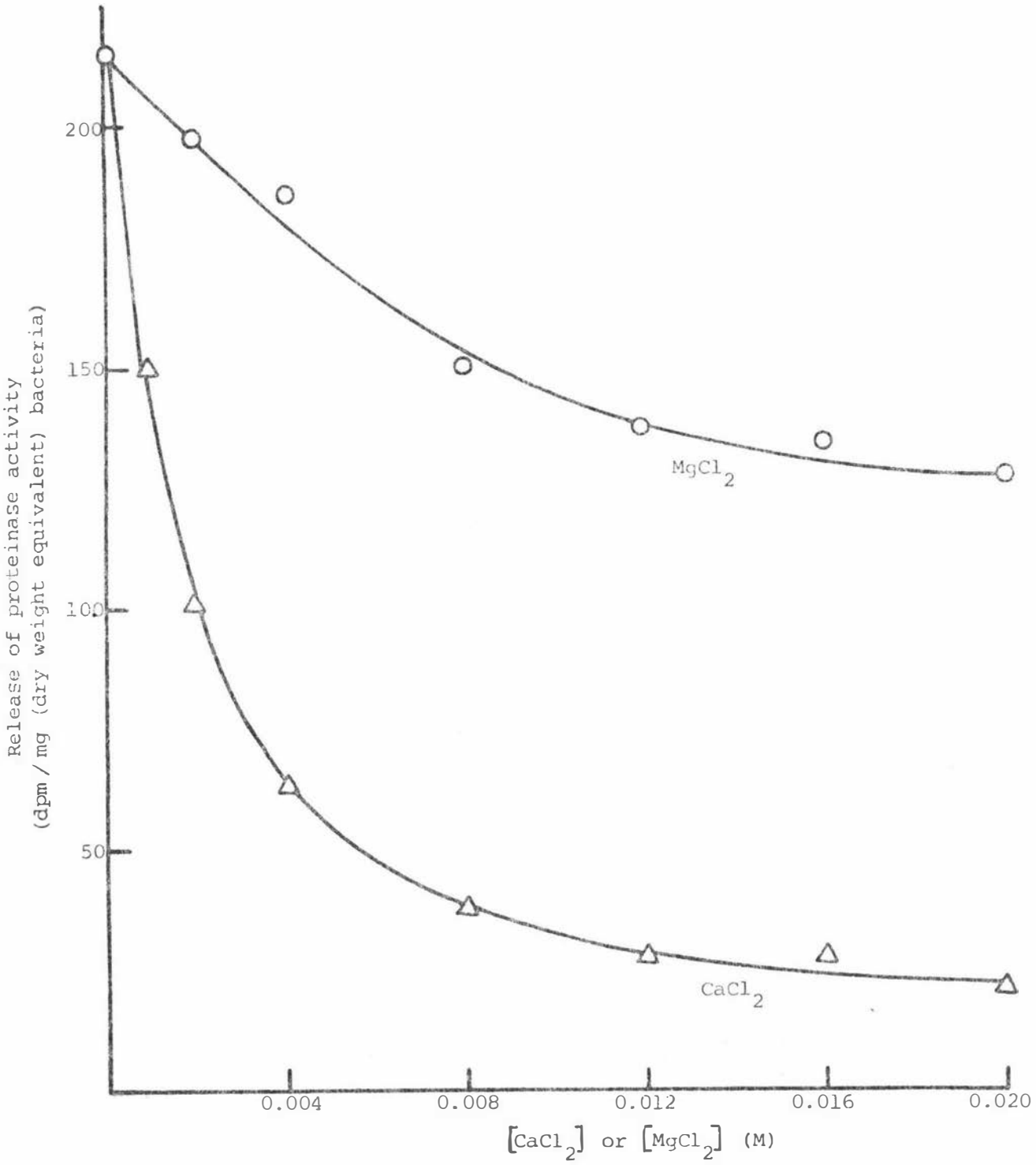

FIGURE 23: Effect of Ca^{2+} and Mg^{2+} on the release of proteinase from S. cremoris 266. Cells were suspended at 22.0 mg (dry weight) / ml in 0.05 M Tris buffer (pH 7.8). Calcium chloride or $MgCl_2$ was added to the buffer prior to resuspension of the pellet after washing, to give the desired final concentration. The suspensions were maintained at 25°C for 120 min, centrifuged and the supernatants assayed for proteinase activity as described under Experimental.

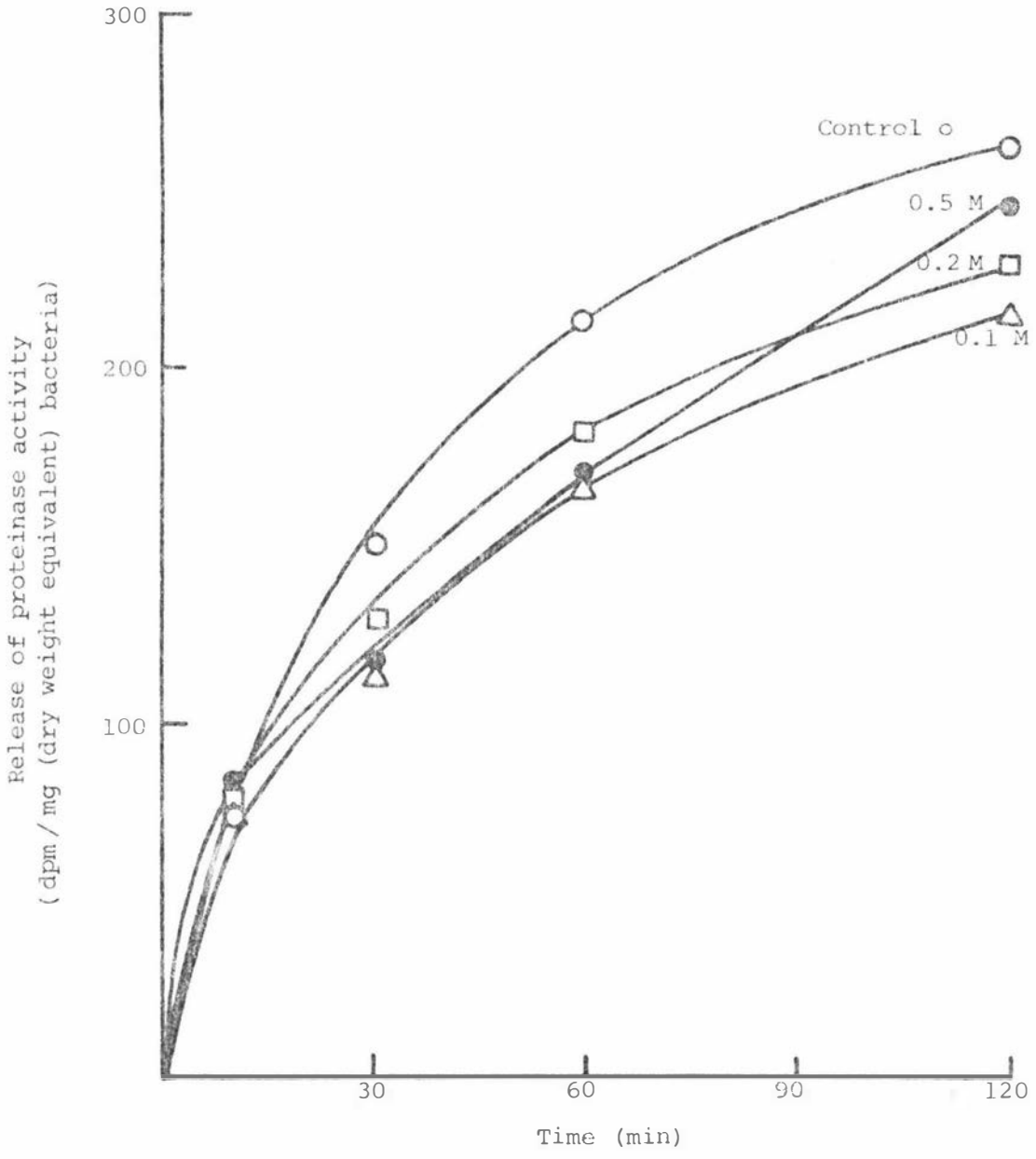

FIGURE 24: Effect of NaCl on the release of proteinase from S. cremoris 266. Cells were suspended at 22.5 mg (dry weight) / ml in 0.05 M Tris buffer (pH 7.8) containing 0.1, 0.2 or 0.5 M NaCl. The suspensions were incubated at 25°C. Samples were taken at the times indicated, centrifuged and the proteinase activity in the supernatants was determined as described under Experimental.

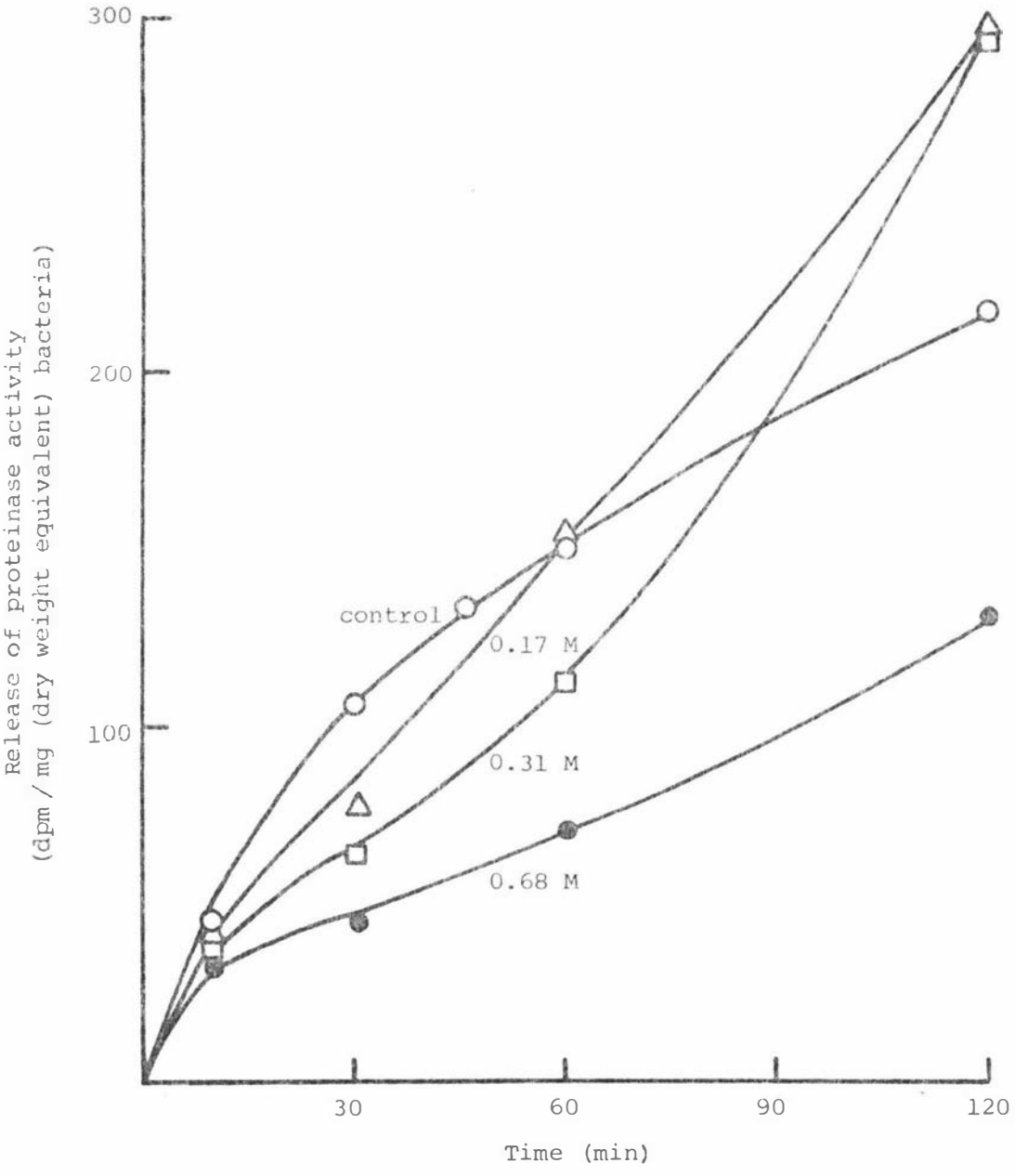

FIGURE 25: Effect of sucrose on the release of proteinase from S. cremoris 266. Cells were suspended at 24.2 mg (dry weight) / ml in 0.05 M Tris buffer (pH 7.8) containing sucrose at the concentrations shown. The suspensions were maintained at 25°C. Samples were taken at the times indicated, centrifuged and the proteinase activity of the supernatants was determined as described under Experimental.

the proteinase in the assay was reduced by 6%.

Stability of proteinase:

No decrease in activity was detected when proteinase was held in 0.05 M Tris buffer (pH 7.8) for 120 min at 25°C.

Estimation of cell lysis:

None of the conditions used during the investigation of spontaneous release of proteinase from 266 resulted in more than 1% cell lysis and proteinase solutions were, therefore, considered to be free of appreciable amounts of intracellular proteinases and peptidases.

15.3 Survey of a Range of Lactic Streptococci for Spontaneous Release of Proteinase from Intact Cells

Only strains which could be sedimented from milk cultures by centrifugation (Thomas & Turner, 1977) were examined. Of 26 strains tested, 14 gave greater than 80% sedimentation of the cells. The proteinase released after 120 min at 0°C and 25°C in the absence of Ca^{2+} and also at 25°C in the presence of 0.02 M $CaCl_2$ was measured with 10 of these strains (Table 12). All strains released proteinase at 25°C in the absence of $CaCl_2$, although the activity released by ML_1 was very small. A considerable reduction in the amount of proteinase released was obtained for all strains, except ML_1, by adding 0.02 M $CaCl_2$ to the buffer or by lowering the temperature to 0°C.

15.4 Proportion of Total Cell Wall Proteinase Released

Intact cells of strains 266, AM_2 and ML_3 were washed in water at 0°C and resuspended in 0.05 M Tris buffer (pH 7.8) at 25°C. After holding for 0 and 4 h the suspension was centrifuged and both the supernatant and intact cells were assayed for proteinase activity using 0.05 M Mes buffer (pH 6.5). The time allowed for release of proteinase was extended to 4 h to accentuate any decrease in the activity of intact cells. To stop any proteinase release during assay, $CaCl_2$ (0.02 M) was also added to the Mes buffer. The proportion of the total cell wall proteinase released was

TABLE 12: The Effect of $CaCl_2$ and Temperature on the Release of Proteinase from Lactic Streptococci. Cells were Suspended in 0.05 M Tris Buffer (pH 7.8) at 25°C in the presence and absence of $CaCl_2$ and at 0°C. After 120 min the Suspensions were Centrifuged and the Supernatants Assayed.

Strain	Proteinase Activity in Supernatant (dpm/mg (dry weight equivalent) bacteria)		
	25°C	25°C 0.02 M $CaCl_2$	0°C
S. cremoris			
114	91	11	18
266	236	40	29
AM_2	108	25	42
BR_4	159	61	23
E_8	57	8	25
KH	332	31	43
ML_1	16	24	10
P_2	177	31	33
S. lactis			
ML_3	141	17	30
ML_8	58	15	10

strain dependent (Table 13). After 4 h intact cells of AM_2 had lost 81% of their original activity while strain 266 had lost only 33%. The proteinase released was also assayed in the presence of 0.02 M $CaCl_2$ and the decrease in activity of intact cells did not correspond with the activity present in the suspension buffer after 4 h. When the three supernatants were assayed in the absence of $CaCl_2$ they all exhibited lower activities. This Ca^{2+} activation was most pronounced with AM_2 proteinase, where 0.02 M Ca^{2+} produced a five-fold increase in activity. The effect of Ca^{2+} on the activity of proteinase from 266 and ML_3 was less marked with increases of 19% and 72%, respectively.

15.5 Location of Proteinase in the Cell

Washed cell walls were prepared using low temperatures and a buffer containing Ca^{2+} (see Experimental), conditions which prevented the release of proteinase from intact cells. Cell walls and intact cells of 266 had proteinase activities of 592 and 603 dpm/mg (dry weight) bacteria (or equivalent), respectively, indicating that the activity displayed by intact cells is cell wall-associated. Proteinase was released from cell walls held at 25°C in buffer (pH 7.8) lacking Ca^{2+}, conditions which caused release of proteinase from intact cells.

TABLE 13: Proportion of Total Cell Wall Proteinase Released after 4 h in 0.05 M Tris Buffer (pH 7.8) at 25°C. Intact Cells were Assayed in 0.05 M Mes Buffer (pH 6.5) in the Presence of $CaCl_2$ (0.02 M).

Strain	Proteinase Activity (dpm/mg (dry weight) bacteria)	
	0 h	4 h
S. cremoris AM_2	489	93 (81)[a]
S. cremoris 266	528	353 (33)
S. lactis ML_3	479	182 (62)

[a] Percentage decrease in activity of intact cells in brackets.

CHAPTER 16

DISCUSSION

The rate of release of proteinase from intact cells of 266, ML_3 and P_2 in the presence of lytic enzymes was no faster than the rate of spontaneous release, indicating that wall solubilization was slow. Phage-associated lysin accelerated the release of proteinase from E_8, suggesting that the cell wall in this strain was more readily solubilized. With all strains, spheroplasts were formed indicating some cell wall digestion by the lytic enzymes although wall solubilization may not have been extensive. The comparable rates of spontaneous release and that found with lytic enzymes illustrates the need for a control when attempting to release cell wall-associated enzymes by solubilization of the wall. It should also be recognized that the cell-free proteinase preparation examined in the present study may not contain all the cell wall-associated enzyme(s). Phage-associated lysin accelerated the release of proteinase from the walls of broth-grown S. lactis C10 cells (Thomas et al., 1974). Exterkate (1975), during proteinase localization studies, suggested that all the enzyme from cell walls of S. cremoris HP was released with lysozyme. In the absence of a control, the possibility remains that lysozyme was ineffective and proteinase was released spontaneously.

A range of lactic streptococci were examined for the spontaneous release of proteinase from intact cells held in buffer at pH 7.8. Although the conditions used for proteinase release and assay may not have been optimum for each strain, significant amounts of proteinase were released from seven strains of S. cremoris and two strains of S. lactis. Release of proteinase was retarded considerably by adding Ca^{2+}, by lowering the temperature to $0^{o}C$, or by lowering the pH to 5.5. Release was retarded to a lesser extent by adding NaCl or sucrose. A simple explanation for these findings is that the composition of the suspending medium affects the permeability of the cell wall to proteins, while the temperature

may influence their rate of diffusion through the cell wall. Conditions which produce swelling of the cell wall may allow loosely entrapped proteinase to escape. Ou & Marquis (1970) suggested that the swelling and contraction observed with cell walls of some Gram positive cocci could be attributed to changes in electrostatic interaction between charged polymers of the cell walls during pH changes or the addition of NaCl. Shrinkage of cell walls was also achieved by the addition of sucrose. In the present study the effect of pH, NaCl and sucrose on the release of proteinase tends to support the hypothesis that these factors influence the permeability of the wall to proteins. The effect of Ca^{2+} in preventing release is not simply the result of screening of electrostatic charge, as might occur with NaCl, since the effect is relatively specific compared with that of Mg^{2+}. Calcium ions may be directly involved in binding proteinase to the cell wall, although the results do not exclude the possibility that they may alter the structure of the cell wall or the proteinase molecule, thereby affecting the rate of diffusion of the proteinase. The present study demonstrates the need, when investigating the proteinase activity of lactic streptococci, to be aware that proteinase may be released during growth and cell washing if attention is not given to the cell environment, in particular to the pH, temperature and Ca^{2+} concentration.

Release of proteinase from cells during growth in milk is probably suppressed by the Ca^{2+} present. Milk contains 0.03 M calcium (Jenness & Patton, 1959) and although most of this occurs as colloidal calcium phosphate or is bound to casein micelles, at least 0.0027 M is present as Ca^{2+} when the cells are harvested at pH 5.8 - 6.0 (K N Pearce, private communication). Of this available Ca^{2+}, some 20 - 30% will be chelated by the β-glycerophosphate added to buffer the experimental milk medium used in this study. Moreover the decrease in pH during growth, and the consequent increase in Ca^{2+} ions released from the micelles, make it unlikely that significant amounts of proteinase are released during the growth of starter bacteria in milk. The suppression of release by Ca^{2+} might be advantageous to the cell in that the products of proteolysis are formed close to the cell and may be more

readily used than would otherwise be the case if the proteinases were released into the milk.

SECTION IV

EFFECT OF THE LEVEL OF STARTER PROTEINASE ON BITTERNESS DEVELOPMENT IN CHEDDAR CHEESE

CHAPTER 17

INTRODUCTION

Bitter compounds in cheese were first identified as peptides in Gouda cheese (Raadsveld, 1953) and more recently in Cheddar cheese (Harwalker & Elliott, 1971; Richardson & Creamer, 1973; Hamilton et al., 1974). Several proteolytic enzymes are present in cheese. Natural milk proteinases, rennet and the proteinase / peptidase systems of starter (and possibly non-starter) bacteria all break down casein during cheese ripening but their respective contributions to the release, or removal, of bitter peptides is unclear. This complex mixture of enzymes and their interactions make experimental design difficult and most information has been of an indirect nature coming from cheesemaking trials with different (i) starter strains, (ii) concentrations of rennets, (iii) manufacturing conditions, especially different cooking temperatures (which alter the final concentration of starter bacteria in the cheese), and salt-in-moisture levels (see Crawford & Zwaginga, 1977). It was suggested (Czulak, 1959) that all bitter peptides were produced solely by residual rennet in the cheese and the removal of bitter peptides, in the case of transient bitterness, was due to breakdown by starter and non-starter enzymes. The hydrolysis of bitter peptides to non-bitter products was proposed by Emmons et al. (1962) to be the property which characterized 'non-bitter' starter strains. 'Bitter' starter strains lacked this property. Lowrie & Lawrence (1972) showed that the main pathway to the production of bitter peptides in Cheddar cheese is via the rennet hydrolysis of casein to high molecular weight (mostly non-bitter) peptides which are subsequently hydrolyzed to low molecular weight bitter peptides by starter. All strains were shown to be potentially 'bitter' since bitterness resulted when the cell density in the curd at salting was increased by cooking at a lower temperature. Non-bitter cheeses are produced when there is insufficient starter proteinase activity to degrade high

molecular weight non-bitter peptides to bitter peptides. These workers also attributed transient bitterness to the degradation of bitter peptides by 'non-bitter' starters. This mechanism was supported by Jago (1974) who suggested a number of measures to be taken to avoid bitterness in cheese.

It is clear that starter bacteria have a direct role in bitterness development in Cheddar cheese since use of higher cooking temperatures leading to relatively low cell densities reduced bitterness development (Lowrie et al, 1972). A lower cell density in the cheese will result in reduced levels of both cell wall-associated starter proteinase (Thomas et al, 1974; Exterkate, 1975) and intracellular proteinases and peptidases (Exterkate, 1975; Mou et al, 1975). It is presumed that starter bacteria lyse during cheese ripening and these intracellular enzymes are released into the curd matrix.

To establish the importance of the various bacterial enzymes in producing bitterness it would be useful to alter the concentration of one of the enzymes in the curd while the others remain constant. The present report describes use of Prt^- cells which are deficient in cell wall proteinase (Pearce et al, 1974; Exterkate, 1976a) but contain the same peptidase activities as the parent cell (Exterkate, 1976a), to specifically assess the role of starter cell wall proteinase in bitterness development in Cheddar cheese. Starter strains chosen were all relatively temperature-insensitive. This means they all grow to some extent during cooking ($38^{o}C$) and therefore the control cheese will contain a high cell density in the curd at salting and consequently should develop bitterness.

The rennet concentration used in Cheddar cheesemaking has an important influence on the development of bitterness, especially when temperature-insensitive strains are used. Lawrence et al (1972) showed that with these strains, increased rennet levels gave more bitter cheese. Since the objective of the present trials was to determine the effect of the level of cell wall proteinase on bitterness development, a relatively low level of rennet with a relatively high setting temperature was used.

CHAPTER 18

EXPERIMENTAL

18.1 Bacteria

Stock cultures of *Streptococcus cremoris* strains 108, 104 and P_2 and *S. lactis* H_1 and ML_8 were obtained from the New Zealand Dairy Research Institute culture collection. Stock cultures of Prt^+ and Prt^- cells were prepared as previously described (page 21).

18.2 Cheese Starter Preparation for Cheesemaking

Stock cultures of Prt^+ and Prt^- cells were incubated for 16 h at 22°C then inoculated (0.5, 1.0 and 2.0%) separately into three tubes containing RSM. The RSM had been autoclaved (116°C, 20 min), centrifuged (10,000 x g, 5 min) to remove denatured protein and buffered with β-glycerophosphate (final concentration 0.073 M, pH 7.1). The three tubes of RSM for growth of Prt^- cultures contained Trypticase (5 mg / ml). After 16 h incubation at 22°C, the pH of each culture was determined and the one from each series nearest pH 5.1 was selected. The cell densities in these separate Prt^+ and Prt^- cultures were determined turbidimetrically at 480 nm and cultures for bulk starter inoculation were prepared by mixing so that Prt^- cells comprised 40, 70, 85 or 90% of the total population. The control culture contained only Prt^+ cells. Bulk starter milk was steamed for 1.5 h and cooled to 22°C before inoculation. The level of inoculation depended on the growth rate of the particular starter strain, however, the total number of cells (Prt^+ + Prt^-) inoculated into each bulk starter medium was the same within each trial. The bulk starter was incubated for 16 h at 22°C.

18.3 Cheese Manufacture

Cheddar cheese was manufactured from pasteurized (72°C, 15 s) whole milk by conventional commercial methods using open

vats. The same level (16 ml / 100 l milk) of calf rennet (New Zealand Standard Strength Rennet Extract, New Zealand Co-operative Rennet Co. Ltd) and starter inoculum (2%) were used in all trials. The setting temperature was 34°C and the period after the addition of rennet until cutting was kept constant (∿ 45 min) within each trial. After cutting with a 9 mm knife the temperature was raised to 37.5 - 38°C over a 35 min period. Whey was drained from the vats when the titratable acidity (TA) showed a rise of 0.03% lactic acid from that at cutting. The TA at milling for all trials was between 0.50 - 0.60% and that at salting between 0.63 - 0.72%. In each trial milling and salting was carried out at the same TAs in all vats. Attempts were made to obtain similar salt-in-moisture values by dry stirring and varying the rate of salt addition between 2.5% and 2.95% (w/w curd at milling). Rectangular, 20 kg, blocks of cheese were pressed overnight from the salted curd. These were wrapped in wax-coated film and stored at 11°C for six months.

18.4 Determination of the Ratio of Prt^+ to Prt^- Cells and Starter Colony Counts

Milk samples were taken from the stirred vats after inoculation and chilled. An aliquot (0.1 ml) was blended with sterile β-glycerophosphate buffer (100 ml, 0.01 M, pH 7.0) in an AtoMix blender (Measuring and Scientific Equipment Ltd, London) to reduce streptococcal chains to uniform size (Martley, 1972). The cell suspension was further diluted with the same buffer. Five replicates (0.1 ml) of 10^{-4} dilution were surface-spread on MAG plates for the determination of the ratio of Prt^+ to Prt^- cells. These plates were then incubated for three days at 22°C.

18.5 Proteinase Assay

Proteinase positive and Prt^- cells were grown separately in RSM at 30°C. Cells were harvested and washed by the method previously described (page 22) and the proteinase activity of intact cells was determined using the standard method (page 52).

18.6 Bacteriophage Assay

Whey samples taken from the vat prior to salting were spotted undiluted and at decimal dilutions on lawns of the starter organism prepared by soft agar overlay on M17 agar plates containing 0.02 M $CaCl_2$ (Terzagi & Sandine, 1975).

18.7 Analysis of Cheeses

Plug samples were taken when the cheeses were removed from the press (one day analysis). Moisture was determined by drying at 100°C for 16 h, fat was determined by the Babcock method and salt by the Volhard method.

18.8 Determination of Free Amino Acids and Peptides

Cheese samples (10 g) were taken at 1 day, 3 months and 6 months and homogenized for 5 min in trisodium citrate (90 ml, 2% (w/v)) at 45°C using a Colworth Stomacher 400 (A J Seward & Co, London). An aliquot of homogenate (10 ml) was mixed with TCA (40 ml, 15% (w/v)) and filtered after 15 min through Whatman No. 42 filter paper. The free amino acid and peptide content was then determined by the Folin-Ciocalteu method (Lowry *et al*, 1951) using tyrosine as the reference standard.

Curd samples were taken immediately prior to salting and chilled. A shredded portion (10 g) was homogenized with sterile trisodium citrate (90 ml, 2% (w/v)) at 4°C in the blender. The homogenate was diluted with sterile β-glycerophosphate buffer. Six replicates (0.1 ml) of the 10^{-6} dilution were surface-spread on MAG plates for the determination of the ratio Prt^+:Prt^- as above. Simple colony counting was not applicable to *S. lactis* ML_8 as both Prt^+ and Prt^- colonies were very small on MAG plates. The ratios were, therefore, obtained by picking all the colonies off plates containing 50 - 100 colonies and testing them for coagulation time in sterile skim milk at 22°C.

For total counts, duplicate samples (1 ml) of 10^{-7} dilution of homogenate were mixed with liquid M17 - agar medium and the plates incubated at 30°C for three days.

18.9 Bitterness Evaluation

A screening test for selecting tasters sensitive to bitterness in cheese was devised. A paste of uniform consistency was prepared by grinding bland cheese (8 g of one month old Cheddar) with a mixture (3 ml) of whole milk and a bitter solution of hydrolyzed milk protein. The level of bitterness was varied by changing the ratio of hydrolyzed milk protein to whole milk. Bitter milk protein solution was derived from either whole milk incubated with extracellular proteinase from *Pseudomonas fluorescens* (gift from B C Richardson) or with thermolysin (Calbiochem). Prospective panellists were required to place four cheese pastes in order of decreasing bitterness. The test was conducted on three consecutive days and tasters who had the correct order at least twice were retained for the panel.

The method of evaluating cheese samples was based on the simple paired comparison test. Each panellist was presented with two pairs of samples and two cheese pastes. The cheese pastes were given as examples of blandness and bitterness. Each pair of cheese samples was from a single trial after six months ripening and comprised a sample of the control cheese (100% Prt^+) and of the cheese with the maximum percentage of Prt^- cells. Panellists were required to state whether bitterness was present in each sample. If bitterness was detected in both samples, then panellists were required to indicate which sample was the more bitter. Cheeses from each trial were tasted at least twice by not less than ten tasters. Panel results were statistically analyzed as described for paired-comparison testing (Amerine *et al.*, 1965). One cheese was taken as being significantly more bitter than the other when the probability of it being the same (p) was ≤ 0.05.

CHAPTER 19

RESULTS

19.1 Proteinase Activity of Starter Cultures

The proteinase activity of Prt^+ cells of the five strains of lactic streptococci was 347 to 574 dpm / mg (dry weight) bacteria. The activities of these cells were at least 30 times greater than those of Prt^- cells (Table 14). It is unlikely that different conditions of assay would produce significantly different results for Prt^- cells which for the purpose of the present work can be considered as deficient in proteinase.

19.2 Cell Densities in Curd at Salting

Cell densities at salting were similar for all vats within each trial for Trials 1 to 7 (Table 15) and were, therefore, independent of the proportions of the two cell types. Two vats in Trials 8 and 9 had cell densities which were 60% of the other vats. The actual cell densities were strain-dependent and ranged from an average of 1.0×10^9 colony-forming-units (cfu) / g curd for P_2 to 4.2×10^9 cfu / g curd for strain ML_8. With starters H_1, 108 and P_2 similar densities were obtained in duplicate trials. Under the same cheesemaking conditions Lowrie *et al* (1972) observed a cell density of 4.4×10^9 cfu / g for ML_8 and 1.0×10^9 cfu / g for *S. cremoris* HP (which is the parent strain of P_2, Pearce, 1978). In all the present cheesemaking trials, phage levels in the whey at salting were less than 10^5 plaque-forming-units (pfu) / ml except for Trial 6 where whey from the vat contained 7×10^6 pfu / ml. However, this level of phage infection appears not to have made a significant difference as the cell density in the curd was the same as that for the three experimental vats.

TABLE 14: Proteinase Activity at pH 6.5 and 30°C of Prt^+ and Prt^- Cells Grown in Skim Milk

Strain	Proteinase Activity (dpm / mg (dry weight) bacteria)	
	Prt^+	Prt^-
S. lactis		
H_1	347	7
ML_8	384	2
S. cremoris		
108	530	12
104	570	18
P_2	574	18

TABLE 15: Composition and Cell Density of Starter During Cheddar Cheesemaking with Mixtures of Prt^+ and Prt^- Cells

Trial No.	Strain	Cheese	Starter Composition, % Prt^- (Prt^+ Balance)			Cell Density in Curd at Salting ($\times 10^9$ cfu/g)
			Bulk Starter Inoc	Cheese Vat Inoc	Curd at Salting	
	S. lactis					
1	H_1	C[a]	0	0	0	3.6
		E1	40	21	26	2.8
		E2	70	70	59	3.3
		E3	85	86	75	3.3
2	H_1	C	0	0	0	3.7
		E1	40	36	38	3.0
		E2	70	62	59	3.3
		E3	85	88	73	3.2
3	ML_8	C	0	0	0	4.1
		E1	40	16	17	4.2
		E2	70	53	30	4.6
		E3	90	71	45	3.9
	S. cremoris					
4	108	C	0	0	0	1.8
		E1	40	37	36	2.3
		E2	70	55	57	2.0
		E3	85	76	75	1.8
5	108	C	0	0	0	2.0
		E1	40	29	b	1.8
		E2	70	54	54	1.3
		E3	85	69	64	2.0
6	104	C	0	2	3	1.4
		E1	50	38	34	1.5
		E2	80	67	65	1.2
		E3	90	77	72	1.3
7	104	C	0	5	6	2.4
		E1	90	70	65	2.0
8	P_2	C	0	0	8	1.1
		E1	40	36	36	1.1
		E2	70	65	58	0.7
		E3	85	77	69	1.4
9	P_2	C	0	0	0	0.7
		E1	90	77	70	1.1

[a] C, control; E, Experimental; b, not determined

19.3 Proportions of Prt^+ and Prt^- Cell Types

In all trials there was a shift in the proportion of the two cell types, firstly during incubation of the bulk starter and secondly in the cheese vat. Generally a lower percentage of Prt^- cells resulted (Table 15). The mean standard deviation for the plate-counting method of determining the ratio of cell types was 4.7%. For ML_8, however, the error in determination is probably larger because of the different method used (see Experimental).

19.4 Cheesemaking Times

During the manufacture of the control cheese the set-to-dry time was reasonably consistent at 2 h 40 min to 2 h 50 min whereas the dry-to-salt time covered the range 1 h 55 min to 3 h (Table 16). This variation is not unexpected since cheese was made over a dairying season and with a variety of different strains. As the proportion of Prt^- cells increased there was a general trend towards an increase in both the set-to-dry and especially the dry-to-salt times. Set-to-dry times for the vat with the highest proportion of Prt^- cells (Table 16) were an average of 11% longer than for the control vat. The corresponding dry-to-salt times, however, were an average of 53% longer. Overall this made the slowest vat an average of 90 min behind the control vat. Manufacturing time was appreciably increased when Prt^- cells exceeded about 40% of the population of the vat inoculum (that is in Trials 1 to 6 and in Trial 8 with cheeses E2 and E3; in Trials 7 and 9 with cheese E1 - see Tables 16 & 17).

19.5 Compositional Analysis

Analysis indicated that all cheeses were within the limits for moisture in the non-fat substance (52 to 56%) for export Cheddar as proposed by Gilles & Lawrence (1973) and accepted by the New Zealand dairy industry. Although it was hoped to achieve the same salt-in-moisture levels for all four vats in each trial, the progressive decrease in rate of acid production, as the proportion of Prt^- cells in the starter increased made this difficult. Despite the use of additional dry stirs

TABLE 16: Manufacturing Times and Salt-in Moisture Value of Cheddar Cheese made with Mixtures of Prt^+ and Prt^- Starter Cells

Trial No.	Strain	Cheese	Cheesemaking times (h:min)			Salt-in-Moisture (%)
			Set-to-Dry	Dry-to-Salt	Total	
	S. lactis					
1	H_1	C[a]	2:45	2:00	4:45	5.17
		E1	2:45	2:15	5:00	4.77
		E2	2:50	2:50	5:40	4.80
		E3	2:50	3:15	6:05	4.63
2	H_1	C	2:50	2:05	4:55	5.42
		E1	2:50	2:25	5:15	5.52
		E2	3:00	3:00	6:00	4.86
		E3	3:10	4:15	7:25	4.15
3	ML_8	C	2:40	1:55	4:35	4.64
		E1	2:45	2:00	4:45	5.10
		E2	2:55	2:05	5:00	5.07
		E3	3:30	2:45	6:15	4.41
	S. cremoris					
4	108	C	2:45	2:50	5:35	4.52
		E1	2:45	2:50	5:35	4.44
		E2	2:45	3:15	6:00	4.63
		E3	2:50	4:20	7:10	3.51
5	108	C	2:45	2:55	5:40	4.94
		E1	2:45	2:55	5:40	5.09
		E2	2:55	3:05	6:00	4.97
		E3	3:00	4:20	7:20	4.74
6	104	C	2:45	3:00	5:45	5.37
		E1	2:45	3:05	5:50	5.03
		E2	2:45	3:15	6:00	5.03
		E3	3:05	3:35	6:40	4.72
7	104	C	2:50	2:25	5:15	5.16
		E1	3:05	3:40	6:45	5.51
8	P_2	C	2:45	2:50	5:35	5.15
		E1	2:45	3:15	6:00	5.21
		E2	2:50	3:25	6:15	4.87
		E3	3:10	4:05	7:15	4.57
9	P_2	C	2:45	2:40	5:25	5.24
		E1	2:50	3:55	6:45	5.06

[a] C, Control; E, Experimental.

The four cheeses differ in starter composition (see Table 16)

TABLE 17: Level of 12% TCA-Soluble Amino Acids and Peptides During Ripening of Cheddar Cheese Made with Mixtures of Prt^+ and Prt^- Starter Cells

Trial No.	Strain	Cheese	Ripening Period: Curd at Salting	Three Months	Six Months
			(mg Tyr equivalent / g cheese)		
	S. lactis				
1	H_1	C[a]	0.56	2.06	3.17
		E1	0.52	2.06	3.38
		E2	0.44	2.00	3.23
		E3	0.37	1.88	3.02
2	H_1	C	0.71	2.08	3.03
		E1	0.66	2.02	3.00
		E2	0.65	1.95	3.02
		E3	0.50	1.87	2.82
3	ML_8	C	0.39	2.29	2.89
		E1	0.36	2.21	2.73
		E2	0.42	2.19	2.67
		E3	0.37	2.28	2.77
	S. cremoris				
4	108	C	b	2.01	2.79
		E1	0.45	2.01	2.79
		E2	0.41	1.98	2.82
		E3	0.38	1.98	2.86
5	108	C	0.46	1.85	2.67
		E1	0.47	1.79	2.62
		E2	0.38	1.80	2.79
		E3	0.44	1.82	2.68
6	104	C	0.70	2.18	2.82
		E1	0.63	2.04	2.60
		E2	0.64	1.97	2.67
		E3	0.58	1.97	2.52
7	104	C	0.47	2.03	b
		E1	0.51	1.96	b
8	P_2	C	0.36	2.27	2.73
		E1	0.36	2.36	2.63
		E2	0.35	2.21	2.45
		E3	0.35	2.18	2.46
9	P_2	C	0.30	1.86	b
		E1	0.30	1.54	b

[a] C, Control; E, Experimental. The four cheeses differ in starter compositions (see Table 16)
b, Not determined

and an increased salting rate the usual trend was a decrease in salt-in-moisture value as the proportion of Prt^- cells in the starter was increased (Table 16).

19.6 Free Amino Acids and Peptides in Cheese

In all cheesemaking trials the level of free amino acids and peptides soluble in 12% TCA increased with ripening (Table 17). Little or no difference was found between the experimental and control cheeses at the same sampling time suggesting that the level of starter peptidase was similar in control and experimental cheeses and that peptidase activity was not limited by substrate concentration in the experimental cheeses (where the level of starter proteinase was reduced.

19.7 Bitterness Evaluation

Preliminary tasting trials showed that the range of bitterness intensity developed, when one strain was used at four different ratios of Prt^+ to Prt^- cells, was too narrow for the taste panel to show statistically significant differences between cheeses. Therefore, a simple comparison of bitterness intensity in the two cheeses with extremes of starter composition was made. The panel judged that the control cheese was significantly more bitter than the experimental cheese in six out of nine trials (Table 18). One exception was Trial 4 using strain 108. However, this result could be explained by the unusually low salt-in-moisture level in the experimental cheese (3.51%, compared with 4.52% in the control) since this would encourage bitterness development (Lawrence & Gilles, 1969). The other exceptions were the duplicate trials with strain P_2 (Trials 8 and 9). In both trials the cell densities were lower than with the other strains and bitterness was not detected in the control cheese in 35% of the tastings.

TABLE 18: Bitterness Comparison of Control and Experimental Cheeses after Six Months Ripening

Trial No.	Strain	Cheese	Bitterness Comparison
	S. lactis		
1	H_1	C[a]	S[b]
		E3	
2	H_1	C	S
		E3	
3	ML_8	C	S
		E3	
	S. cremoris		
4	108	C	NS
		E3	
5	108	C	S
		E3	
6	104	C	S
		E3	
7	104	C	S
		E1	
8	P_2	C	NS
		E3	
9	P_2	C	NS
		E1	

[a] C, Control; E, Experimental

[b] S, Control cheese significantly more bitter than experimental cheese (probability that the two cheeses are equally bitter ≤ 0.05); NS, no significant difference in bitterness between the two cheeses.

CHAPTER 20

DISCUSSION

20.1 Role of Starter Proteinase in Bitterness Development

Using five strains of lactic streptococci, Cheddar cheese was made so that while the actual bacterial density in the curd at salting was constant for a given strain, the proportion of Prt^- cells was altered. In this way the level of cell wall-associated starter proteinase in the experimental cheese was reduced to a minimum of 25% of the control level where only Prt^+ cells were present. In six trials the experimental cheese was significantly less bitter ($p \leq 0.05$) than the control while in the remaining three trials no significant difference was found. Taking the data from all nine trials, the experimental cheeses were less bitter than the controls ($p < 0.001$) suggesting that the cell wall-associated proteinase has a role in bitterness development. It is likely from the present results that a weakness in previous research on bitterness in cheese has been the lack of data concerning the presence of Prt^- variants in the cultures used. This could be especially important where mixed cultures of undefined composition were used since they often contain predominantly Prt^- cells (Thomas & Lowrie, 1975).

20.2 Cheesemaking with Cultures Containing Prt^- Cells

Only small changes in the proportions of the two cell types were normally found during growth of experimental cultures in both the bulk starter and the cheese vat. The rates of acid produced by both control and experimental cultures were similar up to running (set-to-dry, Table 16) but acid production by experimental cultures was markedly slower after removal of the whey (dry-to-salt). Whey removal results in a marked reduction in the availability of soluble nutrients, including amino acids and peptides, so that the supply of nitrogen for growth would be more dependent on

the cell wall-associated proteinase. This could account for the slower rate of acid production where the culture contained Prt$^-$ cells.

Use of cultures containing a high proportion of Prt$^-$ cells may represent a practical method for reducing bitterness development in cheese. Mixed cultures of this sort appear to be used for Gouda cheese production (Stadhouders & Hup, 1975), where the rate of acid production is usually not so critical (see review of Lawrence & Thomas, 1979). For Cheddar cheese manufacture, however, the rates of acid production would need to be faster than was found with the cultures used in the present study.

20.3 Role of Starter Peptidase in Bitterness Development

While the present study provides only indirect data on starter peptidase activities, discussion of the possible role of this system in bitterness development seems appropriate. Bitter peptides are produced by the action of proteolytic enzymes (starter proteinase and / or rennet) while their removal could be brought about by the action of starter peptidases (Czulak, 1959; Lowrie & Lawrence, 1972), which are located primarily in the cytoplasm and membrane of the bacteria (Exterkate, 1975; Schmidt et al, 1977). Whereas the cell wall-associated proteinase will have access to substrate when cells are intact, the intracellular peptidase activity must be released by cell lysis before peptides which are too large to enter the cell can be hydrolyzed. In all the published work on bitterness development, with the exception of Law et al (1976a), no measurements of peptidase levels in cheese and the rate of lysis of starter cells have been made. Determination of viable cell numbers does not necessarily give a measure of cell lysis, since starter cells may be structurally intact and yet unable to grow on an agar recovery medium (Thomas et al, 1969). Premature cell lysis was induced when cheese was made with lysozyme-sensitized cells and the subsequent release of peptidase possibly accounted for the lower incidence of bitterness in this cheese even though the density of starter bacteria had been markedly increased (Law et al, 1976a). A similar ex-

planation may account for the lower incidence of bitterness when phage multiplied to high titres (Lowrie et al., 1974) since premature cell lysis would again be expected. The rate of acid production up to the time of milling was only slightly affected by phage, indicating that the maximum cell density attained in the curd was not reduced as much as Lowrie et al.(1974) suggested. The major effect of phage is more likely to be the induction of premature lysis, possibly in concert with the action of salt (Lowrie et al., 1974). Therefore, bitterness development appears to result where high densities of starter bacteria persist in the cheese as intact cells. Differences between strains may result partly from different susceptibilities to autolysis which may also be influenced by addition of salt. The degradation of bitter peptides in cheese may also depend on the actual starter strain used since the activity of peptidases is strain dependent (Sullivan et al., 1973).

It is concluded from the present work that cell wall-associated proteinase has a role in the formation of bitter peptides while earlier data (Lowrie et al., 1974; Law et al., 1976a) are interpreted as showing that intracellular peptidases can have an important role in removing bitter peptides, especially when these enzymes are released from the cells at an early stage in cheese ripening.

APPENDIX I

EFFECT OF GROWTH MEDIUM ON THE PROTEINASE ACTIVITY OF INTACT CELLS

INTRODUCTION

This Appendix describes a preliminary examination of the effect that some components of the growth medium have on the level of proteinase activity of intact cells. A more detailed investigation was intended but this was curtailed following publication of a recent report (Exterkate, 1979). A minimum level of cell-bound proteinase activity for S. cremoris AM_1 was found when cells were grown in a broth medium containing amino acids as the nitrogen source and no calcium (Exterkate, 1979). It was also shown that proteinase activity increased with incubation time when these cells were inoculated into a broth medium containing $CaCl_2$. Proteinase activity increased more quickly as the initial concentration of amino acids in the medium decreased. As the total concentration of calcium increased, in media containing various fractions of milk (serum, acid precipitated casein and casein micelles), then the proteinase activity of intact cells increased. It was, therefore, of interest to study the effect of growth in broth medium compared with that in milk on the level of cell-bound proteinase activity of some different strains of lactic streptococci. Also the effect of the concentration of peptides and amino acids in the growth medium was determined by adding these compounds to milk. The effect of Ca^{2+} on the level of proteinase activity was studied with the use of pH-stat cultures since this avoided the presence of Ca^{2+}-chelating buffers.

EXPERIMENTAL

Proteinase Activity of Cells Grown in Broth

Deep frozen stocks were thawed, inoculated (1%) into M17 broth (Terzaghi & Sandine, 1975), and incubated at 30°C for 16 h. Cells were subcultured (1%) in this medium and incubation repeated. These cultures were used to inoculate (3%) the experimental M17 medium. Experimental cultures were grown and harvested as given on page 22 and the proteinase activity of intact cells determined (page 52).

Effect of Amino Acid and Peptide Addition to Experimental RSM on the Level of Proteinase Activity of Intact Cells

Sterile solutions of Casamino acids (vitamin-free, acid hydrolyzed casein, 14% (w/w) NaCl, DIFCO Laboratories) and Trypticase (pancreatic digest of casein, BBL) were added to experimental RSM (5%, v/v) to give final concentrations of up to 8.0 mg / ml Casamino acids or 7.5 mg / ml Trypticase. The inoculum cultures contained the same concentration of Casamino acids or Trypticase as in the experimental RSM medium. Cells were grown and harvested in the usual way (page 22) and the proteinase activity of intact cells determined (page 52).

Proteinase Activity of Cells Transferred from Broth to Experimental RSM

Cells of 266 were grown in M17 medium, harvested, washed and resuspended in sterile water at 5 mg (dry weight) bacteria / ml. Experimental RSM was inoculated (1%) with this suspension and incubated at 30°C. Cells were harvested after 1, 2, 3.5 and 5 h, washed, resuspended to 20 mg (dry weight) bacteria / ml and assayed for proteinase activity.

pH Stat Cultures

The apparatus consisted of a jacketed vessel (100 ml) maintained at 30°C by circulating water. A pH electrode and

two syringe needles were inserted through holes in the lid which also incorporated a sampling port. All parts of the apparatus were flushed with hypochlorite solution (1%, v/v) and rinsed with sterile water. The combination glass electrode was connected to a Radiometer PHM26 pH meter to which a TTT11 auto-titrator (Radiometer, Copenhagen, Denmark) was attached. The auto-titrator controlled a magnetic valve which regulated the flow of 6 M NaOH into the culture through one syringe needle to hold the pH at 6.5. An atmosphere of 5% CO_2 in N_2 was maintained by bubbling the gas into the solution through the other syringe needle. The composition of experimental broth (EB) medium used was the same as M17 with the omission of β-glycerophosphate. The Ca^{2+} level was adjusted by the addition of sterile 1 M $CaCl_2$ solution. Total calcium concentration of media was determined by the method of Pearce (1977). The inoculum culture grown overnight in EB medium contained the same concentration of $CaCl_2$ as in the EB medium used in the pH-stat culture. Experimental cultures were inoculated (1%) and incubated for 5 h when the growing cells were harvested, washed and assayed in the usual way.

RESULTS

Proteinase Activity of Cells Grown in Broth or Milk

Cells of 266, E_8 and ML_3 grown in M17 had levels of proteinase activity which were only 15 to 18% of the levels with milk-grown cells (Table 1). Of the four strains grown in milk, AM_2 had the lowest level of proteinase and this level was not as markedly affected by growth in broth (Table 1).

Effect of Amino Acid and Peptide Addition to Experimental RSM on the Level of Proteinase Activity of Intact Cells

With the addition of 8 mg Casamino acids / ml of experimental RSM there was no significant change (< 5%) in the proteinase activity of intact cells of 266 and E_8 but with strains AM_2 and ML_3 the level of proteinase activity decreased by 30%

TABLE 1: Effect of Growth Medium on the Proteinase Activity of Intact Cells of Lactic Streptococci

Strain	Proteinase Activity (dpm/mg (dry weight) bacteria)	
	M17	Experimental RSM
S. cremoris		
266	84	475
AM_2	174	281
E_8	97	627
S. lactis		
ML_3	80	465

and 47%, respectively, (Table 2). Addition of amino acids made no difference to the growth rates (data not shown).

Proteinase activity of intact cells of 266 decreased as the concentration of Trypticase in the experimental RSM increased (Fig. 1). The reduction in the level of proteinase with 7.5 mg / ml added Trypticase represents a 35% decrease in proteinase activity of cells. The growth rate of cultures also increased as the Trypticase concentration of the medium was increased. The maximum generation time for the control culture of 266 was 65 min compared with 50 min for the culture which contained 7.5 mg / ml added Trypticase. The effect of the addition of Trypticase to experimental RSM on the level of proteinase activity of intact cells of AM_2, E_8 and ML_3 was investigated at a single Trypticase concentration (7.5 mg / ml). Strains showed a 29 to 37% decrease in the level of proteinase activity (Table 3).

Proteinase Activity of Cells Transferred from Broth to Experimental RSM

The proteinase activity of intact cells of 266 increased from 84 dpm / mg (dry weight) bacteria to about 340 dpm / mg (dry weight) bacteria in approximately four generations (Fig. 2). Values for repeat experiments were ± 10% of those in Fig. 2.

Effect of the Addition of Calcium to Broth on the Level of Proteinase Activity of Intact Cells

The proteinase activity of intact cells of E_8 increased more than twelve-fold as Ca^{2+} concentration increased from 2×10^{-4} M to 0.027 M (Fig. 3). A value of 600 dpm / mg (dry weight) bacteria was obtained at 0.027 M Ca^{2+}. With AM_2 activity increased by only 25% (Fig. 3).

DISCUSSION

All strains of lactic streptococci tested had much lower cell-bound proteinase activities when grown in broth medium than when grown in experimental RSM (Table 1). A major

TABLE 2: Effect of Added Casamino Acids (8.0 mg / ml) on the Level of Proteinase Activity of Intact Cells of Lactic Streptococci Growing in Experimental RSM

Strain	Proteinase Activity (dpm/mg (dry weight) bacteria)	
	Control	Casamino Acids Added
S. cremoris		
266	409	391
AM_2	360	251
E_8	662	631
S. lactis		
ML_3	420	224

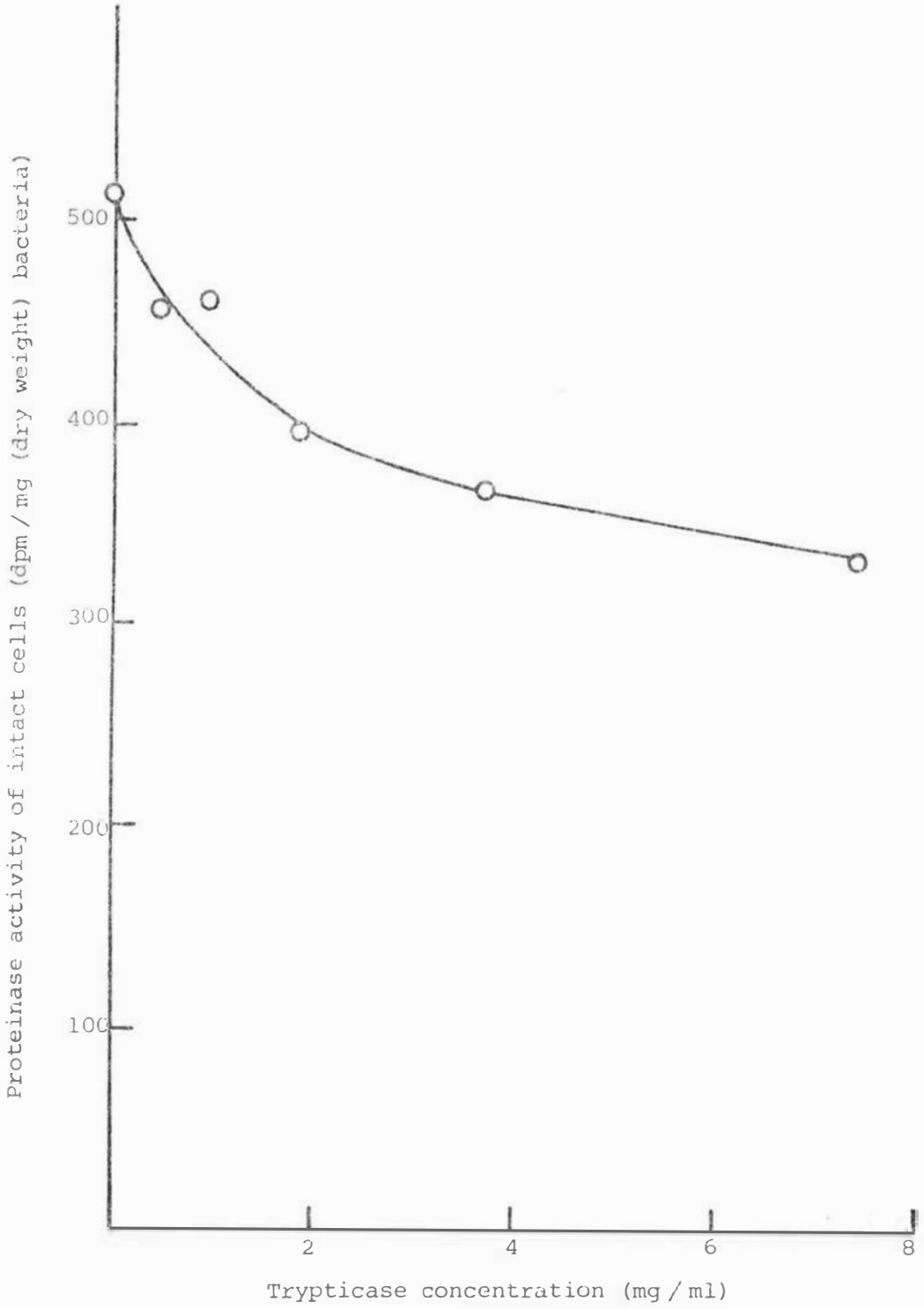

FIGURE 1: Effect of added Trypticase on the level of proteinase activity of intact cells of S. cremoris 266 growing in experimental RSM. Cultures were incubated at 30°C and growing cells harvested after five generations when the culture pH was ∿ 5.0.

TABLE 3: Effect of Added Trypticase (7.5 mg / ml) on the Level of Proteinase Activity of Intact Cells of Lactic Streptococci Growing in Experimental RSM

Strain	Proteinase Activity (dpm/mg (dry weight) bacteria)	
	Control	Trypticase Added
S. cremoris		
266	514	333
AM_2	258	163
E_8	491	350
S. lactis		
ML_3	464	302

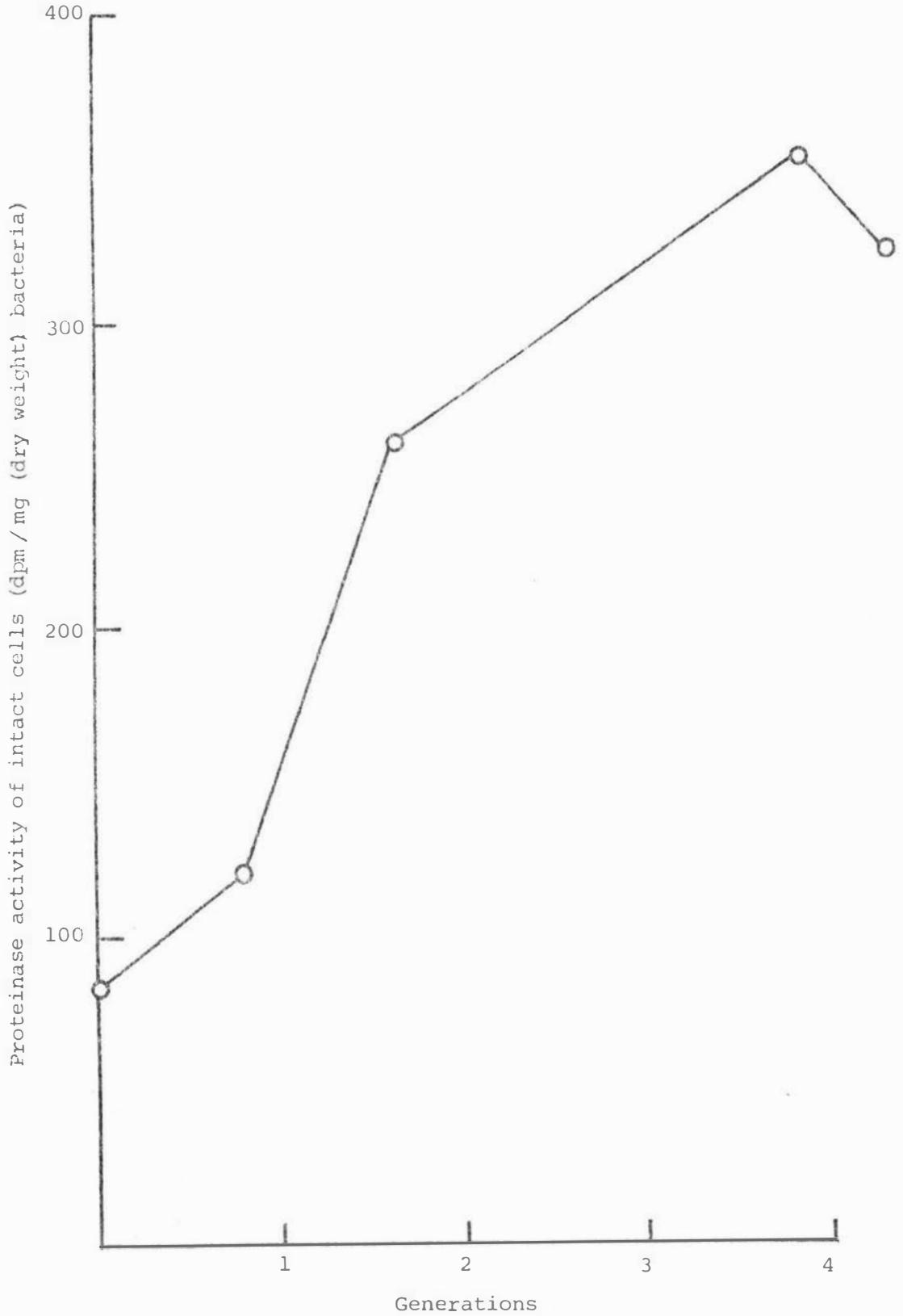

FIGURE 2: Level of proteinase activity of intact cells of S. cremoris 266 transferred from M17 broth into experimental RSM. The culture was incubated at 30°C.

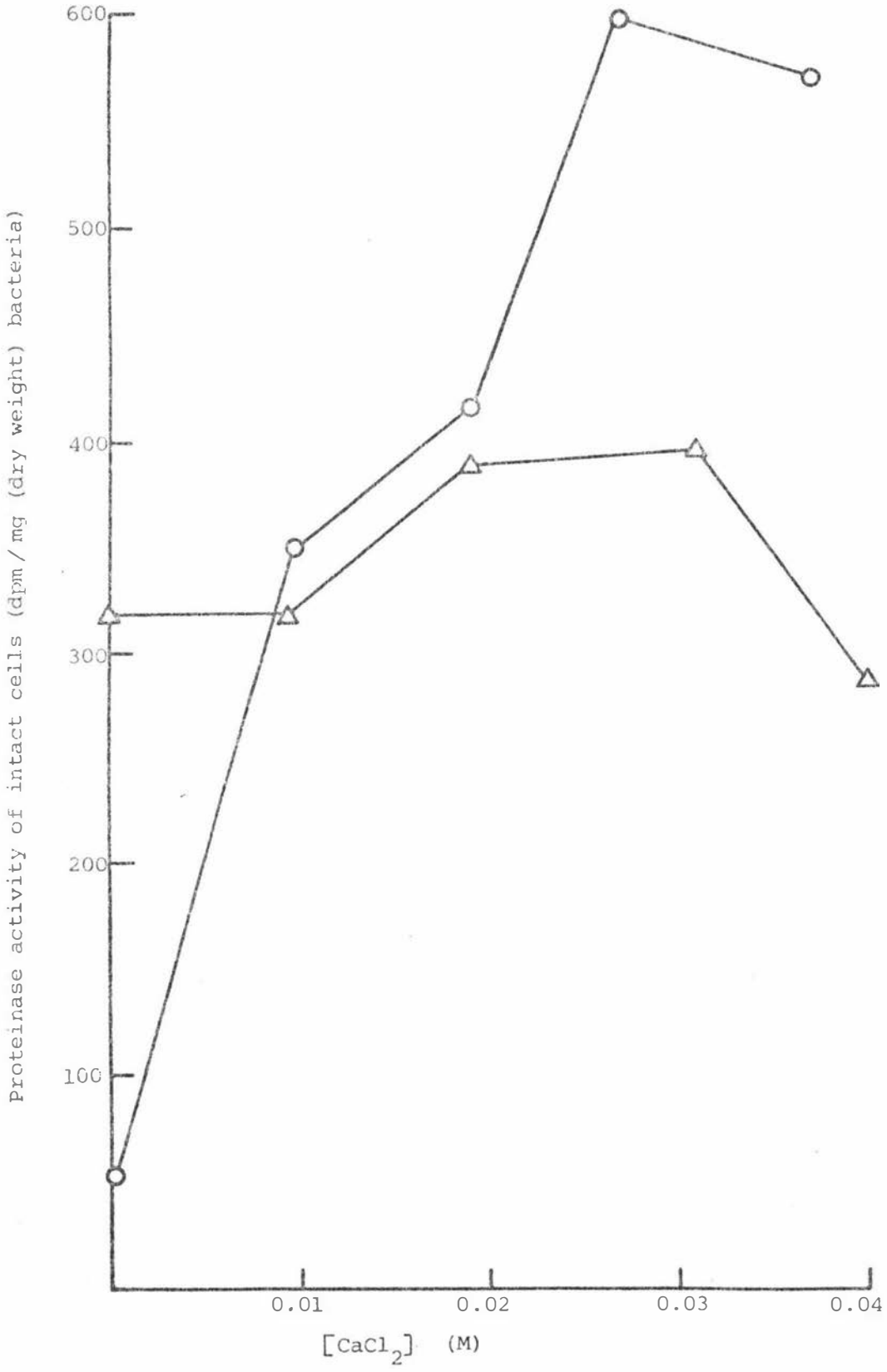

FIGURE 3: Effect of $CaCl_2$ on the level of proteinase activity of S. cremoris E_8 (o) and AM_2 (Δ) grown at 30°C in experimental broth (see text) with pH control.

difference between broth and milk is the compositon of the nitrogen sources. The broth medium contains high levels of amino acids and low molecular weight peptides whereas these materials are present at relatively low levels in milk and protein is the major potential source of nitrogen. When the level of amino acids in experimental RSM was increased the cell-bound proteinase activity of the four strains examined decreased (Table 2), particularly in the case of AM_2 and ML_3. Increase in the Trypticase level of experimental RSM, however, also caused a decrease in the cell-bound proteinase activity of all strains examined (Table 3). When cells were transferred from broth to experimental RSM more than four generations (see Results) was required before maximum proteinase activity ($\sim$ 470 dpm / mg (dry weight) bacteria) was regained (Fig. 2). The composition of nitrogen sources, and especially the concentration of low molecular weight nitrogen, is therefore an important factor in determining the proteinase activity of intact cells. Preliminary experiments involving the addition of $CaCl_2$ to cultures of E_8 and AM_2 (Fig. 3) confirm the finding of Exterkate (1979) that Ca^{2+} concentration is also important in determining the level of proteinase activity, at least with some strains. Cells to be used in a study of the proteinases of lactic streptococci should clearly be grown in milk where the low concentration of low molecular weight nitrogen, together with the high Ca^{2+} level give rise to maximum levels of proteinase activity.

A P P E N D I X I I

HYDROLYSIS OF NATIVE AND METHYLATED CASEINS BY CHYMOSIN AND TRYPSIN

EXPERIMENTAL

Preparation of Methylated Caseins

Pure α_{s1}- and β-caseins were prepared by DE 52 chromatography of acid precipitated whole casein by the method previously described (page 6). The lysine residues were methylated using formaldehyde (Lin et al., 1969). Complete methylation was observed when the lysine peak had disappeared from the amino acid analyzer trace of the casein hydrolysate and the expected amount of methylysine had appeared.

Hydrolysis

Pure crystalline chymosin (10^4 units / g, Berridge (1955)) was a gift of the New Zealand Rennet Co. Ltd (Eltham, New Zealand). Trypsin (Type I) and trypsin inhibitor (Type I-S) were obtained from the Sigma Chemical Co.

Casein solutions (0.02%, w/v) were incubated with proteinases at 30°C in 0.2 M phosphate buffer (pH 6.5). Trypsin was used at a concentration of 0.12 µg / ml for native and methyl α_{s1}-caseins and 0.42 µg / ml for native and methyl β-caseins. Chymosin was used at 28.5 µg / ml for all caseins. Samples were taken at 0, 5, 10, 20, 60 and 120 min. Hydrolysis by chymosin was stopped by shaking the sample (2 ml) with urea (1 g) and 2-mercaptoethanol (0.05 ml) until the urea had dissolved. The trypsin reaction was stopped by the addition of trypsin inhibitor (0.02 ml, 2.18 mg / ml). Samples (0.3 ml) were then electrophoresed on polyacrylamide disc gels.

RESULTS

The rates at which α_{s1}- and methyl α_{s1}-caseins were degraded by trypsin were similar although the product which had a lower mobility than α_{s1}-casein was not produced from methyl α_{s1}-casein (Fig. 1a, b). The degradation of β-casein by trypsin was much more rapid than was the degradation of methyl β-casein (Fig. 1c,d). There was about the same amount of normal β-casein left at 10 min as there was methyl β-casein at 60 min. The number of bands formed from β-casein was greater than from the methylated derivative.

Chymosin degraded methyl α_{s1}-casein at a greater rate than normal α_{s1}-casein but the products and sequence in which they appeared were similar for both substrates (Fig. 2a, b). The degradation of β-casein and methyl β-casein was similar in all respects (Fig. 2c, d).

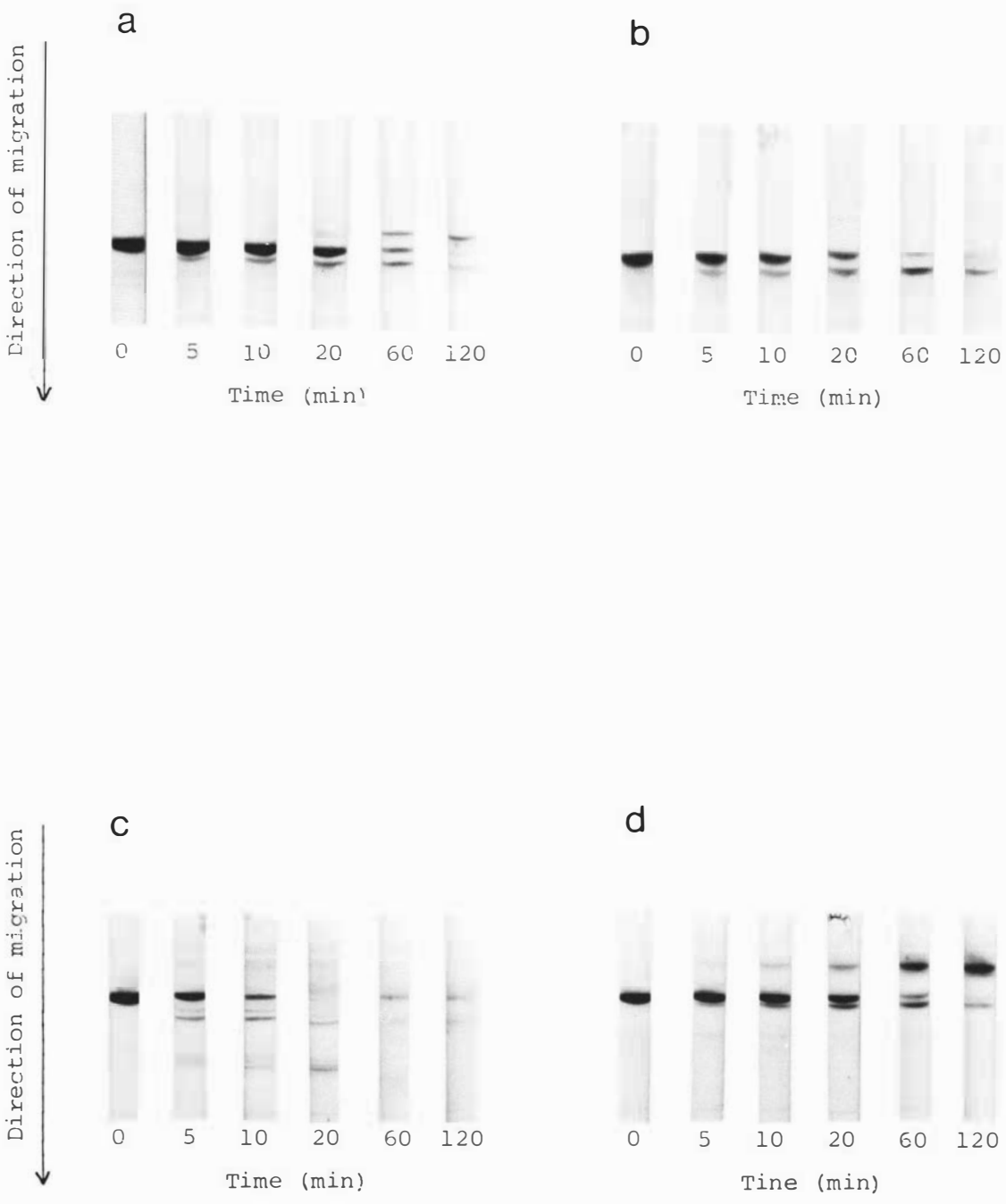

FIGURE 1: Disc gels showing time-course for trypsin hydrolysis of α_{s1}- and β-casein and their methylated derivatives. a, α_{s1}-casein; b, methyl α_{s1}-casein; c, β-casein; d, methyl β-casein.

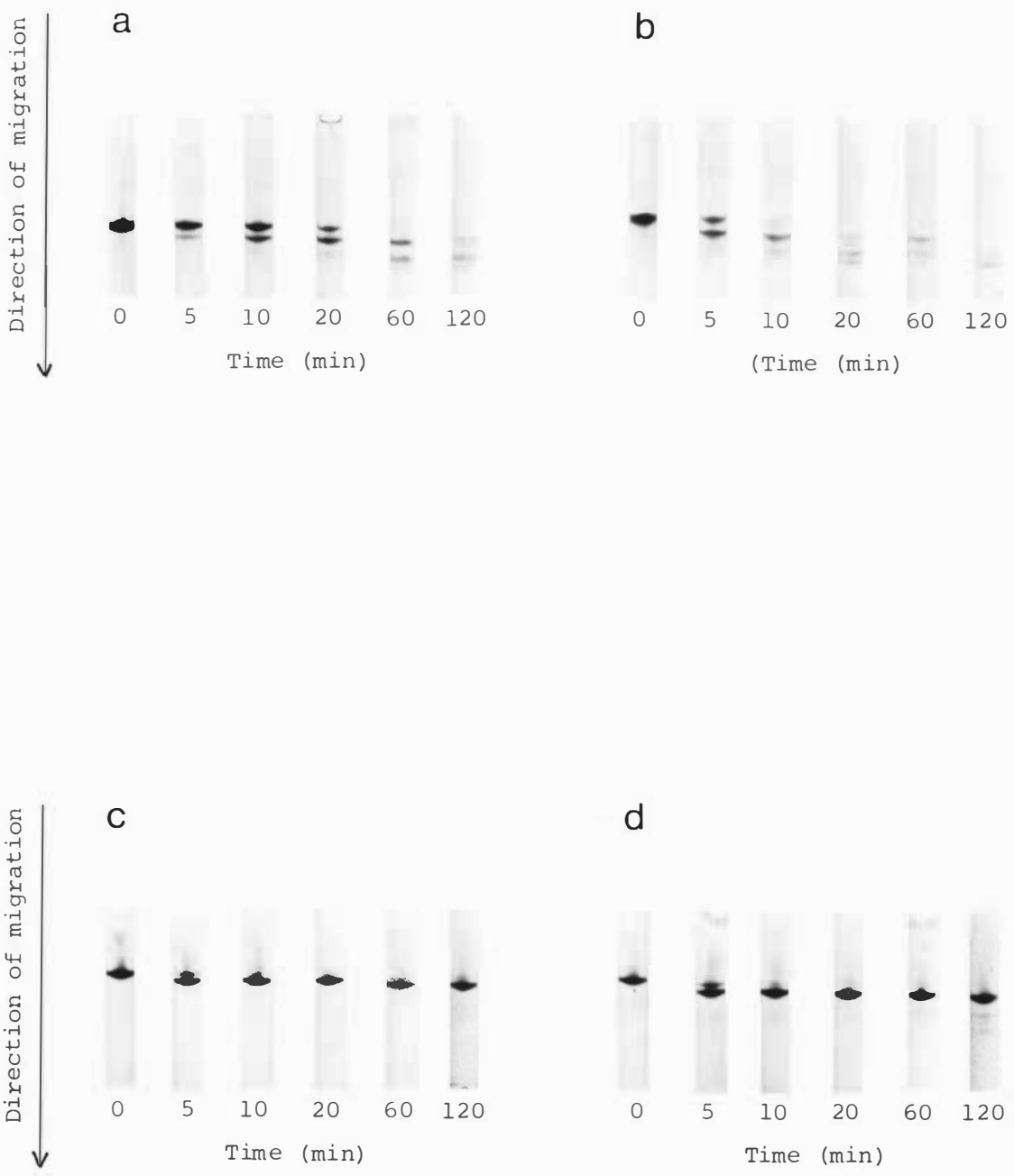

FIGURE 2: Disc gels showing time-course of chymosin hydrolysis of α_{s1}- and β-casein and their methylated derivatives. a, α_{s1}-casein; b, methyl α_{s1}-casein; c, β-casein; d, methyl β-casein.

APPENDIX III

HYDROLYSIS OF β-CASEIN WITH CELL WALL-ASSOCIATED PROTEINASE FROM S. CREMORIS 266

EXPERIMENTAL

Pure β-casein was prepared by DE 52 chromatography of acid precipitated whole casein using the method previously described (page 6).

Cell wall-associated proteinase from S. cremoris 266 was prepared from cells grown in experimental RSM. Washed cells were resuspended in 0.1 M phosphate buffer (pH 7.0) containing $MgCl_2$ (0.01 M) and sucrose (0.5 M). Phage-associated lysin (page 72) was added and the suspension held at 32°C for 40 min. Phase contrast microscopy showed that most of the cells were converted into spheroplasts indicating cell wall removal. The release of lactate dehydrogenase from cells was used to estimate cell lysis (page 74). Since less than 5% of cells lysed, there was not appreciable contamination of cell wall proteinase with membrane-bound or cytoplasmic peptidases or with intracellular proteinases. Cell wall-associated proteinase was contained in the supernatant after centrifugation (35,000 x g, 10 min) of the spheroplast suspension. A solution, buffered with 0.1 M phosphate (pH 6.5), containing cell wall-associated proteinase (2 mg (dry weight equivalent) bacteria / ml) and β-casein (0.5%, w/v) was maintained at 30°C. Samples were taken at 0, 1, 2, 4 and 6 h and the reaction stopped by addition of urea (9 M, final concentration). The quenched samples were electrophoresed on polyacrylamide disc gels.

RESULTS AND DISCUSSION

Four major products and several minor ones were observed on the gels (Fig. 1). Only relatively large peptides are fixed

in the gel by staining and the intensity of the band will depend on the molecular weight and the affinity for stain of these peptides. Of the four major products, two (a and b) did not appear to change in concentration between 2 and 6 h while products c and d both increased in concentration with time. Small peptides, soluble in 6% TCA, produced during a proteinase assay will probably not appear on polyacrylamide gels.

Incubation for 6 h at 30°C during the hydrolysis of β-casein causes only slight inactivation of the cell-free proteinase (see page 56).

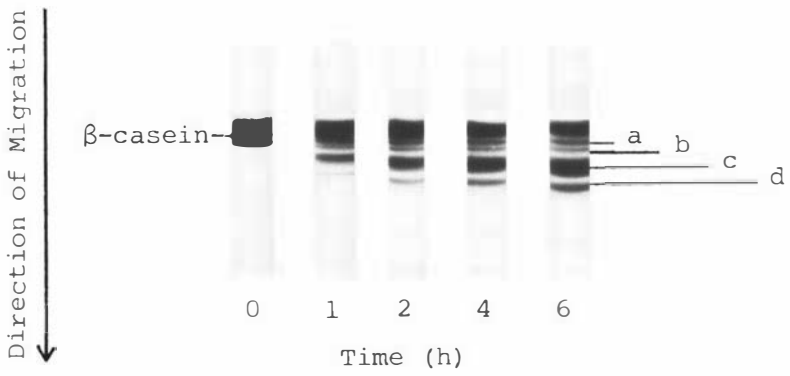

FIGURE 1: Polyacrylamide disc electrophoresis showing the hydrolysis of β-casein (0.5%, w/v) by cell wall-associated proteinase of S. cremoris 266 (2 mg (dry weight equivalent) bacteria / ml) in 0.1 M phosphate buffer (pH 6.5) at 30°C.

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