PROPERTIES OF SOME ANIMAL DERIVED MILK COAGULATING ENZYMES

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ABSTRACT

Extracts of milk coagulating enzymes were obtained, adult bovine from the abomasums of pasture fed cattle and lamb rennet from the abomasums of partially milk fed lambs slaughtered between three and six months of age. Both rennets were, in the presence of sodium chloride, most stable at 10°C or lower at pH 4.7 for adult bovine and pH 4.1 for lamb. Heat treatment in a sodium chloride solution (200 mg/ml) at 68°C for 60 minutes destroyed less than 35% of the activity of each rennet. Under the same conditions calf rennet was completely inactivated.

The cheesemaking properties of adult bovine and lamb rennets were compared with calf rennet. Adult bovine and calf rennets responded similarly to changes of pH however lamb rennet appeared less active than calf rennet at higher pH's when measured in caseinate solution but appeared more active than calf rennet when measured in milk by a curd tension method. The optimum temperature for milk coagulating activity was 40°C for adult bovine and calf rennets but only 30°C for lamb rennet when measured in caseinate solution. The curd tension of milk coagulated with the three rennets increased with time but both adult bovine and lamb rennets appeared more sensitive to milk calcium levels than calf rennet. Whole casein, α_s-, β- and k-caseins were hydrolysed in a similar manner by the three rennets. Adult bovine rennet was the most proteolytic on whole and β- caseins while calf rennet hydrolysed α_s- and k-caseins more rapidly than the other rennets.

Two pepsins, of similar amino acid compositions, were isolated, one from adult bovine rennet and one from lamb rennet which also contained a rennin. All three enzymes were purified so that lamb rennin and pepsin each produced only a single band on polyacrylamide gel

electrophoresis but the adult bovine pepsin produced two bands and appeared to be heterogeneous.

Cheese made with adult bovine, lamb or a 50/50 mixture of calf-adult bovine rennets were compared with cheese made with calf rennet and found to be similar in manufacturing characteristics, flavour and body after three and six months storage at 6 or 13°C. Polyacrylamide gel electrophoretograms of these cheese after one to twelve months storage at 6°C showed that the milk coagulant had no effect on the casein degradation products produced in the cheese although the rate of degradation varied slightly.

Perchloric acid was found to be a good protein precipitant for quenching casein-rennet reactions and was utilized in a method which was developed for assaying milk coagulating activity. Rennet was added to sodium caseinate solution and one minute later the reaction quenched with perchloric acid. The quantity of peptides hydrolysed from the casein was measured at 217 nm and was directly proportional to rennet activity over a limited range of activity.

A method for removing mucoproteins, the major impurity in rennet, was developed and a commercial scale plant commissioned. A diethylaminoethyl cellulose based resin, equilibrated with a citrate buffer, retained the rennet enzymes while the mucoproteins passed through uninhibited. The enzymes were eluted with the same buffer made 1.0 M with sodium chloride. The eluent was more active than the original rennet, was crystal clear in appearance and remained so during twelve months storage when normally mucoproteins would have precipitated out of solution to form a cloud in the rennet.

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CHAPTER I

INTRODUCTION

One of the first steps in the manufacture of most varieties of cheese is the use of milk coagulating enzymes to precipitate casein and so form a gel. Until the last decade virtually all cheese was made with rennet extracted from the abomasum of young calves. However in the early 1960's the slaughter of young calves declined due to smaller dairy herds throughout the world and the increase in the number of calves being raised for beef production. For example in the United States of America the number of calves slaughtered dropped from 12.5 million in 1956 to 6.9 million in 1963 and further to 4.1 million in 1970 (USDA reports). In New Zealand the number of calves slaughtered was more stable but dropped from 1.24 million in 1964 to 0.95 million in 1973 (N.Z. Dairy Board Annual Reports, 1965 & 1974). During this same era world cheese production was estimated to have increased from 3.8 to 4.2 million tons from 1966 to 1970. Thus the increase in rennet requirements from 1.59 to 1.78 million gallons per year was approximately a 10% increase for the five year interval (Coward, 1972).

The decrease in the number of calf abomasums at the same time as cheese production was increasing led to an unavoidable shortage of calf rennet but other enzymes capable of coagulating milk have been forthcoming to fill the gap. Most, if not all, proteases are capable of coagulating milk under suitable conditions but they may break down caseins in the cheese curd too rapidly and too extensively. An alternative milk coagulating enzyme must be similar to calf rennet in having high milk coagulating and low proteolytic activities (Fox, 1969, b).

A large number of micro-organisms and plants have been proposed as sources of milk coagulating enzymes (Sardinas, 1972). The most sucessful of these have been enzymes from two molds Mucor pusillus Lindt (Tsugo et al, 1964) and Mucor miehei (Aunstrup, 1968), both of which are in commercial use in many countries. However microbial rennets are usually more proteolytic than animal rennets (Hansen, 1970) and hence a greater interest has been shown in milk coagulating enzymes from animal sources. Porcine pepsin has been used extensively as a milk coagulating enzyme (Maragoudakis, Young & Stein, 1961; Melachouris & Tuckey, 1964) both alone and in mixtures with calf rennet (Green, 1972). However porcine pepsin will not readily coagulate milk above pH 6.68 (Ernstrom, 1961; Fox, 1969 b) and is not as active at pH values close to 6.6 as calf rennet or bovine pepsin (Fox, 1969, b). Bovine pepsin from older calves or adult cattle has been used with calf rennet but it was not until 1969 that Fox (1969, b) reported an investigation of bovine pepsin as such. Ovine pepsin, an extract of milk coagulating enzymes from the abomasums of older lambs, has not been used for cheesemaking but its suitability as a rennet substitute was reported recently (O'Leary & Fox, 1973).

In New Zealand there are approximately 23 million four to twelve month old lambs and 1.3 million cattle over three months of age slaughtered annually (N.Z. Meat Producers Board 52 Annual Report, 1974). Thus there are a large number of abomasums from these two species available for the extraction of milk coagulating enzymes and the New Zealand Co-operative Rennet Co. Ltd., Eltham, New Zealand, has established commercial methods to extract milk coagulating enzymes from the abomasums of both lambs and cattle.

This thesis reports an investigation of the properties of the enzymes from lamb and adult bovine extracts and their use for cheesemaking. A commercial method for the purification of milk coagulating enzymes from a crude extract is also described.

CHAPTER II

RENNET ASSAYS

REVIEW OF LITERATURE

The coagulation of milk

The addition of one part of rennet to 5,000 to 10,000 parts of milk will cause the milk to gel in 10 to 20 minutes at pH 6.6 and 30°C. The process has three defined kinetic stages, which Tuszynski (1971) has called enzymatic, flocculation and gelification. The first two stages are interdependent (Foltman, 1959) and calcium ions are necessary for the last two stages. The gel, which is formed by rennin modified casein, traps most of the milk constituents and becomes firmer with time until it is cut to expose a large surface area for moisture expulsion. This is the basis for the manufacture of most varieties of cheese.

The primary phase. The primary phase of rennin action on casein is the release of highly acidic peptides, including glycopeptides, from k-casein (Alais, 1956; Armstrong et al., 1967; Wake, 1959) by hydrolysis of a phenylalanine-methionine bond (Jolles, Alais & Jolles, 1968). This destroys the micelle stabilising properties of the k-casein (Wake, 1959). The coagulation of milk is largely determined by this primary phase of rennin action and hence by the amount of k-casein susceptible to attack by rennin (Wheelock & Penney, 1972). The action on purified k-casein liberates a restricted number of peptides soluble in trichloroacetic acid (Armstrong et al., 1967) but under normal milk coagulating conditions such compounds are not split from other milk protein fractions by rennin (Wake, 1959). Modification in k-casein of any one of the following amino acids, histidine (Hill & Laing, 1965), lysine (Hill & Craker,

1968) and arginine (Hill, 1970) inhibits coagulation of rennin treated casein. These three amino acids are considered to form part of a positively charged region of k-casein that is important in the coagulation of casein (Hill, 1970).

The flocculation of whole milk by rennin is first observed about the time the quantity of soluble glycopeptides reaches a maximum (Castle & Wheelock, 1972) and the flocculation time is inversely proportional to both the total amount of glycopeptides released and the initial velocity of their release from that milk (Wheelock & Penney, 1972). The total amount of glycopeptides released from any one milk is constant but the initial velocity of their release is proportional to rennin concentration at low levels of rennin (Castle & Wheelock, 1972). The variation in flocculation times for different milks is probably due to the above factors which affect the rate of the primary rennin action on k-casein although the size and structure of the micelles may also be involved (Wheelock & Penney, 1972). Primary rennin action on milk releases peptides which contain sialic acid more slowly than peptides without sialic acid (Wheelock & Knight, 1969) and there is an increase in the ratio of N-acetylneuraminic acid to D-galactose in the glycopeptides as the reaction proceeds (Sinkinson & Wheelock, 1970). This evidence suggests that the structure of the k-casein carbohydrate moiety may also be a factor in determining the rate at which the glycopeptides are released by rennin (Sinkinson & Wheelock, 1970) possibly by affecting the affinity of the enzyme for the substrate (Wheelock & Penney, 1972).

Factors affecting milk coagulation. The flocculation time of renneted milk has been found to be closely related to pH (White & Davies, 1958; McDowell, Pearce & Creamer, 1969) and it appears that weak relationships between the concentrations of milk constituents and flocculation time

can be attributed to the effect of pH on the concentrations of these constituents (White & Davies, 1958). The ionic strength of a solution affects the rate of rennin action which reaches a maximum at an ionic strength equivalent to 0.025-M sodium chloride for a wide range of salts (Kato et al., 1972). However the most necessary ion for rennin coagulation of milk has proved to be calcium. It has been found necessary for the formation of casein micelles (Zittle, 1970; Lin et al., 1972) which bind available calcium until they are essentially isoelectric (Zittle, 1970). Hence the addition of calcium to milk accelerates the secondary phase of milk coagulation (Green, 1972), increases the proportion of casein in the micelles (Green, 1972; Lin et al., 1972) and the amount of calcium bound by the charged groups on the casein (Green, 1972).

Milk treatment prior to rennin action also affects coagulation. For example,

(i) ultra-high temperature treatment of milk causes partial inhibition of rennin action probably due to the formation of a β-lactoglobulin-k-casein complex (Hindle & Wheelock, 1970).

(ii) holding milk before rennin treatment or lowering the milk pH reduces the gel firmness in rennin treated milk although the flocculation time is not affected (Tuszynski, Burnett & Scott-Blair, 1968).

The role of electrostatic forces. It has been suggested that adding calcium to milk reduces the negative charge on the casein micelles, and consequently the repulsive forces between the micelles, thus promoting micelle agregation after rennin treatment (Green, 1972). A similar reduction of electrostatic forces by salts has

been shown to give firmer gels of k-casein than are obtained in salt free solutions (Zittle, 1970). It has been shown that the role of calcium during casein coagulation by rennin is not the formation of bridges between the micelles (Green, 1972) and that the effect of magnesium chloride on renneting time is similar to that of calcium ions (Pearce & Creamer, 1974). Both of these facts support the idea of cations reducing electrostatic forces between micelles.

Other observations of milk behaviour and coagulation have also been explained in terms of electrostatic forces between micelles. Even though rennin hydrolyses k-casein at a reasonable rate at low temperatures the milk does not coagulate until warmed (McKenzie, 1971). During the cooling of the milk it appears that both β- and k-caseins are released into solution as their calcium complexes. This increases the negative charge on the micelles. Therefore repulsive forces between both native and rennin treated micelles are higher at lower temperatures and are sufficient to prevent aggregation of rennin treated micelles. An increase in milk temperature reduces the negative charge on the micelles, and hence the repulsive forces between them, and rennin treated micelles will aggregate (Green & Crutchfield, 1971).

A model. The mechanism of milk coagulation can perhaps be best explained by the use of a model of a casein micelle (Garnier & Ribadeau Dumas, 1970) which explains most of the properties of micelles. The model comprises an average repeating unit of one k-, two α_s- and two β- casein subunits assembled in a porous three dimensional polymer in which trimers of k-casein occupy the nodes and copolymers of α_s- and β -caseins the branches of the network. All associations between the subunits are through non-covalent bonds and it is suggested that calcium or calcium phosphate has an affinity for the subunits such that they can arrange them

spatially to promote the early stages of micelle formation (Garnier & Ribadeau Dumas, 1970).

Evidence supporting this model has come from several workers and includes the reports that casein micelles appear to be of uniform composition throughout for both k-casein (Green & Crutchfield, 1969) and the three major caseins (Ashoor et al., 1972). Micelles were found to be porous enough to allow rennin sized molecules to penetrate readily (Ribadeau Dumas & Garnier, 1970) and a micelle framework of α_s-casein in which 30 to 50% of the β-casein has a structural role has been reported (Fox & Guiney, 1973). However other reports suggest that the model does not fit all data. For instance a micelle framework of mainly α_s-casein has been reported (Lin et al., 1972) and the non-availability of the α_s-casein and part of the β-casein for rennin hydrolysis does not support the idea of a completely porous micelle (Fox & Guiney, 1973).

However the micelle model explains and agrees with much of the reported knowledge of milk coagulation. Rennin action transforms all the k-casein to para-k-casein within the micelle by spitting off acidic peptides (Garnier & Ribadeau Dumas, 1970). It has been proposed that the coalescence of the micelles is then due to either a collision of two trimers of para-k-casein on the exterior surfaces of the micelles (Garnier & Ribadeau Dumas, 1970) or that the attractive forces between the micelles are greater than the repulsive forces because of a lower charge on rennin treated micelles than on native micelles (Green & Crutchfield, 1971) or a combination of both (Green, 1972). After coagulation a close packed network of copolymers of the caseins is formed (gelification) either by mechanical disruptions of micelle structure bringing trimers of para-k-casein into contact and possible linkage (Garnier & Ribadeau Dumas, 1970) or electrostatic interactions between micelles cause bonding between the relatively acidic α_s- and β-caseins and the relatively basic para-k-casein

(Green, 1972; Talbot & Waugh, 1970) or a combination of both (Green, 1972). The strength of the network depends upon the number of linkages between subunits which become more tightly packed with each new bond expelling the internal aqueous solution (whey) i.e. syneresis occurs (Garnier & Ribadeau Dumas, 1970). Acid production in cheese curd aids syneresis, probably by a further reduction of replusive forces between subunits. Thus rennin causes the coagulation of the casein in milk and aided by acid production and usually heat, both of which reduce repulsive forces between micelles, force whey from the cheese curd. Hence the basis of cheese manufacture.

Rennet assays

Rennet is manufactured, sold and used on an activity basis and therefore a reliable assay method is most necessary. There have been several assays proposed but most do not relate assay conditions to those used in cheesemaking.

Comparison of coagulation times. The most widely reported assay for milk coagulating enzymes uses a calcium enriched reconstituted skim milk as substrate and is based on the comparison of coagulation times for solutions of unknown with solutions of known enzyme activity (Berridge, 1955; Ernstrom, 1958). A similar assay using k-casein as substrate has been described (Douillard & Ribadeau Dumas, 1970). Both of these methods rely upon operator judgement of the same end-point for several samples coagulating over a period of some minutes. A major disadvantage is the need to maintain a reference rennet of constant activity and the need of a specially adapted water bath. In addition the assay is accurate only if the coagulation times are similar to that of the reference rennet and as mentioned the assay conditions are not the same as found in cheese-

making, for which the rennet is produced. This complicates the comparison of different enzymes unnecessarily.

Recorded end-points. Several methods with recorded end-points which eliminate operator end-point judgement errors have been proposed. However one method requires overnight incubation (Lawrence & Sanderson, 1969) while others require synthetic substrate preparation (Hill, 1969; Raymond et al., 1973; Polzhofer, 1972). These methods are all very time consuming. Another method (Everson & Winder, 1968) measures the increase in the velocity of sound waves passing through a milk-rennet mixture when coagulation occurs but this method requires elaborate equipment. Methods that measure the increase in soluble casein after enzyme action (Kato et al., 1972; Gavin & Nick, 1971; Zittle, 1965) are not as sensitive as a milk coagulation assay (Berridge, 1955; Ernstrom, 1958), and those that follow rennet action by monitoring the turbidity produced in k-casein solutions do not appear to be suitable as an assay for rennet activity (Lawrence & Creamer, 1969). Other methods measure rennet activity from the hydrolysis of the β -chain of insulin (Fish, 1957) and haemoglobin (Herriott, 1955) but these methods rely upon the general proteolytic activity of rennet and not its specificity for the phenylalanine-methionine bond of k-casein.

A proposed assay. It can be concluded that there exists a need for a rapid simple assay which uses conditions similar to those employed in cheesemaking and which has a measured end-point. The basis of the method described in this thesis is that the rennin coagulation time of bovine milk is inversely proportional to the amount of peptides released and to the initial velocity of the release of peptides from casein (Wheelock & Penney, 1972). The concentration of the peptides released is measured by their absorption at 217 nm and

it has been shown that a linear relationship exists between the increase in absorbance at 217 nm and the increase in nitrogen soluble in 0.73-M trichloroacetic acid (Castle & Wheelock, 1972). However trichloroacetic acid absorbs strongly at 217 nm and therefore a direct estimate of peptide concentration can not be made when trichloroacetic acid is used to halt rennin-casein reactions. To overcome this problem perchloric acid has been used as a protein precipitant for enzyme incubation mixtures (Dawson et al., 1969) as it absorbs only slightly at 217 nm. Under New Zealand conditions rennet is used at pH 6.4 to 6.7 and at 30 to 33°C to produce a firm coagulum in 30 to 40 minutes (Gilles, 1972).

In the proposed assay sodium caseinate is used as the substrate. After enzyme action the caseinate is precipitated with perchloric acid and the peptides estimated by measuring the supernatant absorbance at 217 nm.

METHODS

Rennet source

The three rennets used in this study were supplied by the New Zealand Co-op Rennet Co. Ltd., (Eltham, New Zealand). The rennets were extracted from the abomasums of calves, older cattle or lambs and were called calf rennet, adult bovine rennet and lamb rennet respectively. Calf rennet was from the abomasums of animals slaughtered between 4 and 10 days of age whose diet would have been predominantly milk. Adult bovine rennet was from the abomasums of pasture fed cattle slaughtered at an age usually in excess of one year. Lamb rennet was from the abomasums of lambs slaughtered at three to six months of age and their diet would have still included some milk.

In all cases the abomasums were frozen as soon as possible after removal from the animal. Rennet was extracted from the abomasums and prepared at the New Zealand Co-op Rennet Co. Ltd. by their usual methods. The rennets supplied for this study contained 190 g/l of sodium chloride, 7.5 g/l of sodium benzoate and approximately 100 rennin units per ml (RU/ml) of rennet activity. Sodium chloride and sodium benzoate were removed from each rennet by dialysis against water at 4^{o}C. The resultant solutions were freeze dried and the rennet powder stored at 4^{o}C until required. Rennet powders were reconstituted in either sodium chloride or buffer solutions to the required activity level.

Precipitants for casein-rennet reaction mixtures

Preparation of sodium caseinate. Skim milk at room temperature was acidified to pH 4.55 $\pm$ 0.05 in 40 to 50 minutes by adding M-hydrochloric acid beneath the surface of the agitated skim milk (McKenzie, 1971). Casein was separated by centrifuging at 1500 rpm for 10 minutes and

A rennin unit, as used in this discussion, may be defined as that quantity of milk coagulating enzyme activity required to produce a firm coagulum in 100 ml of normal cheese milk at pH 6.55 and 32^{o}C in 30 minutes.

was washed twice with water before acetic acid extraction at pH 4.0 to remove milk proteases (McMeekin, Hipp & Groves, 1959). The casein was washed twice more with water at room temperature and then dissolved by adding M-sodium hydroxide to raise the pH to 6.55. The solution was left overnight at 4°C and the pH readjusted, if necessary, before freeze drying.

Preparation of substrate. Sodium caseinate was dissolved in a buffer (pH 6.55) containing 0.024-M potassium dihydrogen orthophosphate and 0.009-M disodium hydrogen orthophosphate (Long, 1961), to give a final concentration of 60 mg/ml. The substrate was held at 4°C for 4 to 20 hours before use.

Preparation of rennet solutions. Rennets were diluted in the same phosphate buffer used for substrate preparation so that when equal volumes of caseinate and rennet solutions were mixed there was 1.25 rennin units per gram (RU/g) of casein for calf rennet and 2.4 RU/g of casein for adult bovine and lamb rennets. These levels were three times greater than used in cheesemaking.

Effect of precipitants. Equal volumes of each rennet and substrate were mixed and the reaction allowed to proceed at 32°C. After reaction times of 0, 1, 5, 10, 30 and 60 minutes aliquots of each reaction mixture (1 ml) were added to 0.36-M perchloric acid, 0.15-M trichloroacetic acid or 0.88-M trichloroacetic acid (5 ml). Air was removed from the precipitated casein by applying a vacuum for 10 minutes and the mixture then centrifuged at 2600 rpm for 10 minutes. Absorbance of the perchloric acid supernatant was measured at 217 nm while the trichloroacetic acid supernatants were dialysed against water for 14 days at 3°C (Castle & Wheelock, 1972) before absorbance at 217 nm was measured. Absorbance values were corrected for changes in volume during dialysis.

Assay

Caseinate substrate at pH 6.55 was prepared, as described above, and held at 4°C for 4 to 20 hours before use. Rennet solutions of suitable activity were prepared in the pH 6.55 phosphate buffer (described above) from solutions of rennet (New Zealand Co-op Rennet Co. Ltd.), assayed by a milk coagulation method similar to that of Ernstrom (1958) with a substrate pH of 6.35.

Aliquots of substrate (0.5 ml) were pipetted into centrifuge tubes which were placed in a 32°C water bath for 20 minutes. The rennet solutions were equilibrated to 32°C and aliquots (0.5 ml) added to the substrate and mixed. After one minute 0.36-M perchloric acid (5 ml) was added to the reactants and mixed. While the rennet and the substrate were reacting a blank was prepared for each rennet solution by mixing substrate (0.5 ml) with 0.36-M perchloric acid (5 ml) before adding the rennet solution (0.5 ml). The supernatants were prepared as described above and their absorbance read at 217 nm against the corresponding blank. Rennet activity was then read from a standard curve which was prepared for each batch of sodium caseinate.

RESULTS

Precipitants for casein-rennet reaction mixtures

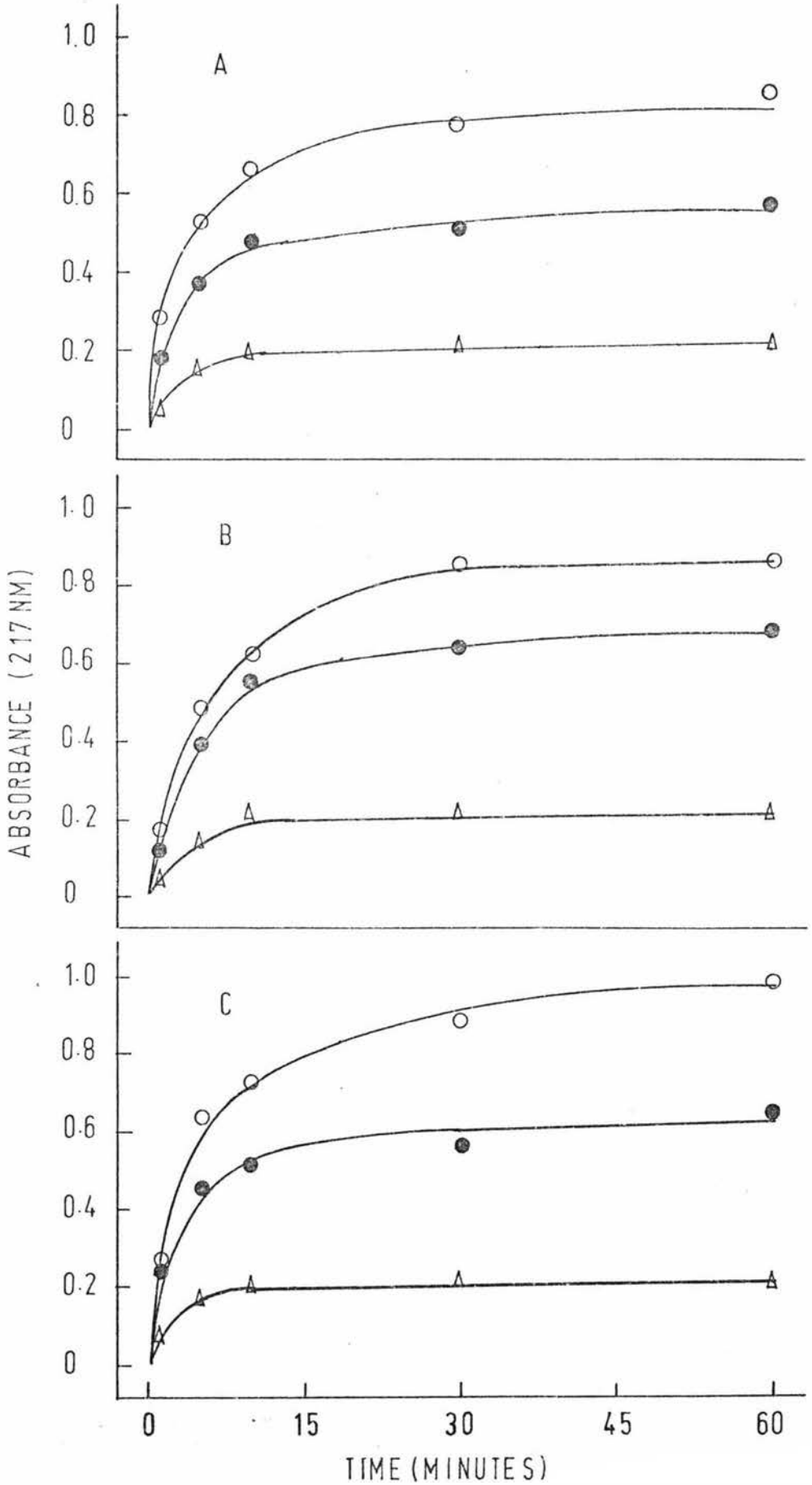
The reaction products from casein hydrolysed by adult bovine, calf and lamb rennets were soluble in the different precipitants in similar quantities (Figure 1). Each rennet released products soluble in 0.73-M trichloroacetic acid in the first 10 minutes of the reaction and products soluble in 0.12-M trichloroacetic acid and 0.30-M perchloric acid were also released rapidly during this period but then more slowly until after 30 minutes there was little change in absorbance at 217 nm.

Each rennet produced more products soluble in 0.30-M perchloric acid than in 0.12-M trichloroacetic acid at all stages of the reactions but the ratio of products soluble in these two precipitants was reasonably constant for each rennet. The average ratio of supernatant absorbance, between 5 and 60 minutes, at 217 nm in 0.30-M perchloric acid to that in 0.12-M trichloroacetic acid were 1.45 (range 1.38 to 1.52) for adult bovine rennet, 1.24 (1.12 to 1.36) for calf rennet and 1.49 (1.39 to 1.58) for lamb rennet. These ratios varied slightly between rennets indicating that the peptides released from casein by each rennet differed. However it appeared that for each rennet all the products soluble in 0.12-M trichloroacetic acid were also soluble in 0.30-M perchloric acid and that most of the products found in both solutions would be from the primary reaction of rennet enzymes on k-casein (Wake, 1959).

Assay

The relationship between absorbance at 217 nm (A^{217}) and rennin units per ml (RU/ml) were compared for each rennet and the data, presented in Figure 2, was obtained

Figure 1. The comparison of 0.30-M perchloric acid (O), 0.12-M (●) and 0.73-M (Δ) trichloroacetic acid as precipitants for casein-rennet reaction mixtures using adult bovine (A), calf (B) and lamb (C) rennets.


A
B
C
1.0
0.8
0.6
0.4
0.2
0
ABSORBANCE (217 NM)
0
15
30
45
60
TIME (MINUTES)

on two days with each point being the average of duplicate determinations. For each rennet there was a linear relationship between 0.01 and 0.09 RU/ml. Below 0.01 RU/ml there was evidence of rennet inhibition, possibly due to protein denaturation at interfaces of dilute protein solutions (Doulliard & Ribadeau Dumas, 1970). The A^{217} value obtained at zero rennet activity could only be due to a more complete caseinate precipitation when perchloric acid was added to 60 mg/ml caseinate in the blank than when perchloric acid was added to 30 mg/ml caseinate in the assay. Above 0.09 RU/ml the curve flattened out as substrate became the limiting factor in the reaction.

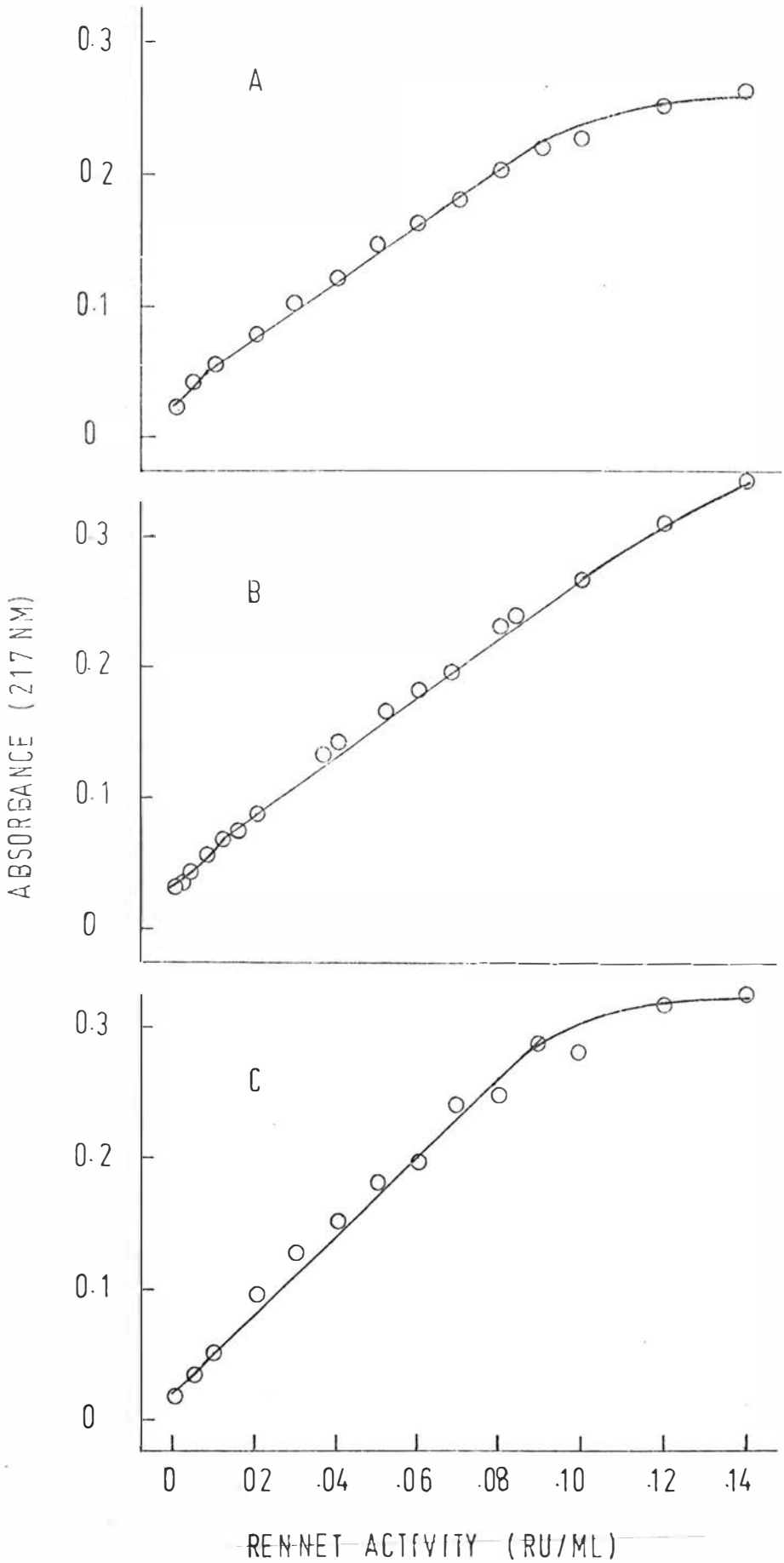
A regression equation and standard deviation for data between 0.01 and 0.09 RU/ml was calculated for each rennet and are presented in Table I.

Table I. A regression equation and standard deviation for the comparison of A^{217} and RU/ml between 0.01 and 0.09 RU/ml for adult bovine, calf and lamb rennets.

Rennet	Regression equation	Standard deviation
Adult bovine	$RU/ml = 0.467\ A^{217} - 0.015$	0.0018
Calf	$RU/ml = 0.416\ A^{217} - 0.016$	0.0016
Lamb	$RU/ml = 0.363\ A^{217} - 0.012$	0.0037

The accuracy of the assays varied with the enzyme activity and was most accurate when the increase in A^{217} was greatest but still on the linear part of the curve. At 0.08 RU/ml accuracy was $\pm$ 2.3% for adult bovine rennet, $\pm$ 1.9% for calf rennet and $\pm$ 4.6% for lamb rennet. (Accuracy was expressed as one standard deviation divided by rennet activity). The high standard deviation for lamb

Figure 2. The comparison of A^{217} and RU/ml for adult bovine (A), calf (B) and lamb (C) rennets.


A
B
C
0.3
0.2
0.1
0
ABSORBANCE (217 NM)
0
.02
.04
.06
.08
.10
.12
.14
RENNET ACTIVITY (RU/ML)

rennet may have been due to the rennet used as it had a high ratio of A^{217} per unit of rennet activity and consequently A^{217} readings were higher, and hence less accurate, for the same rennet activity than the other two rennets.

The slopes of the linear part of the curve of the comparison of A^{217} and RU/ml for each rennet were of the same order. The slope differences may have been due to the different rates of release of peptides at pH 6.55 compared with the milk coagulation activity at pH 6.35 of each rennet. The release of peptides measured the rate of the primary rennet action on caseinate and was not affected by conditions that may have influenced the secondary phase of milk coagulation. It was also possible that the peptide products from the caseinate varied with each rennet and hence their solubility in 0.30-M perchloric acid would also have varied.

The relationship for each rennet between rennet activity and absorbance at 217 nm, as shown in Figure 2, is actually a curve but has been considered to be a straight line, over a limited range of rennet activity, in the above discussion.

DISCUSSION

The comparison of 0.30-M perchloric acid with 0.12-M and 0.73-M trichloroacetic acid as precipitants for casein-rennet reactions has shown that perchloric acid quenches the reactions similarly to 0.12-M trichloroacetic acid. Thus perchloric acid can be used as a precipitant to measure the rate of hydrolysis of the labile phenylalanine-methionine bond of k-casein. The ratio of reaction products soluble in 0.30-M perchloric acid to those soluble in 0.12-M trichloroacetic acid is greater than 1.0 and therefore a larger absorbance reading is obtained when perchloric acid is used as the precipitant for casein-rennet reactions. The larger absorbance value gives a steeper curve against time and hence a more accurate estimate of the stage of the reaction. It appears that perchloric acid is a most suitable precipitant for casein-rennet reactions and allowed the development of the assay method described above.

The assay estimated rennet activity by measuring the quantity of peptides released in one minute from sodium caseinate under controlled conditions and has several advantages over other methods of assaying rennet activity. In particular the method utilises a measured end-point which is more reliable than the observed end-point of several other methods (Berridge, 1955; Ernstrom, 1958; Doulliard & Ribadeau Dumas, 1970) and can measure rennet activity one fortieth that normally measured by a milk coagulation assay at least as accurately. No reference rennet is required as one batch of substrate is calibrated against another batch and as rennet activity is measured directly it is not complicated by the secondary phase of milk coagulation. Conditions for the assay are similar to those found in commercial use of rennet and this is of value when comparing different enzymes for cheesemaking properties.

Measurement of rennet activity is obtained over a range of activities which are wide enough to allow a reliable estimate of activity from serial dilutions of a rennet of unknown activity. The assay can be performed in large numbers quite rapidly and requires no special equipment.

All rennet assays reported in this thesis were conducted with the above method unless otherwise mentioned.

CHAPTER III

ENZYME PURIFICATION

REVIEW OF LITERATURE

Enzyme purification

Enzyme sources. Most proteolytic enzymes can coagulate milk under suitable conditions and therefore milk coagulating enzymes have been obtained from virtually every class of living organism (Ernstrom, 1965). As the traditional milk coagulating enzyme, rennin, is extracted from the abomasums of young milk-fed calves (Placek, Bavisotto & Jadd, 1960; Clarke, 1969; Calvy, 1972) the stomachs of other animals have been widely investigated as a source of milk coagulating enzymes. The commercially more feasible sources investigated include cattle (Northrup, 1933; Chow & Kassell, 1968; Fox, 1969, b; Green, 1972), kids (Yamamoto & Takahashi, 1953), chickens (Bohak, 1969; Green, 1972), swine (Rajogaplan, Moore & Stein, 1966) and lambs (Alais, 1963; Oruntaeva & Seitov, 1971; O'Leary & Fox, 1973). The enzymes extracted from calves, kids and young lambs are predominantly rennins while the enzymes extracted from cattle, chickens, swine and older lambs are predominantly pepsins or a more even mixture of both rennins and pepsins depending upon the age of the animal at slaughter and the nature of its diet prior to slaughter (Henschel, Hill & Porter, 1961).

Extraction procedures. Extraction of both rennin and pepsin is from either dried or frozen stomach tissue with aqueous solutions, of a variety of salts, at pH values above 4.7 (Yamamoto & Takahaski, 1953; Placek, Bavisotto & Jadd, 1960; Alais, 1963; Clarke, 1969; Calvy, 1972) or with weak solutions of acids at pH values down to 2.7 (Rajogaplan, Moore & Stein, 1966; Garnot et al., 1974). Rennins and pepsins are both secreted as inactive zymogens

and must be activated by the hydrolysis of a peptide from the precursor molecule to obtain the maximum milk coagulating activity. Therefore the enzymes extracted at high pH in salt solutions are adjusted to pH 4.7 to 5.0 for 1 to 5 days until the milk coagulating activity of the extract reaches a maximum (Placek, Bavisotto & Jadd, 1960; Clarke, 1969). However enzymes extracted at low pH are fully activated during extraction. Milk coagulating enzymes prepared by either of these procedures are marketed usually as a crude aqueous extract or a dried powder of rennins and pepsins stabilised by a salt, usually sodium chloride, and a preservative such as sodium benzoate (Clarke, 1969), propylene glycol, sodium propionate or a mixture of these (Placek, Bavisotto & Jadd, 1960).

Precipitation techniques. The extraction of milk coagulating enzymes from animal stomachs has always been complicated by the simultaneous extraction of muco-proteins from the mucous lining of the stomachs (Placek, Bavisotto & Jadd, 1960; Clarke, 1968) causing an increase in the viscosity of aqueous solutions which leads to a lower recovery of enzymes (Clarke, 1968) and, as they precipitate slowly from solution in finished rennet, a turbid appearance to the product. Their removal from rennet solutions has been achieved by the addition of potassium alum followed by phosphate or hydroxide to form a complex, containing the mucoproteins, which precipitates out of solution (Van der Berg & Van der Scheer, 1937; Berridge, 1945; Ernstrom, 1958). A similar method for treating undried calf abomasums prior to rennet extraction produces a less viscous extract but does not overcome all problems related to efficient mucoprotein removal (Clarke, 1968). The crude milk coagulating enzymes have been purified by precipitation, often after the removal of mucoproteins by alum treatment, with various compounds including magnesium sulphate (Northrup, 1933), acetone (Northrup, 1933; Bohak, 1969), ammonium

sulphate (Chow & Kassell, 1968), alcohol (Rajogaplan, Moore & Stein, 1966), and sodium chloride (Hankinson, 1943; Ernstrom, 1958; Bundy et al., 1964; Castle & Wheelock, 1971).

Column chromatography. More recently column chromatography has been used to purify milk coagulating enzymes. The initial purification is often with ion exchange chromatography, usually on a diethylaminoethyl cellulose resin which retains the enzymes. The column can be washed, if necessary, before elution usually with a sodium chloride gradient in the equilibration buffer (Bundy et al., 1964; Chow & Kassell, 1968; Bohak, 1969; Asato, 1972). By using ion exchange chromatography a concentration of enzyme activity is often possible and further concentration by ultrafiltration has also been used (Chow & Kassell, 1968) prior to gel chromatography usually in dilute buffers between pH 5.6 and 7.0 depending upon whether the zymogens or active enzymes were being purified (Bundy et al., 1964; Chow & Kassell, 1968; Bohak, 1969; Castle & Wheelock, 1971). Gel chromatography after ion exchange chromatography allows the separation, by size, of molecules of similar ion exchange elution characteristics. Purification by these two methods of column chromatography produces electrophoretically pure milk coagulating enzymes (Asato, 1972).

METHODS

Ion exchange chromatography

Cellulose ion exchange resins have been used to purify several milk coagulating enzymes (Bundy et al., 1964; Chow & Kassell, 1968; Bohak, 1969; Asato, 1972) and the method described below is based in part on work reported in these papers.

Whatman DE 52 cellulose (W. & R. Balston, Maidstone, England) was prepared according to the manufacturers instructions and packed into a 30 x 1.5 cm column. The cellulose was equilibrated with a starting buffer consisting of citric acid (0.005-M) adjusted to pH 5.6 with aqueous ammonia and containing 5 ml/l of chloroform as a preservative. Rennet enzyme powders (20 mg/ml) were dissolved in the same buffer and centrifuged to remove any suspended material from the solution. Between 20 and 50 ml of rennet solution was applied to the column at any one time and the elution of protein material from the column was achieved by a linear gradient of sodium chloride in the starting buffer. The gradient was made from 350 ml of starting buffer and 350 ml of limit buffer made 0.7-M with sodium chloride. The absorbance of eluent fractions (12 ml) was measured at 280 nm against the starting buffer and the sodium chloride gradient was determined by measuring the conductivity of a one to twenty-five dilution, in water, of several fractions from each run.

Gel chromatography

Gel chromatography was used to purify and analyse rennet enzymes by the method, described below, based on Fischer (1971).

Sephadex G-100 gel (Pharmicia Fine Chemicals, Uppsala, Sweden) was prepared according to the manufacturers instructions and packed into a 90 x 1.5 cm column. The gel was equilibrated with a buffer consisting of disodium hydrogen orthophosphate (0.004-M) and potassium dihydrogen orthophosphate (0.063-M) at pH 5.6 containing 5 ml/l of chloroform as a preservative. Enzyme solutions, after ion exchange chromatography, were adjusted to the equilibration buffer concentration by the addition of concentrated buffer. From 1 to 3 ml of enzyme solution, containing up to 20 mg of protein, was applied to the top of the column at one time via a three-way valve and elution of the protein from the column was by gravity induced buffer flow at 20 to 30 ml/h. The absorbance of eluent fractions (3.5 ml) was measured at 280 nm against the buffer.

Enzyme purification

Rennet powders were dissolved in a citric acid (0.005-M) buffer, centrifuged to remove any suspended material, and applied to the ion exchange column. Protein peaks, determined from absorbance measurements of eluent fractions at 280 nm, were assayed for milk coagulating activity. Active fractions were pooled, concentrated by ultrafiltration and applied to the gel chromatography column. Active fractions of protein peaks were again pooled and applied to the ion exchange column for the second time.

Once again active fractions containing proteins were pooled, concentrated by ultrafiltration and applied to the gel chromatography column for the second time. Active fractions were pooled and concentrated by ultrafiltration for further study.

Electrophoresis of purified enzymes

Electrophoresis was used to determine the purity and homogeneity of each enzyme isolated from adult bovine and lamb rennets. The method of Davis (1964) as modified by Creamer (1970) for alkaline (pH 8.6) polyacrylamide gel electrophoresis on rod gels was used. A solution of each purified enzyme was adjusted to contain approximately 3 mg/ml, made 9-M with urea and 0.03-M with 2-mercaptoethanol. Aliquots (100 ul) of each enzyme solution were applied to the gels and electrophoresed for 60 to 90 minutes. Gels were stained overnight with amido black and destained electrophoretically before being photographed.

Commercial rennet purification

Equipment and materials. A cylindrical fiber-glass column 0.91 m dia eter and 2.44 m high was adapted as an ion exchange column for commercial rennet purification. Woven stainless steel clothes were supported on frames at the top and bottom of the column to retain the resin and both ends were closed with lids bolted to the column. Liquid connections were placed in both lids to enable up or down flow which was provided by a centrifugal pump. The column was charged with 100 kg (dry weight) of Grantex A 1 resin (Tasman Vaccine Laboratory Ltd., Upper Hutt, New Zealand), which has diethylaminoethyl (DEAE) groups as the active sites on a cross-linked hydrophilic matrix derived from regenerated cellulose. The resin was prepared as suggested by the manufacturer before equilibration with a starting buffer containing citric acid (0.005-M), sodium chloride (0.1-M) and adjusted to pH 5.5 with aqueous ammonia. The column was operated at 7^{o}C to control microbial growth.

Operating conditions. An aqueous solution of calf rennet was prepared at the New Zealand Co-op Rennet Co. Ltd. by their usual methods and adjusted to the starting buffer concentration by additions of dry chemicals and ammonia or hydrochloric acid solutions. This rennet was applied to the column at 680 to 1000 l/h and a batch contained 4,500 to 9,000 l. A minimum contact time between rennet and resin of 20 minutes was required, corresponding to a flow rate of 1440 l/h, but this flow could not be achieved within the maximum workable pressure drop across the resin bed. After all the rennet had been applied to the column it was followed by approximately 1500 l of starting buffer to wash the resin. Proteins were eluted from the column with starting buffer made 1.0-M with sodium chloride and elution was monitored by measuring eluent absorbance at 280 nm against the starting buffer.

Regeneration. The resin was regenerated by back-washing with 900 l of 0.5-M sodium hydroxide, during vigorous agitation, followed by water until the eluent pH was below 9.0. The resin was repacked and equilibrated with down flow of starting buffer.

RESULTS

Rennet enzymes purification

Ion exchange chromatography. Typical chromatograms of adult bovine and lamb rennets shown in Figure 3 have similar patterns. At the void volume of the column the first peak is large, contains no milk coagulating activity and appeared to consist of mucoproteins. As the sodium chloride concentration in the eluent is increased minor protein peaks appear but none of these contain enzyme activity. The adult bovine rennet chromatograph (Figure 3 A) shows that a large peak is eluted at a conductivity of 180 millisiemens (mS) and this peak contains all the recovered enzyme activity. The lamb rennet chromatograph (Figure 3 B) differs from the adult bovine in that two main peaks are eluted at conductivities of 100 and 195 mS respectively. Both peaks show milk coagulating activity and were called lamb 1 and lamb 2 respectively in order of elution. The lamb 1 peak contains 22% of the recovered enzyme activity while the other 78% is in the lamb 2 peak.

For both adult bovine and lamb rennet the fractions indicated by the bars in Figure 3 were pooled and concentrated by ultrafiltration for further purification. The recovery of enzyme activity for both rennets was never less than 95% of the activity applied to the column and solutions of both rennets purified by ion exchange chromatography were quite clear as compared with the turbid appearance of both the initial and reconstituted rennets.

Gel chromatography. The absorbance of eluent fractions was measured at 280 nm against the buffer and protein peaks assayed for milk coagulating enzyme activity. Chromatographs of the adult bovine, lamb 1 and lamb 2

Figure 3. Elution patterns of adult bovine (A) and lamb (B) rennets from ion exchange chromatography on DEAE cellulose.

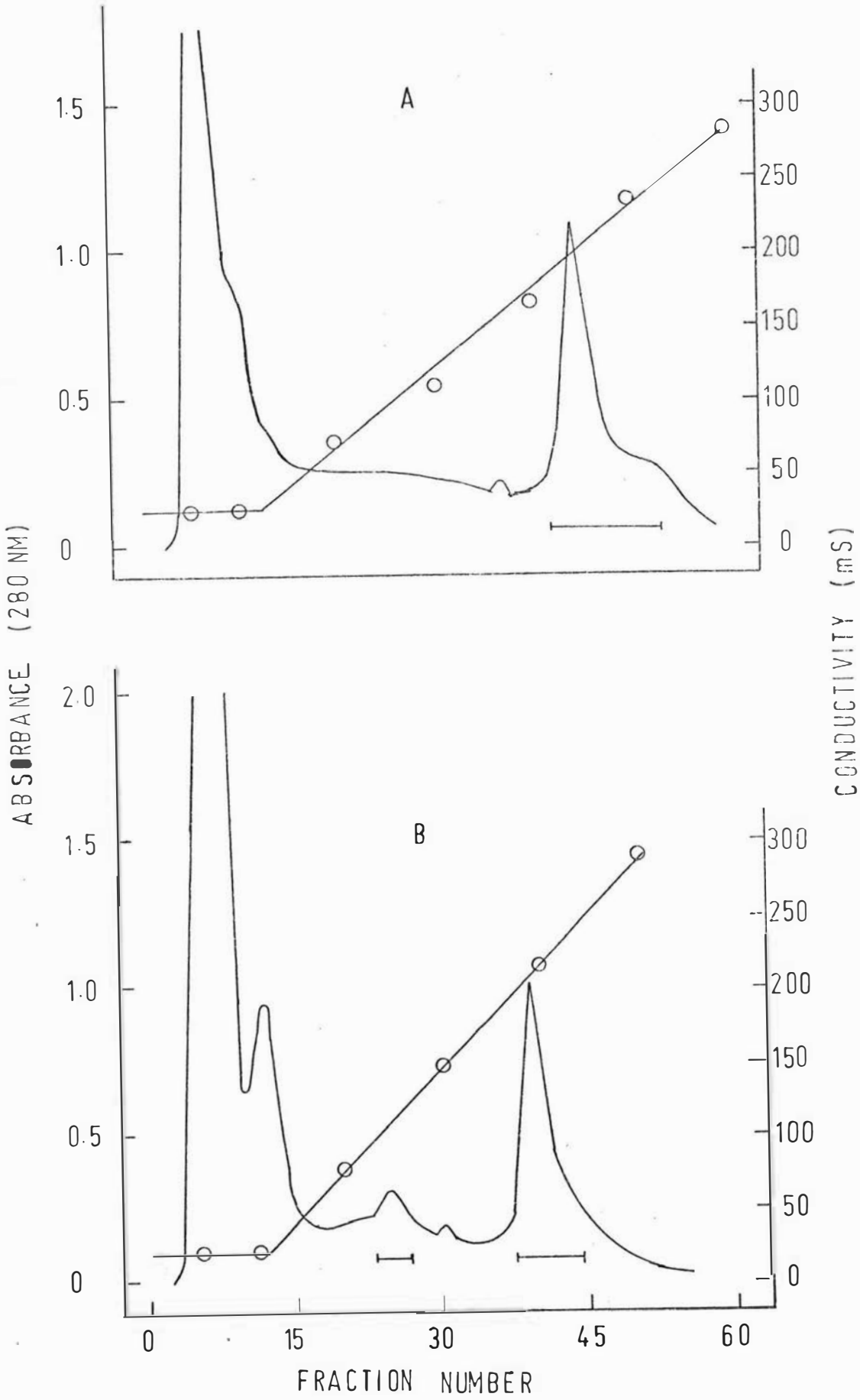
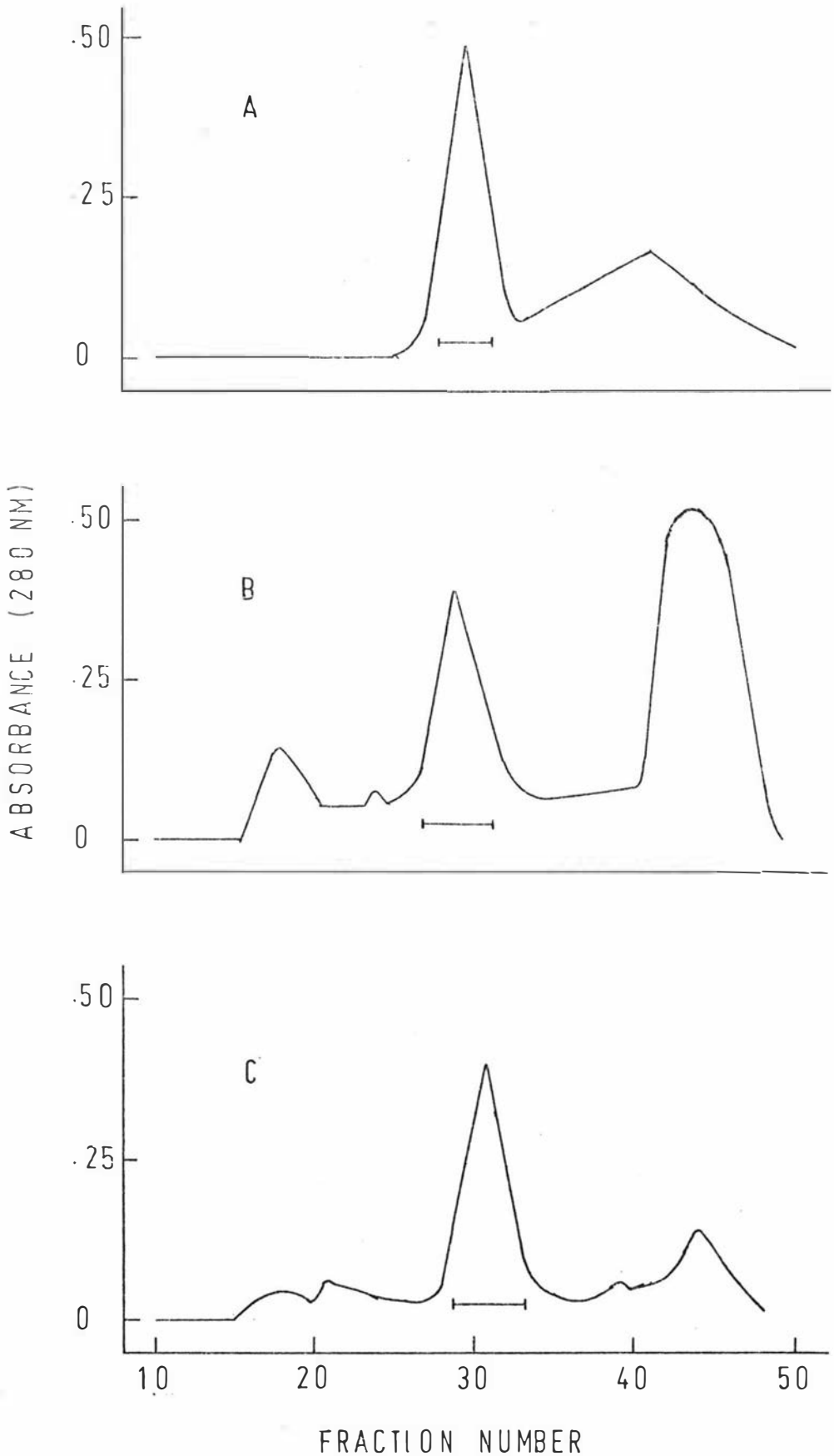

A
B
1.5
1.0
0.5
0
2.0
300
250
200
150
100
50
0
15
30
45
60
ABSORBANCE (280 NM)
CONDUCTIVITY (mS)
FRACTION NUMBER

Figure 4. Elution patterns of adult bovine (A), lamb 1 (B) and lamb 2 (C) enzymes from gel chromatography on Sepha dex G-100 gel.


.50
.25
0
A
B
C
ABSORBANCE (280 NM)
10
20
30
40
50
FRACTION NUMBER

enzymes are shown in Figure 4 and each has a distinct pattern. The adult bovine chromatograph (Figure 4 A) shows two peaks, the first contains all the milk coagulating activity while the second peak is of lower molecular weight inactive material. The lamb 1 chromatograph (Figure 4 B) shows a small peak at the elution volume of the column and is probably mucoprotein material carried over from the ion exchange column and then there is a very small peak of inactive material at fraction 24. A large peak at fraction 29 contains all the milk coagulating enzyme activity while the peak at fraction 44 contains inactive lower molecular weight material. The lamb 2 chromatograph (Figure 4 C) shows a small peak at the elution volume of the column followed by two more small peaks before a large peak at fraction 31 that contains all the milk coagulating enzyme activity. Two other small peaks of inactive material were eluted at fractions 39 and 44. A comparison of the three chromatographs shows that most of the eluted material for the adult bovine and lamb 2 enzymes are active while the lamb 1 enzyme is much less pure at this stage. For each enzyme the fractions indicated by the bars in Figure 4 were pooled and retained for further purification.

Final purification. A second ion exchange chromatography step in the purification of each enzyme followed by concentration of pooled active fractions with ultrafiltration and a second gel chromatography step gave enzyme solutions which produced only one peak by each of these techniques. Each enzyme at this stage was considered to be pure and was stored at $4^{o}C$ in the presence of 5 ml/l of chloroform, as a preservative, for further study.

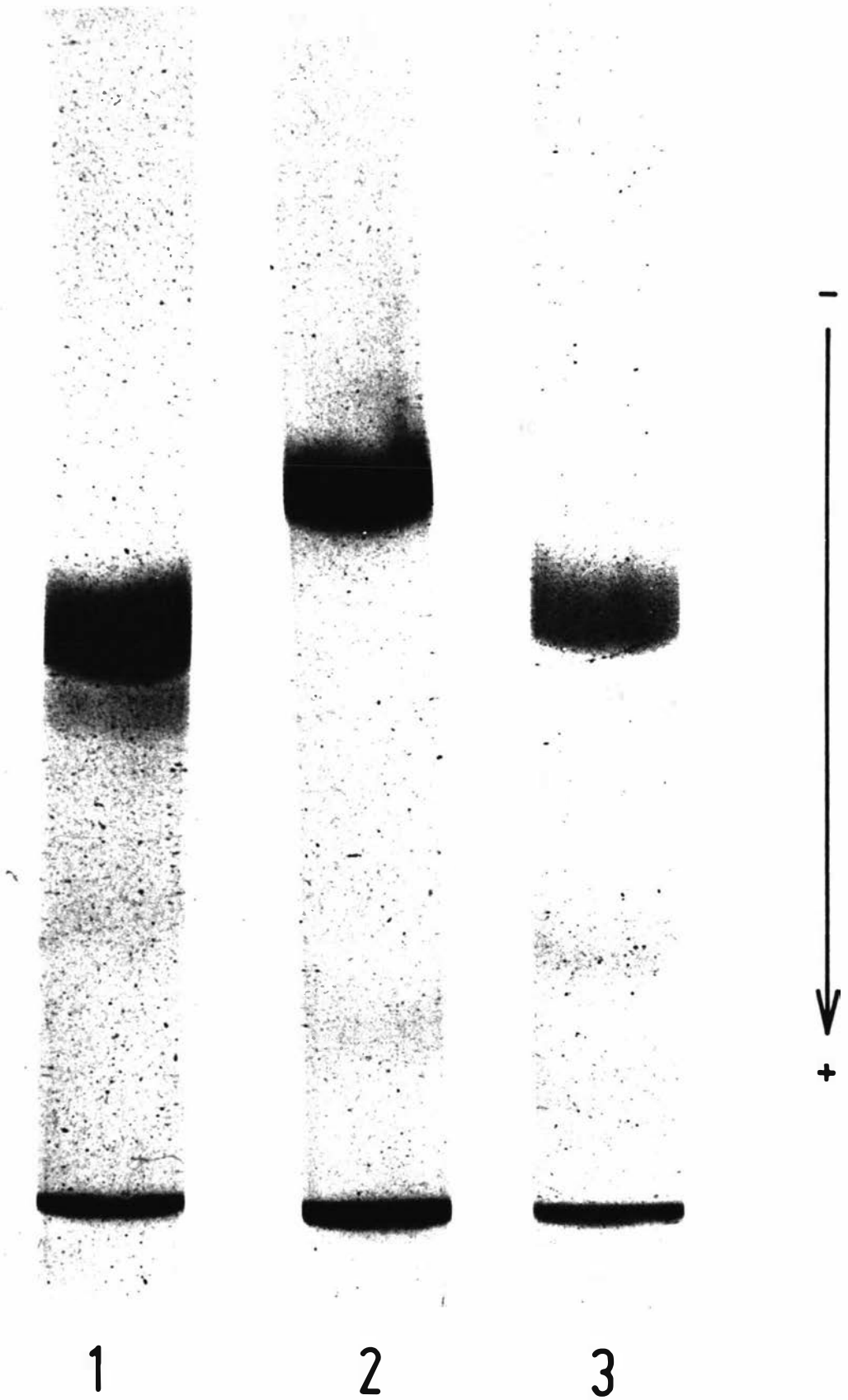
Enzyme purity and homogeneity

Each of the three enzymes isolated from adult bovine or lamb rennets was treated with 2-mercaptoethanol before electrophoresis on alkaline polyacrylamide gels containing urea. A photograph of the gels, presented in Figure 5, shows adult bovine, lamb 1 and lamb 2 enzymes all possess a negative charge at pH 8.6 and that lamb 1 and lamb 2 enzymes each produced a single band demonstrating purity and homogeneity. The adult bovine enzyme produced two bands (one very minor) adjacent to each other, demonstrating either the enzyme was not pure or it was heterogeneous. Heterogeneity has been reported for bovine pepsins (Lang & Kassell, 1971) which differed only in the number of phosphate groups on the same protein molecule. Work reported later in this thesis confirms that the adult bovine enzyme is a pepsin. It was therefore considered that the second and more mobile band of the adult bovine enzyme solution was a similar enzyme with a greater number of phosphate groups rather than an impurity.

Commercial rennet purification

Application of rennet to the column. Calf rennet was prepared for chromatography and at this stage a typical batch contained 4676 l of rennet solution at 15.1 rennin units per ml (RU/ml). The rennet was applied to the ion exchange column initially at 1000 l/h but this was slowed gradually to 700 l/h so as not to exceed a pressure drop of 2.7 m of water across the resin bed. The rennet solution was displaced from the column by washing with 1590 l of starting buffer and all liquid that had passed out of the column at this stage was bulked and the rennet activity found to be less than 0.09 RU/ml. This liquid had the turbid appearance of the original rennet solution and had a similar absorbance at 280 nm, after correction for the dilution by washing, indicating

Figure 5. Electrophoretic patterns of adult bovine (1), lamb 1 (2) and lamb 2 (3) purified enzyme solutions.


-
+
1
2
3

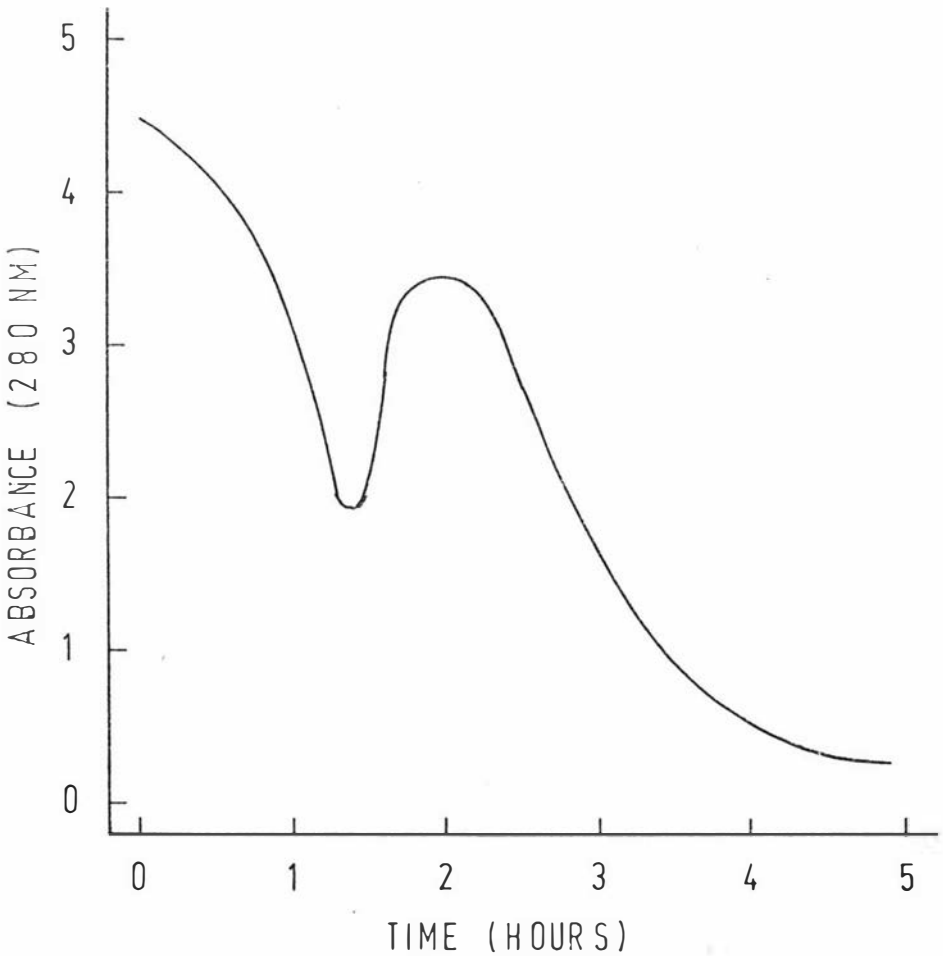
that mucoproteins had passed through the column unobstructed. The absence of rennet activity demonstrated that all the rennet was absorbed onto the ion exchange resin.

Rennet elution. Rennet was eluted from the column with 3600 l of starting buffer made 1-M with sodium chloride. The elution pattern was monitored from the time the elution buffer was applied to the column by measuring the eluent absorbance at 280 nm and the flow rate averaged 820 l/h during elution.

The pattern of elution of rennet from the ion exchange column (Figure 6) indicates that for the first 1.4 hours the starting buffer, containing some mucoproteins, was displaced from the column by the elution buffer. At 1.5 hours the absorbance at 280 nm began to increase indicating that rennet was present in the eluent. The eluent was collected for 4 hours at which time the eluent activity had dropped to less than 1.0 RU/ml. In all 3200 l of eluent were collected which contained 22.0 RU/ml giving an increase in concentration of 1.45 times the original activity. The eluent was further concentrated immediately by ultrafiltration and stabilised by adjustment to 190 g/l of sodium chloride and 7.5 g/l of sodium benzoate. At this stage there were 231 l of rennet at 298 RU/ml which was 97.5% of the rennet activity that was applied to the ion exchange column.

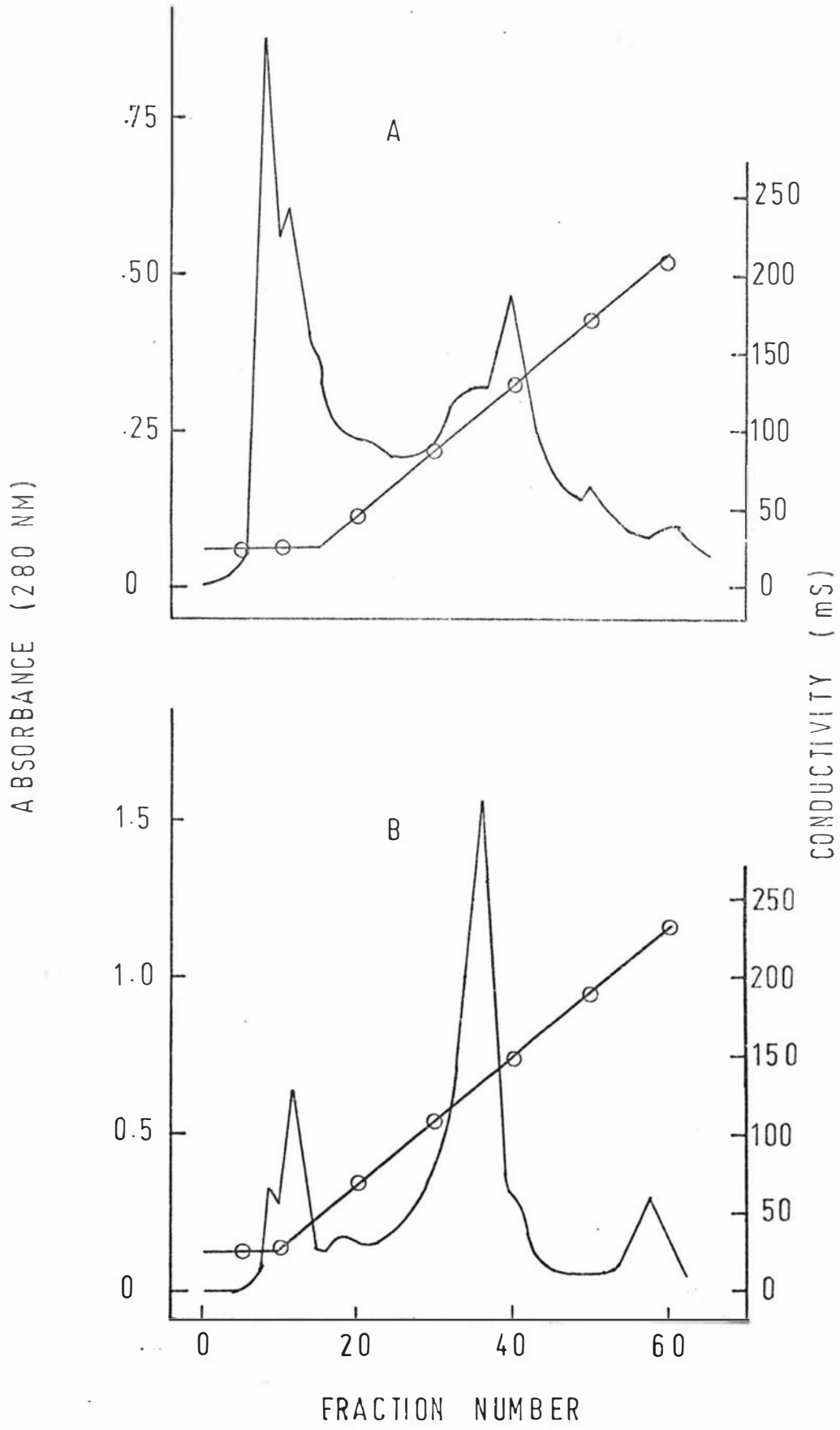
Purified rennet. The rennet obtained after purification by ion exchange chromatography was sparkling clear and has remained so for 12 months since preparation. The degree of purification obtained by commercial ion exchange chromatography was demonstrated, as shown in Figure 7, by the laboratory ion exchange chromatography on Whatman DE 52 cellulose of traditional (Figure 7 A) and purified (Figure 7 B) rennets. The milk coagulating activity was eluted in both cases at conductivities of 115

Figure 6. The elution pattern of calf rennet from a commercial DEAE ion exchange column.


ABSORBANCE (280 NM)
0
1
2
3
4
5
0
1
2
3
4
5
TIME (HOURS)

and 215 mS and were rennin and pepsin respectively (O'Leary & Fox, 1974). The main difference between the two chromatographs was the size of the first peak which in both cases was eluted at the void volume of the column and contained the mucoproteins. In the traditional rennet this first peak was much larger than the rennin peak but in the purified rennet the rennin peak was several times larger than the first peak. Thus relative to the size of the rennin peak the purified rennet contained much less other material than the traditional rennet. The rennet purified by ion exchange chromatography has been at least as stable as the traditional product in that rennet activity loss has been less than 1% per month for 12 months at 4°C.

Figure 7. Elution patterns of traditional (A) and purified (B) calf rennets from ion exchange chromatography on DEAE cellulose.


A
B
.75
.50
.25
0
1.5
1.0
0.5
0
250
200
150
100
50
0
0
20
40
60
ABSORBANCE (280 NM)
CONDUCTIVITY (mS)
FRACTION NUMBER

DISCUSSION

The separation of rennet enzymes from mucoproteins by cellulose ion exchange chromatography opened the way to further purification of the enzymes. At pH 5.6 it appears that the mucoproteins were uncharged as they are not retained on either anionic or cationic cellulose ion exchange resins. On cationic Whatman DE 52 cellulose the mucoproteins passed through the column uninhibited while the milk coagulating enzymes and some other inactive protein material are retained. A linear sodium chloride gradient applied to the column elutes the enzymes distinctly and in good yield.

One milk coagulating enzyme was isolated from adult rennet and two from lamb rennet. The three enzymes were purified to the stage where they were considered to be pure and the lamb enzymes homogeneous but the adult bovine enzyme heterogeneous. In both crude rennets the active enzymes contribute only a small part of the total solution absorbance at 280 nm, the major contributor being the mucoproteins.

An extract of rennet from calf abomasums always contains mucoproteins which increase the viscosity of the rennet extract and give a turbid appearance to the product. Traditional methods for the removal of mucoproteins from solutions are inefficient and usually result in activity losses of 10% or more. The dramatic improvement in the appearance of adult bovine and lamb rennets after an initial ion exchange step indicated that a similar improvement might be possible for calf rennet. Laboratory trials proved successful and the availability of "Grantex" resins at a competitive price opened the way to test the feasibility of a commercial scheme. Pilot plant and then commercial scale production were commissioned and the latter is reported above.

The method for the purification of calf rennet was developed utilising the lack of charge on mucoproteins at pH 5.5. Mucoproteins have always been the major contaminant of rennet solutions and no efficient method for their removal has previously been reported. The main problems encountered in the development of this technique were the maintenance of good flow rates through the resin bed with a low pressure drop across the bed and the efficient cleaning of the resin between batches of rennet. Both problems were closely related and were eventually solved by providing a mechanical stirrer in the column to ensure vigorous agitation during the backwashing stage of the regeneration cycle. This ensured that all resin beads were separated and hence the total surface area of the resin was available for the removal of any foreign material and subsequent regeneration with hydroxide and buffer ions. Since the data reported in this thesis was obtained and the above cleaning procedure developed it has been possible to operate the ion exchange column at the maximum flow rate (1440 l/h) with a pressure drop of approximately one meter of water across the resin bed. In all other aspects the operation is still the same as reported.

It was considered that the development of a method for the removal of most of the mucoproteins from rennet solutions while still retaining almost all the rennet activity could be of commercial significance to the animal rennet industry. The wide scale use of milk coagulating enzymes from microbial sources has introduced to the cheesemaker rennet products that are more consistent in appearance than those usually obtained from animal sources. The method described here will allow the production of animal rennet having an appearance at least as good and as consistent as microbial rennet products.

CHAPTER IV

ENZYME PROPERTIES

REVIEW OF LITERATURE

Enzyme properties

Enzyme sources. The traditional rennet used in the manufacture of cheese is calf rennet and any proposed alternative rennet has invariably been compared with calf rennet for various enzymatic properties and the quality of cheese manufactured using it. Adult bovine rennet (also called bovine pepsin) has been used to manufacture cheese (Fox & Walley, 1971, a; Green, 1972) and the properties of this rennet have been investigated by several workers. Rennet from the abomasum of lambs slaughtered at one to two days of age has also been used to manufacture cheese (Alais, Buthiel & Bosc, 1962; Oruntaeva & Seitov, 1971) but there have been no reports of an extract from older lambs being used for cheese manufacture. However some properties of such an extract from the abomasums of lambs slaughtered at six to twelve months of age (called "ovine pepsin") were reported (O'Leary & Fox, 1973) after work for this thesis had commenced.

Cheesemaking properties. Rennet enzyme extracts from calves, cattle, young lambs and older lambs have been reported and it has been shown that these four rennets have many similarities in cheesemaking properties. For example bovine pepsin and calf rennet are both less proteolytic than ovine pepsin using a caseinate substrate but all three enzymes produced similar breakdown products from α_s- and β- caseins (O'Leary & Fox, 1973). The proteolytic activity of lamb rennet is similar at pH values of 6.45 and 6.8 (Alais, Buthiel & Bosc, 1962) while the ratio of proteolytic activity to milk

coagulating activity is similar for bovine pepsin and calf rennet (Fox, 1969, b; Green, 1972). The effect of temperature on milk coagulating activity (Fox, 1969,b), the chemical reaction causing milk coagulation (Green, 1972) and the rate of destruction of milk coagulating activity at pH 9.0 (Richardson & Chandhari, 1970) are all reported to be similar for calf rennet and bovine pepsin. Cheeses made with bovine pepsin and calf rennet have similar electrophoretic patterns (Fox, 1969, b; Fox & Walley, 1971, a; O'Leary & Fox, 1973) and contain similar quantities of non-protein nitrogen (Fox & Walley, 1971,a). The major difference between calf rennet and any of the other rennet sources in this discussion is the greater pH dependence of lamb rennet (Alais, Buthiel & Bosc, 1962) and ovine pepsin (O'Leary & Fox, 1973) on the coagulation of milk. In all other respects reported it appears that the three alternative rennet extracts are capable of making satisfactory cheese.

Biochemical properties. There have been few reports of the biochemical properties of adult bovine and lamb rennets. Meitner & Kassell, (1971) isolated several bovine pepsinogens which after acid activation produce discrete pepsins that differ only in their organic phosphate content. All of the pepsins have similar proteolytic activity (Lang & Kassell, 1971) and the pepsins with a high phosphate content are converted by an acid phosphatase into pepsins of a low phosphate content (Meitner & Kassell, 1971). Rennin from the abomasum of lambs slaughtered at one to two days of age has been isolated and reported to contain at least three components (Oruntaeva & Seitov, 1971) which differ in amino acid composition and milk coagulating activity. The molecular weight of bovine pepsinogen was estimated to be 38,900 by amino acid analysis and 37,500 by ultracentrifugation (Chow & Kassell, 1968), that of porcine pepsin 34,520 by amino acid analysis (Moravek &

Kostka, 1974), and that of calf rennin 31,000 (Ilie et al., 1966). The amino acid analysis of porcine pepsin (Moravek & Kostka, 1974), bovine pepsinogen (Chow & Kassell, 1968), lamb rennins A & B (Oruntaeva & Seitov, 1971) and calf prorennin (Ilie et al., 1966) are presented in Table II and show that each of these enzymes contains large numbers of acidic residues and relatively small numbers of basic amino acid residues. Hence their general classification as acidic proteases.

Table II. The amino acid composition of bovine pepsinogen, lamb rennins A & B and calf prorennin.

Amino acid	Bovine pepsinogen (residues)	Lamb rennins A (%)	Lamb rennins B (%)	Calf prorennin (residues)	Porcine pepsin (residues)
Aspartic acid	40	11.29	10.99	33	42
Threonine	27	5.13	4.32	21	26
Serine	50	11.66	8.85	31	44
Glutamic acid	32	11.66	12.74	36	26
Proline	15	5.83	5.07	14	15
Glycine	35	5.95	5.96	29	35
Alanine	16	3.77	3.92	15	16
Half-cystine	6	-	-	-	6
Valine	25	5.51	6.42	23	22
Methionine	4	Trace	Trace	7	4
Isoleucine	32	5.62	6.02	19	25
Leucine	25	9.20	9.29	26	26
Tyrosine	18	7.27	8.62	18	16
Phenylalanine	15	6.53	7.67	14	14
Histidine	2	1.15	1.28	5	1
Lysine	8	4.64	4.28	13	1
Arginine	6	3.28	3.08	5	2
Tryptophan	6	-	-	-	5
Total	362			309	326

Apart from the amino acid compositions and the heterogeneity reported for both adult bovine pepsin and lamb rennin there is no data available to compare other biochemical properties of these enzymes.

Hydrolysis of casein fractions

The caseins, which are the proteins precipitated from milk at about pH 4.6, may be separated into three main fractions, called α_s-, β- and k-caseins, as well as several minor fractions. Each of the three main casein fractions has been found to be heterogeneous and genetic variations of each specific casein have been isolated making a large number of discrete proteins in what is commonly referred to as casein. In this discussion, unless otherwise mentioned, casein fractions refer to the heterogeneous groups of α_s-, β- and k-caseins.

Separation of casein fractions. The separation of the three main casein fractions from whole acid casein is accomplished by ion exchange chromatography on DEAE cellulose equilibrated with a buffer containing urea and 2-mercaptoethanol to dissociate the casein molecules (Thompson, 1966). The casein fractions are eluted from the column with a linear 0.0 to 0.3 M-sodium chloride gradient, in the equilibrating buffer. The order of elution is minor caseins, k-casein, β-casein and α_s-casein. This method (Thompson, 1966) gives good separations in good yield of α_s- and β-caseins but k-casein is more readily obtained from a gel chromatography method of Yaguchi, Davies & Kim, (1968). They used Sephadex G-150 gel equilibrated with a buffer containing urea but no 2-mercaptoethanol so that the k-casein was not dissociated and was eluted at the void volume of the column. The k-casein was distinctly separated from the second large peak of α_s- and β-caseins in good yield and purity (Yaguchi, Davies & Kim, 1968).

Factors affecting casein hydrolysis. The susceptibility of caseins to hydrolysis, by proteolytic enzymes such as rennin, is very much influenced by the state of aggregation of the caseins. In milk, where the caseins are largely in the micellar form, α_s- and β-caseins are almost inaccessible to proteolysis even though it has been shown that rennin is probably able to penetrate to the interior of the micelles (Ribadeau Dumas & Garnier, 1970). However the removal of colloidal phosphate from milk, by pH adjustment followed by dialysis against some of the original milk, disrupts the micelles and renders the components accessible to proteolysis (Fox, 1970). In this form the relative suceptibilities of α_s- and β-caseins to rennin proteolysis are affected by temperature with β-casein being more suceptible at low temperatures and α_s-casein more suceptible at higher temperatures (Fox, 1969,a). The pH of the system also affects the proteolysis products from rennin-casein reactions which are considered to be better evaluated by changes in electrophoretic patterns than changes in non-protein nitrogen (Fox, 1969, a). The purity of the rennin used in this type of reaction does not appear to affect the products formed (Fish, 1957; Fox, 1969, a) suggesting that pure enzymes are not necessary to evaluate their proteolytic activity on caseins.

Hydrolysis of casein fractions. The hydrolysis of individual casein fractions in solution has been reported for α_s-casein (Ledford, Chen & Nath, 1968; Fox & Walley, 1971, b) β-casein (Cerbulis, Custer & Zittle, 1960; Ledford, Chen & Nath, 1968; El Negoumy, 1970) and k-casein (Jolles, Alais & Jolles, 1968; Lawrence & Creamer, 1969). Of the four α_s-caseins only α_{s1}-casein is hydrolysed by rennin (El Negoumy, 1970) and only the B genetic variant of the α_{s1}-casein (Creamer & Richardson, 1974). The α_{s1}-casein B is

hydrolysed by rennin at the phenylanine 23 - phenylalanine 24 bond to form two peptides (Hill, Lahav & Givol, 1974). The N-terminal peptide is basic while the C-terminal peptide, called α_{s1}-I by Fox & Guiney (1973), contains residues 24 to 199 (Hill, Lahav & Givol, 1974) or residues 25 to 199 of α_{s1}-casein B or a mixture of both (Creamer & Richardson, 1974). The proteolysis of β-casein B by rennin shows that three bonds are appreciably more sensitive than any others. The rate of hydrolysis is decreased by both increased ionic strength and calcium ion concentration at constant ionic strength (Creamer, Mills & Richards, 1971). Rennin action on k-casein hydrolyses a phenylalanine-methionine bond (Jolles, Alais & Jolles, 1968) releasing a series of discrete macropeptides (Armstrong et al., 1967) which contains all the sialic acid of k-casein (Beeby, 1963). The composition of the carbohydrate moiety of these glycopeptides affects their rate of release (Sinkinson & Wheelock, 1970) and hence the aggregation of para-k-casein which is inhibited by the presence of any of the three major casein fractions in the absence of calcium (Lawrence & Creamer, 1969).

Hydrolysis of casein fractions in cheese. The proteolysis patterns of casein fractions in cheese differ in some respects from those found in solution although the relative rates of hydrolysis of α_{s1}-and β-caseins are affected mainly by the water activity of the system (Creamer, 1971). Although β-casein is readily hydrolysed in solution the addition of 5% sodium chloride inhibits hydrolysis (Fox & Walley, 1971, b) which can not be detected in cheese unless the cheese has high moisture and low sodium chloride levels (Phelan, Guiney & Fox, 1973). However α_s-casein is almost completely hydrolysed in cheese and solutions where there is 5 to 10 % sodium chloride in the liquid phase (Fox & Walley, 1971, b).

The information reported above indicates that rennin has a specific and recognisable action on casein fractions both in solution and in cheese. Therefore any alternative enzyme used to coagulate milk for cheese manufacture should ideally have the same proteolytic specificity as calf rennet under similar conditions.

METHODS

Effect of pH on rennet stability

The effect of pH on the stability of adult bovine, calf and lamb rennets was studied by a method similar to that used by Mickelsen & Ernstrom (1967) for calf rennin.

Buffers. The buffers, of ionic strength 0.05 (Long, 1961), used to maintain the required pH in each rennet solution were;

(i) pH 1.4 to 1.9 potassium chloride-hydrochloric acid.
(ii) pH 2.1 to 3.4 glycine-hydrochloric acid.
(iii) pH 4.0 to 6.0 sodium acetate-acetic acid.
(iv) pH 6.4 to 7.1 disodium hydrogen phosphate-potassium dihydrogen phosphate.

Buffers, of ionic strength 1.0, were prepared by adding sodium chloride to the above.

Method. A solution of each rennet was prepared by mixing sufficient rennet powder with water to achieve an activity of approximately 140 RU/ml. One part of rennet solution was then added to six parts of each buffer solution to give samples containing approximately 20 RU/ml at an ionic strength of 0.04 or 0.86. One set of samples was incubated at temperatures of 3, 10, 20 and 30°C for 96 hours while a control for each set was held at 3°C at the pH at which each rennet was most stable (adult bovine, 4.7, calf 5.4 and lamb 4.1). After incubation the residual milk coagulating activity of each sample was determined, compared with the corresponding control and any loss reported as a percentage of the activity of the control.

Effect of heat on rennet stability

The effect of heat treatment on the milk coagulating activity of adult bovine, calf and lamb rennets was determined with two solutions of each rennet prepared by

dissolving rennet powder in either 17 mg/ml or 200 mg/ml sodium chloride and adjusted to the most stable pH for each rennet (adult bovine 4.7, calf 5.4 and lamb 4.1). Aliquots (2.5 ml) of each rennet were dispensed into 15 ml screw-top bottles and sealed. A set of five bottles for each rennet were placed in a heated water bath which was stirred briskly to ensure a uniform temperature throughout the bath. The time required for the bottle contents to reach the water bath temperature was predetermined and 40% of this time was included in the heating period for each bottle. After heating periods of 5, 10, 20, 30 and 60 minutes one bottle from each set was removed from the water bath and immediately surrounded by ice water to cool the contents as rapidly as possible. The milk coagulating activity was determined for each sample, and compared with a control sample, from each set, which had remained at room temperature during the heat treatment. Any loss of activity was expressed as a percentage of the control.

Variations of rennet activity with pH

The relative milk coagulating activity of adult bovine, calf and lamb rennets at various pH levels in the region found in cheesemaking (pH 6.1 to 7.0) was determined in solutions of sodium caseinate (60 mg/ml), in phosphate buffers of 0.05 ionic strength (Long, 1961), which had been stored at 4°C overnight before use. Stock solutions of each rennet were diluted with phosphate buffers of the same pH as each caseinate solution and their milk coagulating activity determined. The pH of each reaction mixture was measured at room temperature after larger volumes (2 ml) of each reactant had been mixed and held at 32°C for one minute. The activity of the rennet at various pH values was expressed as a percentage of the activity of that rennet at pH 6.55

Effect of temperature on rennet activity

The variation of rennet activity with temperature over a wide range and including those temperatures likely to be used in cheesemaking was determined for adult bovine, calf and lamb rennets by measuring the milk coagulating activity of each rennet with the assay method, described earlier, at temperatures of 25, 30, 35, 40, 45 and 50°C. The same caseinate substrate, at pH 6.55, and one stock solution of each rennet was used for all the measurements in each experiment. Rennets of similar milk coagulating activity at 32°C were used to ensure any comparisons between rennets were unbiased. Stock solutions of each rennet were diluted with phosphate buffer at pH 6.55 and tempered to the reaction temperature for 5 minutes instead of the usual 10 to 15 minutes. This time was sufficient for the dilute rennet to reach the reaction temperature but was kept to a minimum to reduce the possibility of enzyme denaturation at the higher temperatures. The activity of each rennet at various temperatures was expressed as a percentage of the activity of that rennet at 30°C.

Curd tension measurements

The effect of several factors on curd tension were measured, for adult bovine, calf and lamb rennets, by the method of Hehir (1968) as modified by Ellis (1972). The incubation period was recorded as the time interval between the addition of rennet to the milk and the placement of the weight on the curd surface. All milks were prepared from one batch of low heat skim milk powder (100 mg/ml) with additions of dry calcium chloride, M-hydrochloric acid or M-sodium hydroxide to achieve the desired calcium concentration and pH. Where necessary water was added to the milk to ensure the milk solids concentration was the same for each set of milks. The milks were held at room temperature for at least two hours

after reconstitution before use and each rennet was diluted with water just before addition to the milk. The three rennets were used in the same activity ratios as found necessary for cheese manufacture (adult bovine and lamb rennets 1.9 times higher activity than calf rennet). The incubation temperature was 32°C for all measurements and the pH was determined in the coagulum after the curd tension had been measured. The calcium content of the milk was measured by an ethylenediamine tetra acetic acid titration method (Vogel, 1961).

Effect of time on curd tension. The effect of time on the curd tension of milk coagulated by each rennet was determined in milk containing 35.5 mM calcium at 32°C and pH 6.6. Each rennet was incubated with the milk for 25, 32, 39, 46, 53 and 60 minutes.

Effect of pH on curd tension. The effect of pH on the curd tension of milk, containing 43 mM calcium, coagulated with each rennet was determined at 32°C after 30 minutes incubation at various pH values between 6.16 and 6.83.

Effect of calcium concentration on curd tension. The effect of calcium concentration on the curd tension of milk coagulated with each rennet was determined at pH 6.56 after incubation at 32°C for 30 minutes in milk containing 33, 36, 39, 42 and 45 mM calcium.

Proteolysis of whole casein

The proteolytic activity of adult bovine, calf and lamb rennets on whole casein was determined by measuring the increase in soluble nitrogen with increased reaction time. Sodium caseinate (60 mg/ml) was dissolved in a sodium acetate-acetic acid buffer, ionic strength 0.05 (Long, 1961), to achieve a solution at pH 5.55. The caseinate solution was held at 4°C overnight before

aliquots (8 ml) were tempered to 32°C for 20 minutes. Meanwhile each rennet, at the same activity ratios as found necessary for cheesemaking, was diluted in the same buffer and tempered to 32°C before an aliquot (2 ml) was mixed with an aliquot of caseinate solution and incubated for 240 minutes. After reaction times of 1, 15, 30, 60, 120, 180 and 240 minutes an aliquot (1 ml) of each reaction mixture was withdrawn and added to 0.15-M trichloroacetic acid (5 ml). Samples were placed under vacuum for 10 minutes, to remove air from the precipitated casein, and centrifuged at 2600 rpm for ten minutes before the absorbance of the supernatants from each set was measured at 280 nm against the sample of each set prepared after a one minute reaction time. A blank of caseinate solution (8 ml) mixed with buffer (2 ml) was incubated and sampled at the above reaction times to measure any changes in the substrate with time at 32°C.

Casein fractionation

Whole casein was separated into the three main fractions, α_s-, β- and k-caseins, to evaluate the hydrolysis of each casein fraction by adult bovine, calf and lamb rennets. Whole acid casein was prepared from reconstituted skim milk by the method described earlier for sodium caseinate preparation. However instead of adding sodium hydroxide to dissolve the casein it was centrifuged and the casein pellet stored frozen until required.

Isolation of α_s- and β-caseins. The isolation of α_s- and β-caseins was by the ion exchange chromatography method of Thompson (1966) except that the buffer contained 4.5 M urea (Garnier, Ribadeau Dumas & Mocquot, 1964). Prior to application to the chromatography column the casein was dissolved in buffer

and centrifuged to remove any insoluble material. Whatman DE 52 cellulose (W. & R. Balston, Maidstone, England) was prepared according to the manufacturers instructions and packed into a column (30 by 1.5 cm). Flow rates (20 to 30 ml/h) were maintained by a three-channel peristaltic pump (Pharmacia Fine Chemicals, Uppsala, Sweden) which also produced the 0.0 to 0.3-M sodium chloride gradient for column elution. The absorbance of the eluent fractions (10 ml) was measured against the buffer at 280 nm. The sodium chloride gradient was determined by measuring the conductivities of a one to fifteen dilution in water of several fractions from each run.

Isolation of k-casein. The isolation of k-casein was by the method of Yaguchi, Davies and Kim (1968) from whole acid casein on a column of Sephadex G-150 (90 by 1.5 cm). Flow rates (15 to 20 ml/h) were maintained by gravity and the absorbance of eluent fractions (3 ml) measured against the buffer at 280 nm.

After isolation each casein fraction was dialysed against 0.005 M-sodium chloride before concentration by ultrafiltration and further dialysis to remove all the buffer components (Yaguchi, Davies & Kim, 1968). The individual fractions were stored frozen until required.

Hydrolysis of casein fractions

Each of the three main casein fractions, α_s-, β- and k-caseins, were incubated with adult bovine, calf and lamb rennets and the hydrolysis products determined by polyacrylamide gel electrophoresis. The reation between each rennet and the α_s- or β-casein at 32°C and pH 6.5 was followed by the technique of Creamer, Mills & Richards, (1971). Reaction times of 1, 4, 15, 30, 60, 120 and 240 minutes were used and the quenched reaction mixtures electrophoresed on alkaline polyacrylamide gels.

The reaction between k-casein and each rennet was followed as above for reaction times of 0, 15, 30 and 60 minutes at 32°C and pH 6.5. Quenched reaction mixtures were electrophoresed on acid polyacrylamide gels by the method of Peterson & Kopfler (1966) except that the stock buffer was 5% acetic acid and amido black was used to stain the gels. Both alkaline and acid gels were destained electrophoretically and photographed.

Molecular weight determination

The molecular weight (MW) of each purified enzyme was estimated by the gel chromatography method of Fischer (1971). Sephadex G-100 gel, prepared according to the manufacturers instructions, was equilibrated with a pH 5.6 phosphate buffer (described earlier) and packed into a column (90 by 1.5 cm). Standard proteins (Sigma Chemical Co., St. Louis, U.S.A.), used to calibrate the column were bovine serum albumin (MW 67,000), ovalbumin (MW 45,000), myoglobin (MW 17,800) and cytochrome C (MW 12,400) (Andrews, 1970). Samples were dissolved in the equilibration buffer and applied to the column either in pairs (standard proteins) or singly (purified enzymes). Flow rates (10 to 20 ml/h) were maintained by gravity and each fraction of eluent (2.5 ml) was weighed to determine the cumulative volume after correcting for the buffer density. The absorbance of each eluent fraction was measured at 230 nm against the buffer and a graph of absorbance with cumulative eluent volume plotted to determine the elution volume of each protein. The elution volume and the logarithim of the molecular weight of each standard protein was used to calculate a regression equation for the column. This regression equation was then used to determine the molecular weight of each purified enzyme from its elution volume.

Amino acid analysis

The amino acid analysis of each purified enzyme was carried out on aliquots of a solution of each enzyme hydrolysed with 6-M hydrochloric acid at 110°C for 24, 48 and 72 hours. Tryptophan was measured spectrophotometrically by the method of Beaven & Holiday (1952) while the amino acid composition of each hydrolysate was determined with a Locarte Mark IV amino acid analyser. The data obtained was treated similarly to a method described by Richardson, Creamer & Munford (1973). The number of residues of each amino acid present in each enzyme was calculated using the molecular weights determined by gel chromatography data.

RESULTS

Effect of pH on rennet stability

The loss of milk coagulating activity of adult bovine, calf and lamb rennets at ionic strengths of 0.04 and 0.86 between pH 1.4 and 7.1 are presented in Figure 8. For each rennet the stability at 3 and 10°C was similar so that only 3°C data is presented.

At an ionic strength of 0.04 each rennet has a region of maximum stability, adult bovine pH 4.3 to 5.5, calf 5.0 to 6.5 and lamb 3.8 to 4.6 which is the same for all temperatures between 3 and 30°C. The loss of activity at any pH, for each rennet, is temperature dependent and in all cases greater activity losses occur as temperature increases.

Due to the presence of sodium chloride in each buffer, at 30°C adult bovine and lamb are both more stable at an ionic strength of 0.86 than at 0.04 ionic strength. The same applies to calf rennet above pH 4.8 but below pH 4.8 calf rennet is less stable at the higher ionic strength supporting the findings of Mickelsen & Ernstrom (1967) that calf rennin is less stable at pH values below 5.0 in the presence of chloride ions.

Effect of heat on rennet stability

The effect of heat on the stability of each rennet was measured at a different temperature for each sodium chloride concentration, 68°C for 200 mg/ml and 64°C for 17 mg/ml, so that the three rennets could be compared under the same conditions. Results, presented in Figure 9, show that each rennet is more readily inactivated in 17 mg/ml than 200 mg/ml sodium chloride solution.

Both adult bovine and lamb rennets are more stable to heat than calf rennet but this may be due to the rennets used having a higher concentration of mucoproteins and other non-active solids per unit of enzyme activity in the adult bovine and lamb rennets.

Variations of rennet activity with pH

The variation of rennet activity between pH 6.17 and 6.96 was determined for adult bovine, calf and lamb rennets and the results are presented in Figure 10. In cheesemaking rennets are required to coagulate milk between pH 6.4 and 6.7 (Gillies, 1972) and for the lower part of this range adult bovine and lamb rennets acted similarly to calf rennet. However at pH 6.7 adult bovine and lamb rennets are both less active than calf rennet suggesting that when the milk pH increased more adult bovine and much more lamb rennet would be required to coagulate milk in the same time as calf rennet. This data demonstrates that each of the three rennets is active above pH 6.9 unlike porcine pepsin which showed no activity above pH 6.81 (Green, 1972).

Effect of temperature on rennet activity

The rennet activity of adult bovine, calf and lamb rennets was determined at pH 6.55 between 25 and 50°C and results, presented in Figure 11, show that for each rennet there is an optimum temperature. For adult bovine and calf rennet this is 40°C and for lamb rennet 30°C.

Figure 8. The effect of pH on the stability of adult bovine (A), calf (B) and lamb (C) rennets at ionic strengths of 0.04 (3, (○), 20, (●) and 30, (△) °C) and 0.86 (30, (▲) °C).

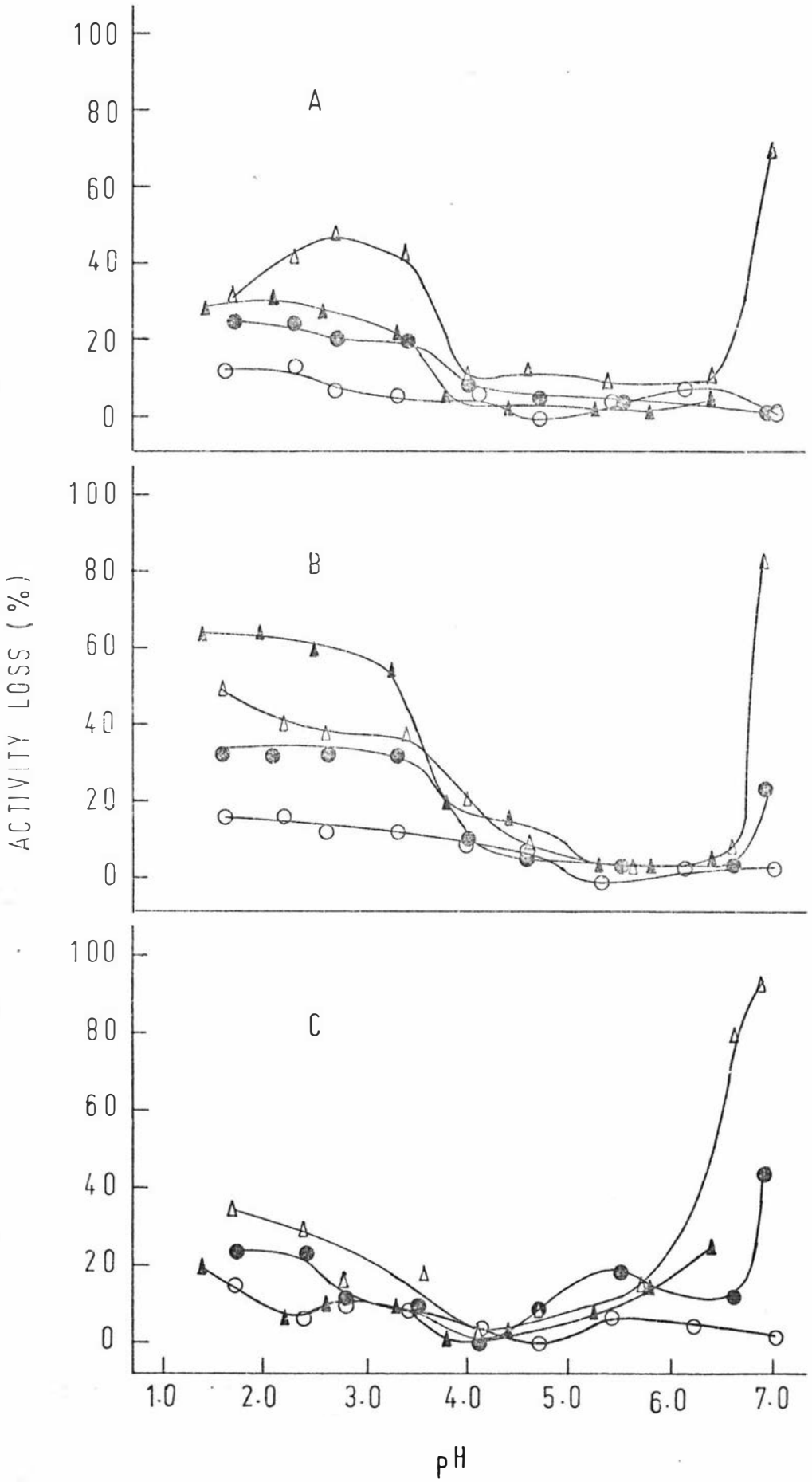

A
B
C
100
80
60
40
20
0
ACTIVITY LOSS (%)
1.0
2.0
3.0
4.0
5.0
6.0
7.0
pH

Figure 9. The effect of heat on the stability of adult bovine (○), calf (●) and lamb (△) rennets in 200 mg/ml (A) and 17 mg/ml (B) sodium chloride solutions.

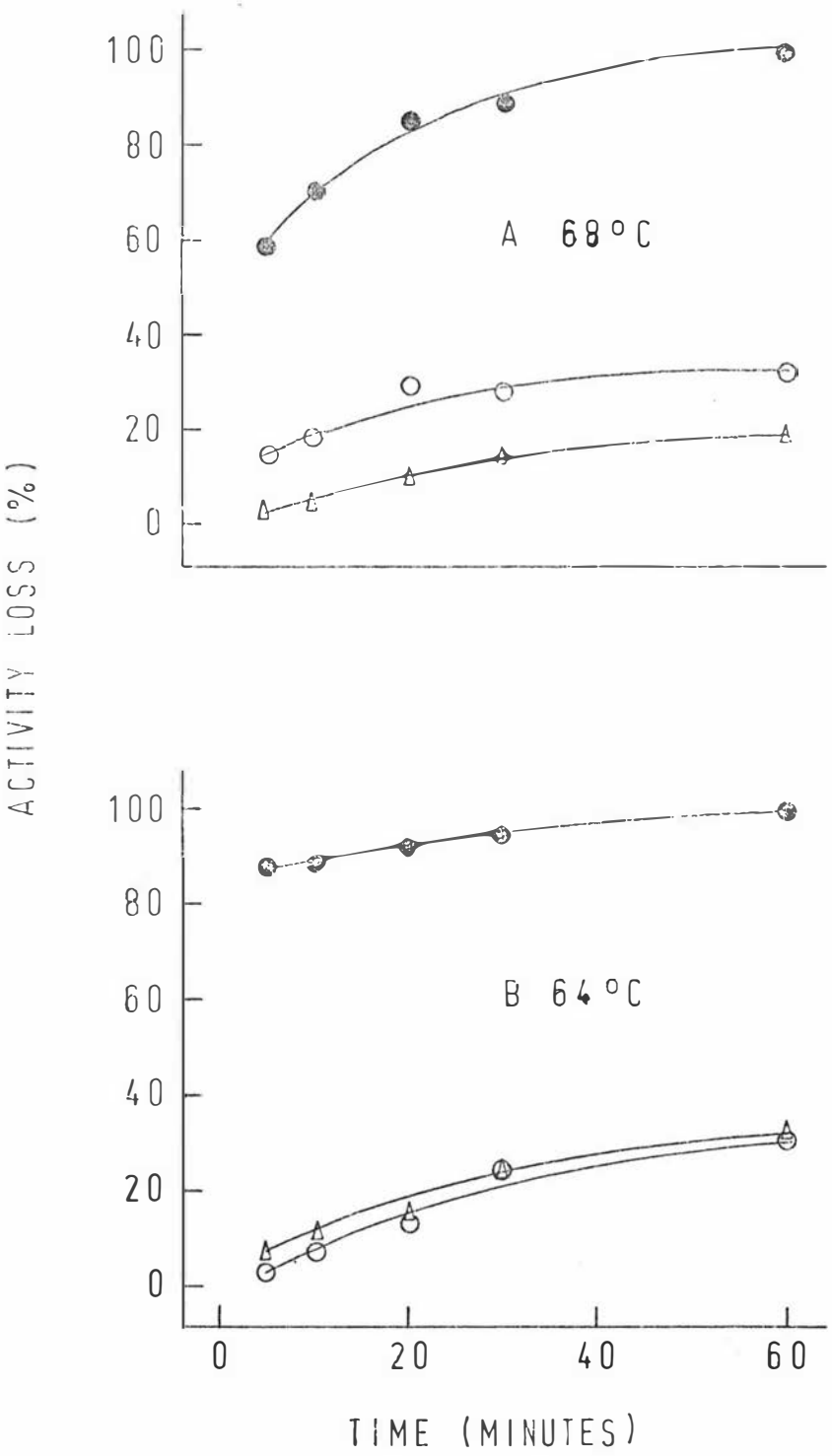

100
80
60
40
20
0
A 68°C
100
80
60
40
20
0
B 64°C
0
20
40
60
ACTIVITY LOSS (%)
TIME (MINUTES)

Figure 10. The variations of milk coagulating activity with pH for adult bovine (○), calf (●) and lamb (△) rennets.

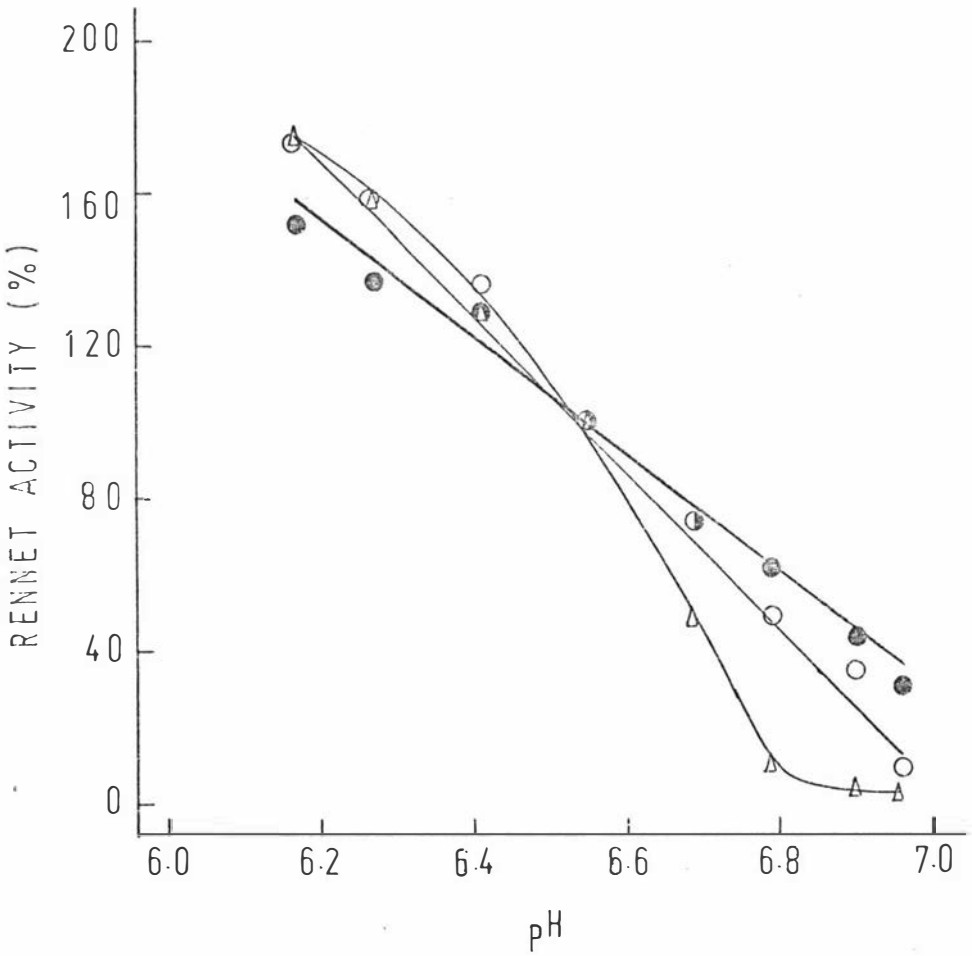
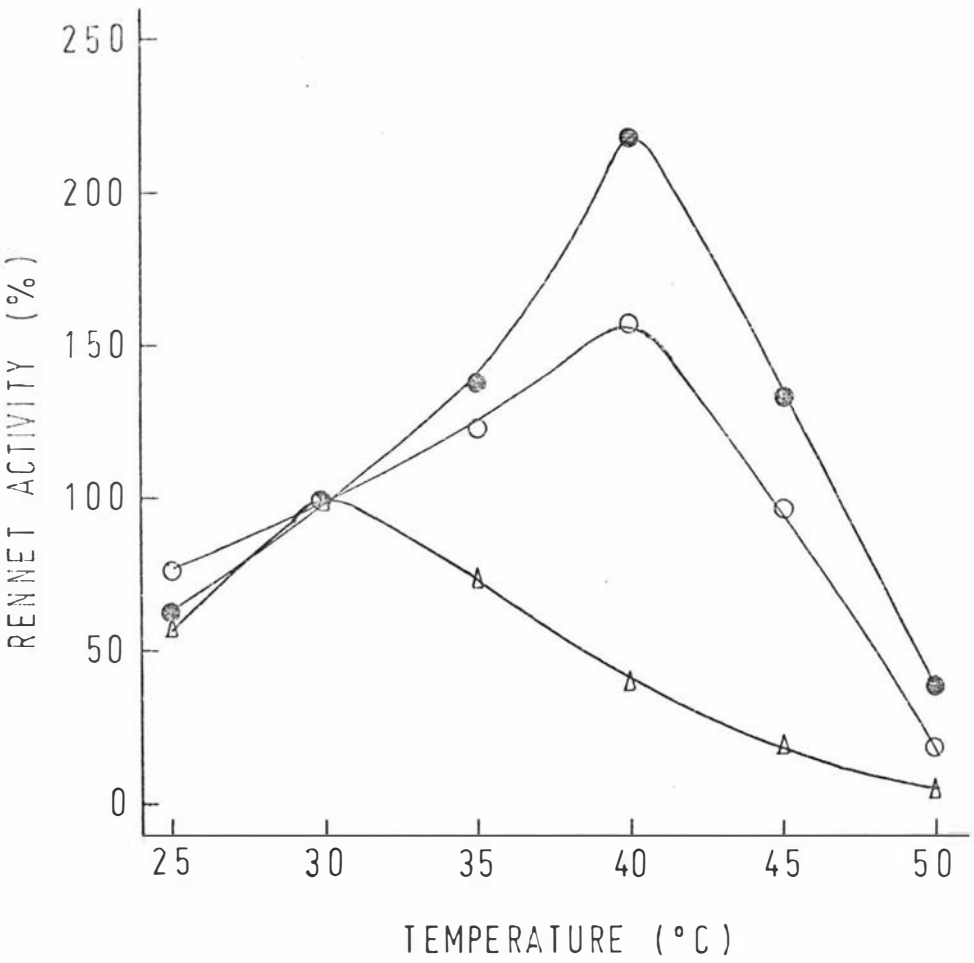

200
160
120
80
40
0
RENNET ACTIVITY (%)
6·0
6·2
6·4
6·6
6·8
7·0
pH

Figure 11. The effect of temperature on the milk coagulating activity of adult bovine (○), calf (●) and lamb (△) rennets.


250
200
150
100
50
0
RENNET ACTIVITY (%)
25
30
35
40
45
50
TEMPERATURE (°C)

The temperature range used in cheesemaking to coagulate milk is 30 to 35°C and in this range adult bovine and calf rennets behave similarly in that they become more active as temperature increases in contrast to the lamb rennet which becomes less active. The different response in activity to temperature increases between adult bovine and calf rennets would not be noticeable in cheesemaking but the difference between lamb and calf rennets would indicate that the setting temperature might need to be limited if the minimum quantity of lamb rennet was to be used.

Effect of time on curd tension

The curd tension of milk coagulated by adult bovine, calf and lamb rennets was measured after incubation times of 25 to 60 minutes and the results, presented in Figure 12, were the average of duplicate determinations.

The three rennets showed a similar response in that the curd tension increased with incubation time between 25 and 60 minutes although the increase was small after 46 minutes. Therefore a cheesemaker could extend the setting time to achieve the desired coagulum firmness when using adult bovine or lamb rennet in the same manner as with calf rennet although extending the setting time beyond 46 minutes could not be expected to produce a significantly firmer coagulum.

Effect of pH on curd tension

The curd tension of milk coagulated with adult bovine, calf and lamb rennets was measured after incubation at pH values between 6.16 and 6.83 and the results, presented in Figure 13, were the average of duplicate determinations.

The three rennets show similar trends in that they all produce a firmer coagulum at lower pH levels than at higher pH levels. Rennets are required to coagulate milk between pH 6.4 and 6.7 (Gill es, 1972) and within this

Figure 12. The effect of time on the curd tension of milk coagulated by adult bovine (○), calf (●) and lamb (△) rennets.

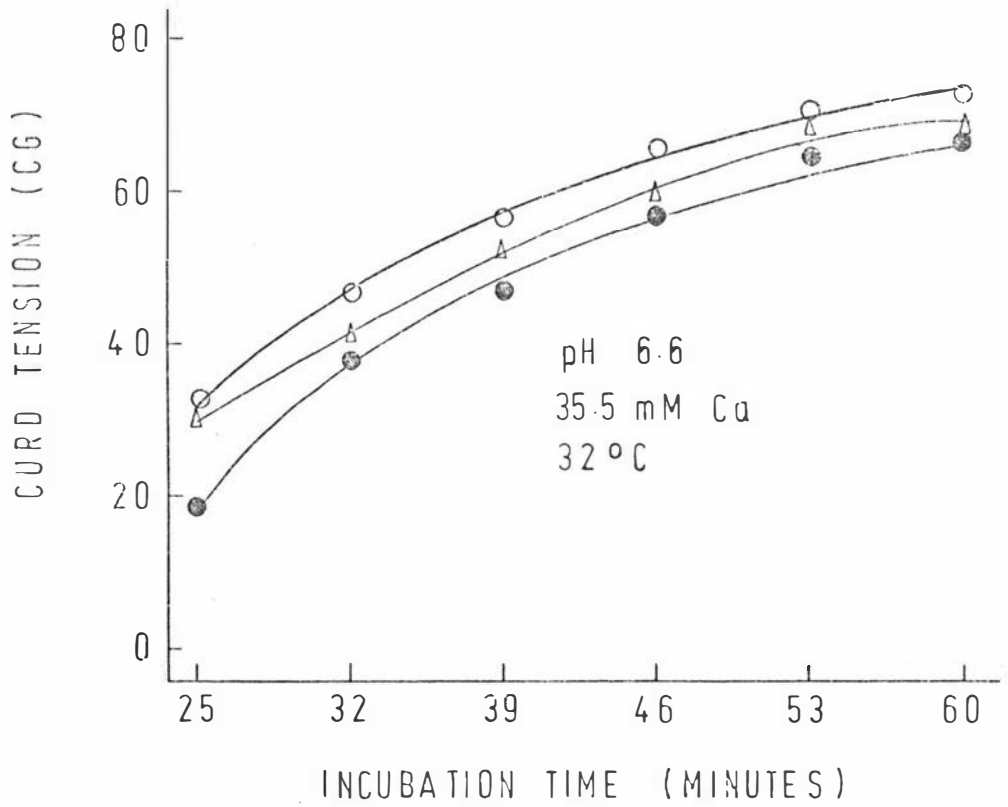
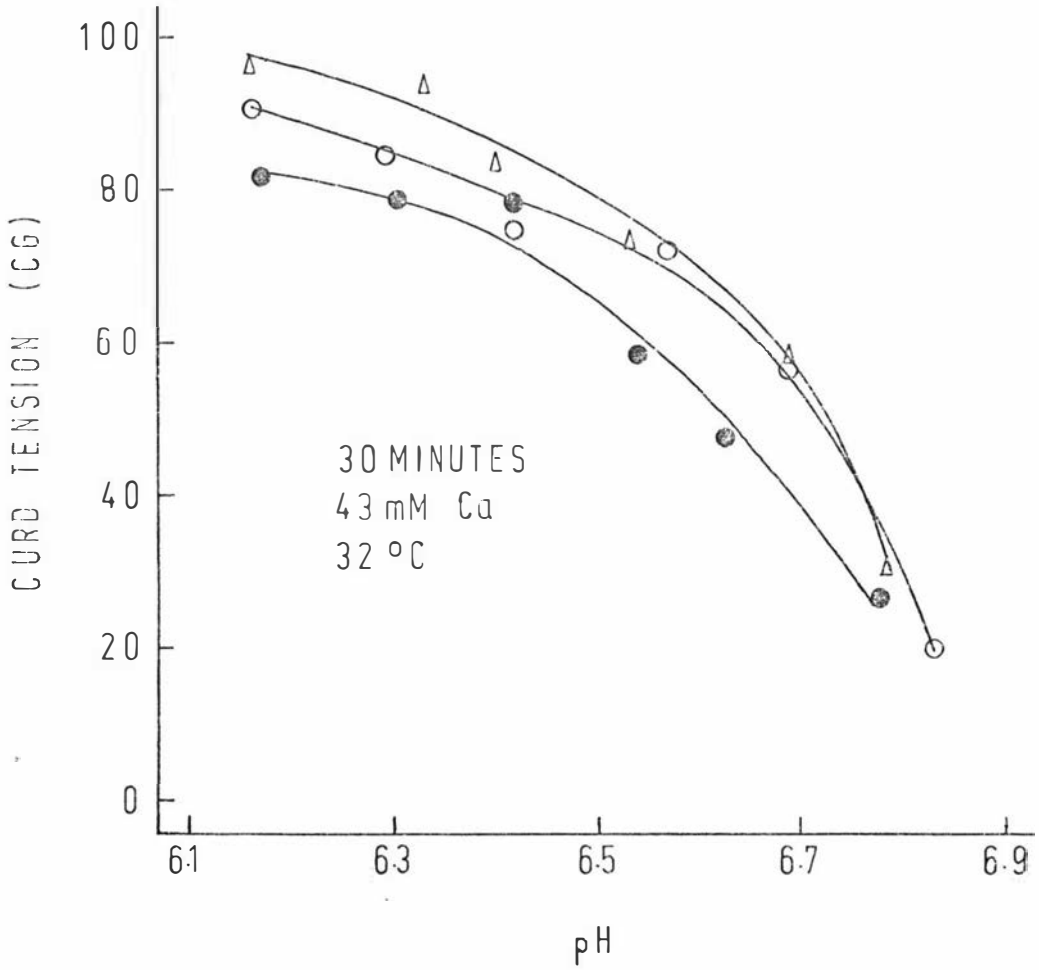

CURD TENSION (CG)
80
60
40
20
0
25
32
39
46
53
60
INCUBATION TIME (MINUTES)
pH 6.6
35.5 mM Ca
32 °C

Figure 13. The effect of pH on the curd tension of milk coagulated by adult bovine (○), calf (●) and lamb (△) rennets.


100
80
60
40
20
0
CURD TENSION (CG)
30 MINUTES
43 mM Ca
32 °C
6.1
6.3
6.5
6.7
6.9
pH

range calf rennet is the most sensitive to pH change. Therefore adult bovine and lamb rennets would be expected to produce slightly firmer coagulums at higher pH levels, relative to calf rennet, if all had been standardised in activity at pH 6.55, A cheesemaker would be able to use adult bovine or lamb rennets in a similar manner to calf rennet when ever milk pH was higher than usual.

Effect of calcium concentration on curd tension.

The curd tension of milk, containing 33.0 to 45.5 mM calcium, coagulated with adult bovine, calf and lamb rennets was measured and the results, presented in Figure 14, were the average of duplicate determinations.

The three rennets showed a similar response in that curd tension increases with calcium concentration. However at lower calcium levels adult bovine and lamb rennets are similar in their response and are both considerably more sensitive to calcium than calf rennet. From a cheesemakers point of view the three rennets respond similarly to the addition of calcium to milk and both the adult bovine and lamb rennets could be used in the same manner as calf rennet. The calcium concentration of milk has little effect on the first stage of milk coagulation and the similarities in the above data suggest that the second stage of milk coagulation is similar for adult bovine, calf and lamb rennets.

Proteolysis of whole casein

The change in soluble nitrogen with time was measured for adult bovine, calf and lamb rennets incubated with a solution of whole casein. Each of the three rennets produced an increase in soluble nitrogen (as measured by absorbance at 280 nm) with time and there was a straight line relationship between these two parameters from 15 to 240 minutes, as shown in Figure 15. There was no change in absorbance with time for the blank, indicating that all the changes in samples was due to enzyme action. The slope of the lines (Figure 15) shows that under these conditions adult bovine rennet is 1.58 times and lamb rennet 1.12 times more proteolytic on whole casein than calf rennet.

Figure 14. The effect of calcium concentration on the curd tension of milk coagulated by adult bovine (○), calf (●) and lamb (△) rennets.

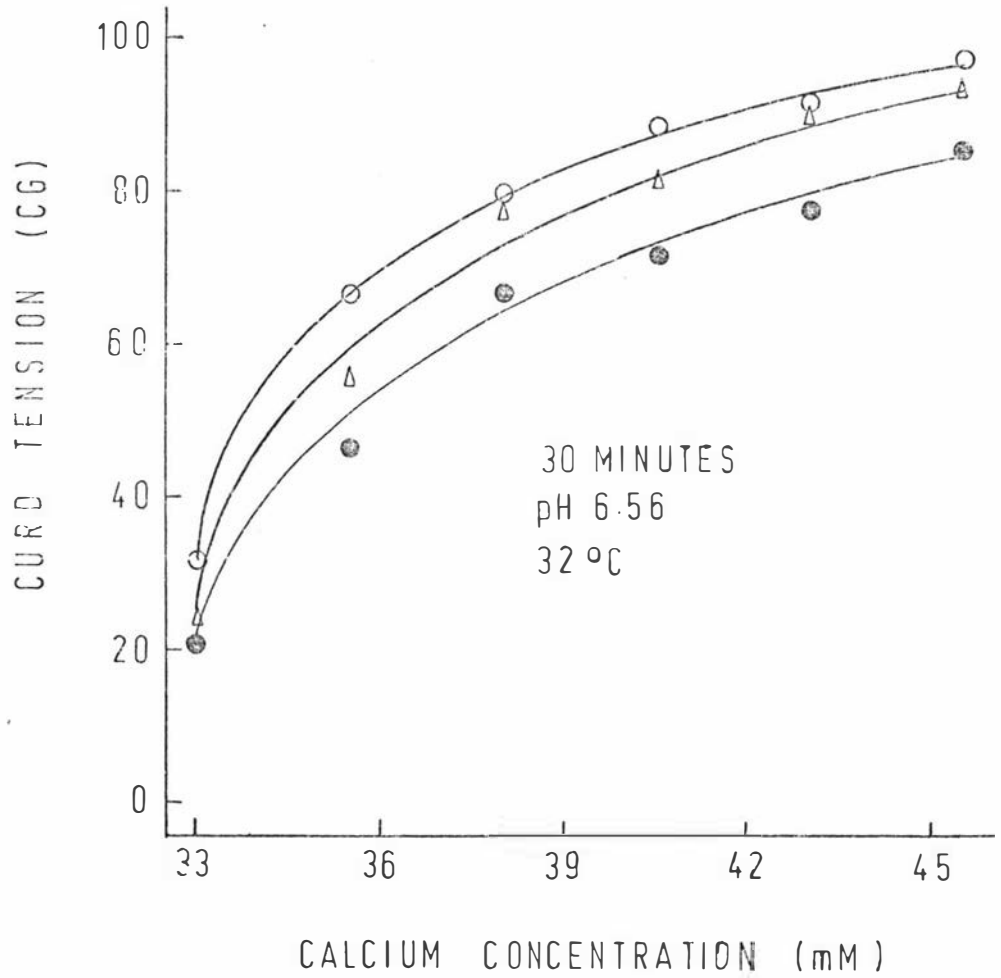
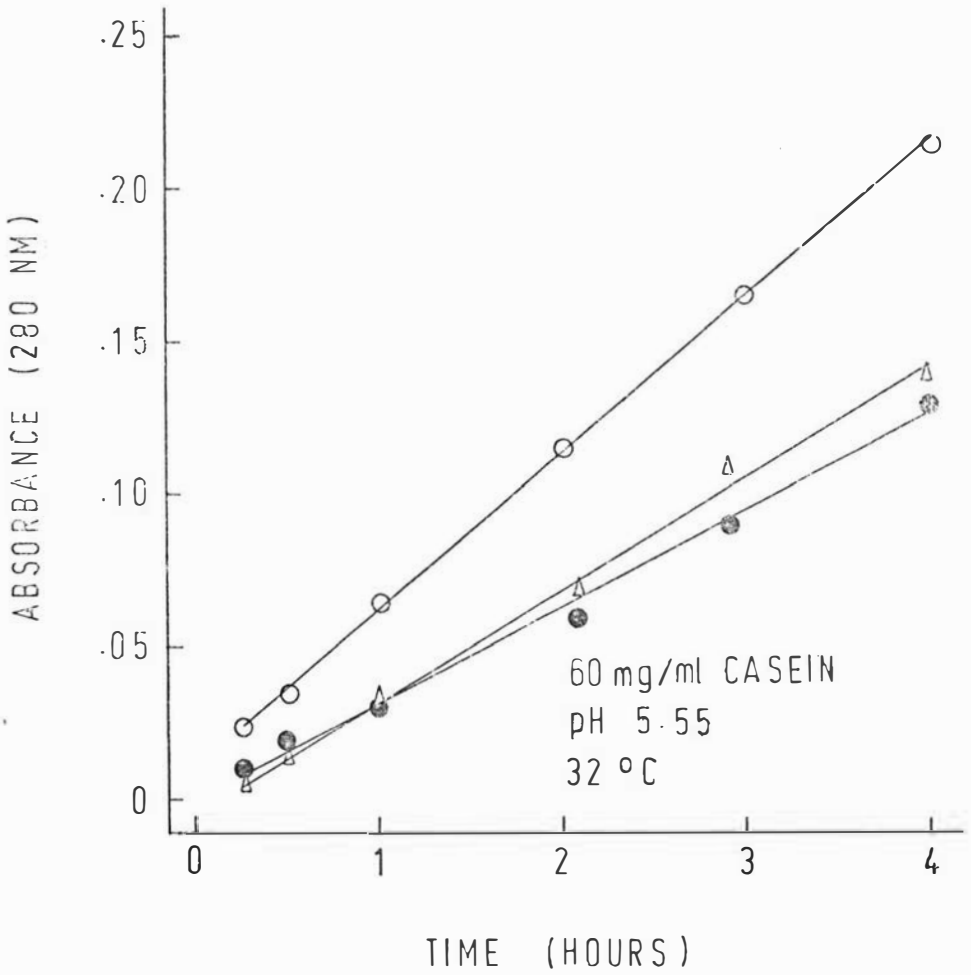

100
80
60
40
20
0
CURD TENSION (CG)
30 MINUTES
pH 6.56
32 °C
33
36
39
42
45
CALCIUM CONCENTRATION (mM)

Figure 15. The proteolysis of whole casein by adult bovine (○), calf (●) and lamb (△) rennets.


.25
.20
.15
.10
.05
0
ABSORBANCE (280 NM)
0
1
2
3
4
TIME (HOURS)
60 mg/ml CASEIN
pH 5.55
32 °C

Casein fractionation

Isolation of α_s- and β-caseins. A typical ion exchange chromatograph, presented in Figure 16, shows that whole acid casein is separated into two main peaks which, in order of elution, are β-casein and α_s-casein (Thompson, 1966). The fractions, containing α_s- or β-caseins, indicated by the bars in Figure 16, were pooled and held at 4°C with 5 g/l of chloroform as a preservative until a sufficient quantity was accumulated.

Isolation of k-casein. A typical gel chromatograph, presented in Figure 17, shows that whole acid casein is separated into two peaks. The first peak, eluted at the void volume of the column, contains k-casein while the second peak consists mainly of α_s- and β-caseins (Yaguchi, Davies & Kim, 1968). The fractions containing k-casein, indicated by the bars in Figure 17, were pooled and held at 4°C with 5 g/l of chloroform as a preservative until a sufficient quantity was accumulated.

The accumulated quantities of each casein fraction were dialysed against 0.005-M sodium chloride, concentrated by ultrafiltration and further dialysed until free of buffers. Solutions were stored frozen until required and the purity of each fraction is demonstrated in the following section.

Hydrolysis of casein fractions

α_s-casein. Electrophoretograms of the hydrolysis products of α_s-casein produced by adult bovine, calf and lamb rennets are shown in Figure 18. Each rennet rapidly converted α_s-casein to the peptide α_{s1}-I (Fox & Guiney, 1973) which was further hydrolysed to two peptides of greater mobility. It can be seen that under the conditions used the rate of hydrolysis of α_s-casein is fastest by calf rennet, then lamb rennet while adult bovine rennet is the slowest. However although the rates varied each of the

Figure 16. Elution pattern of whole acid casein from ion exchange chromatography on DEAE cellulose.

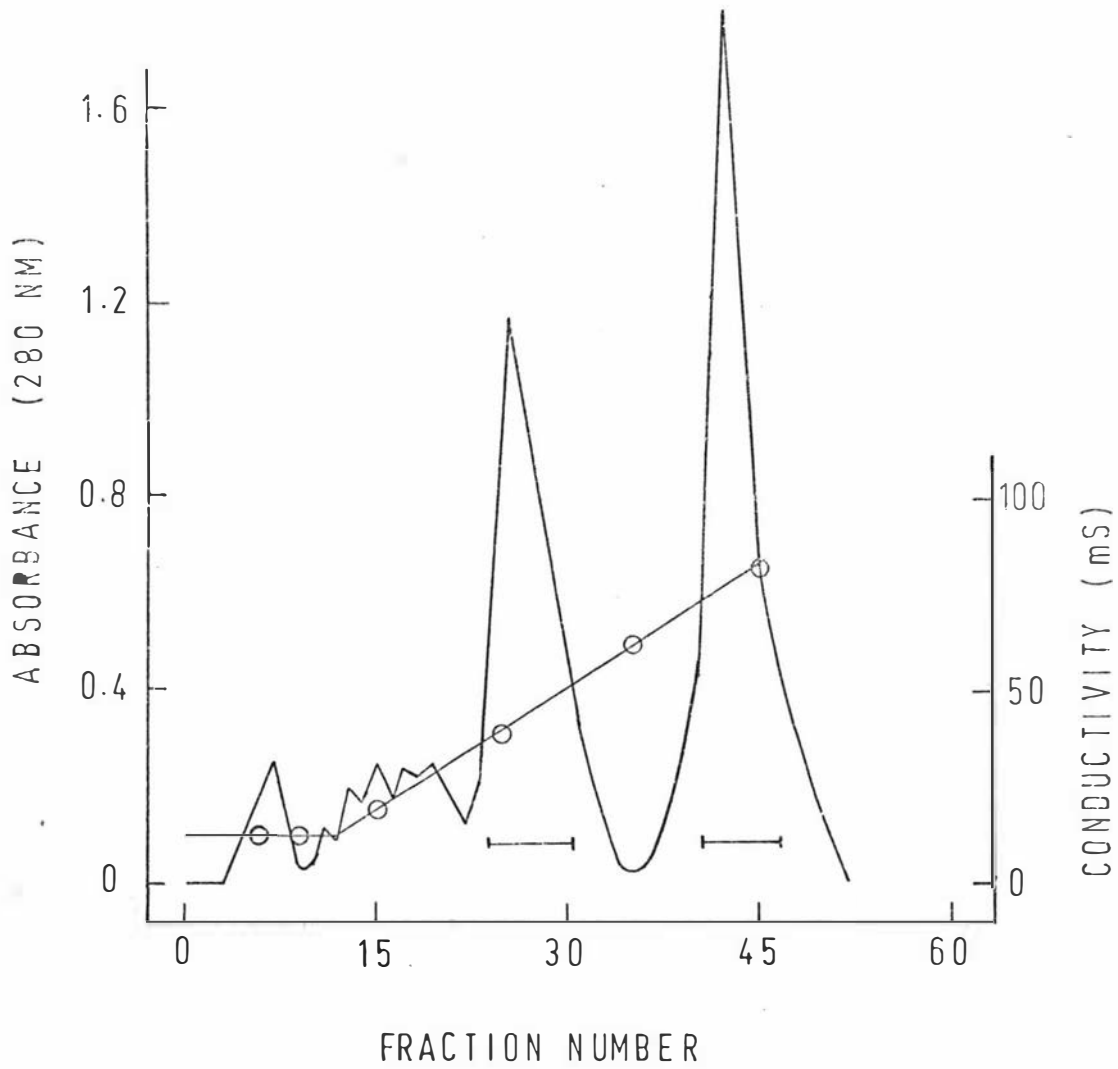

ABSORBANCE (280 NM)
1.6
1.2
0.8
0.4
0
0
15
30
45
60
FRACTION NUMBER
100
50
0
CONDUCTIVITY (mS)

Figure 17. Elution patterns of whole acid casein from gel chromatography on Sephadex G-100 gel.

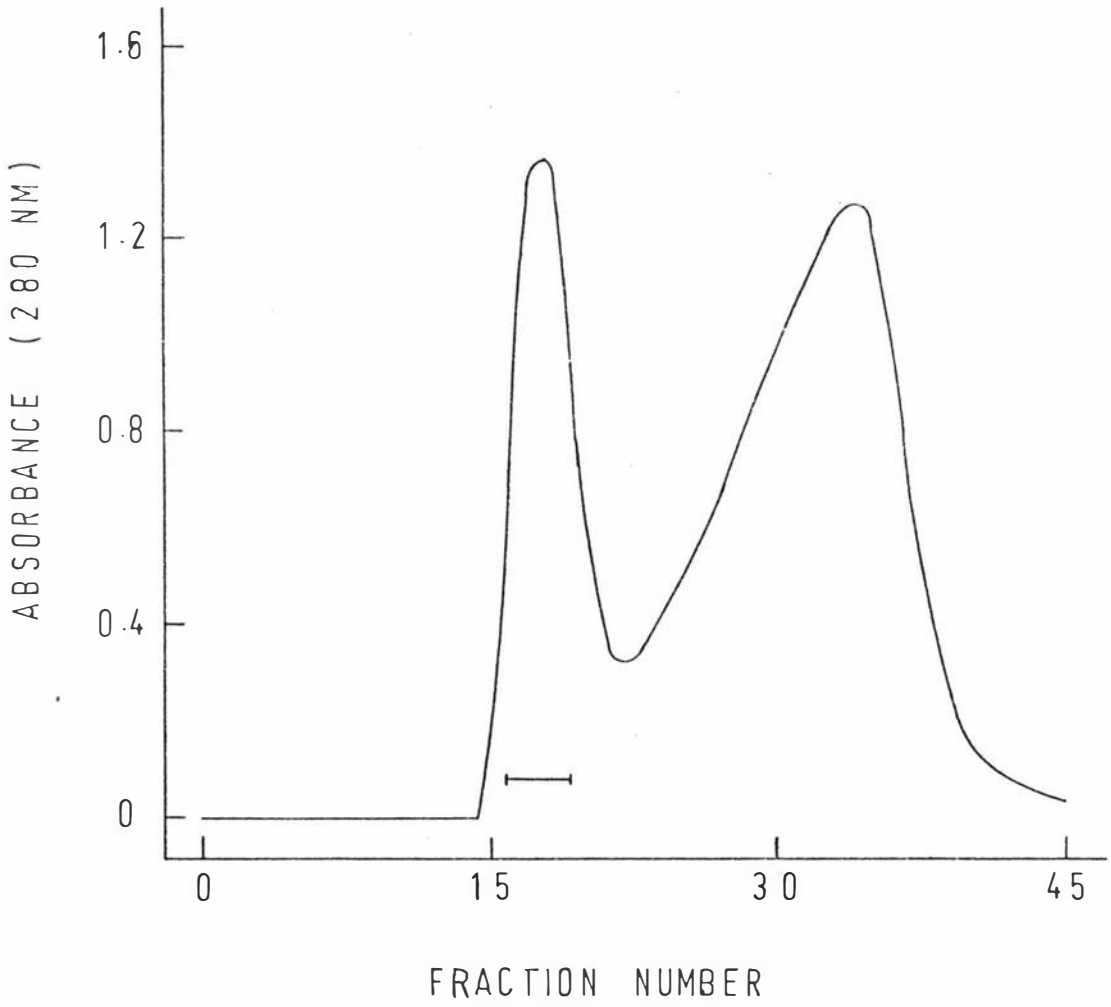
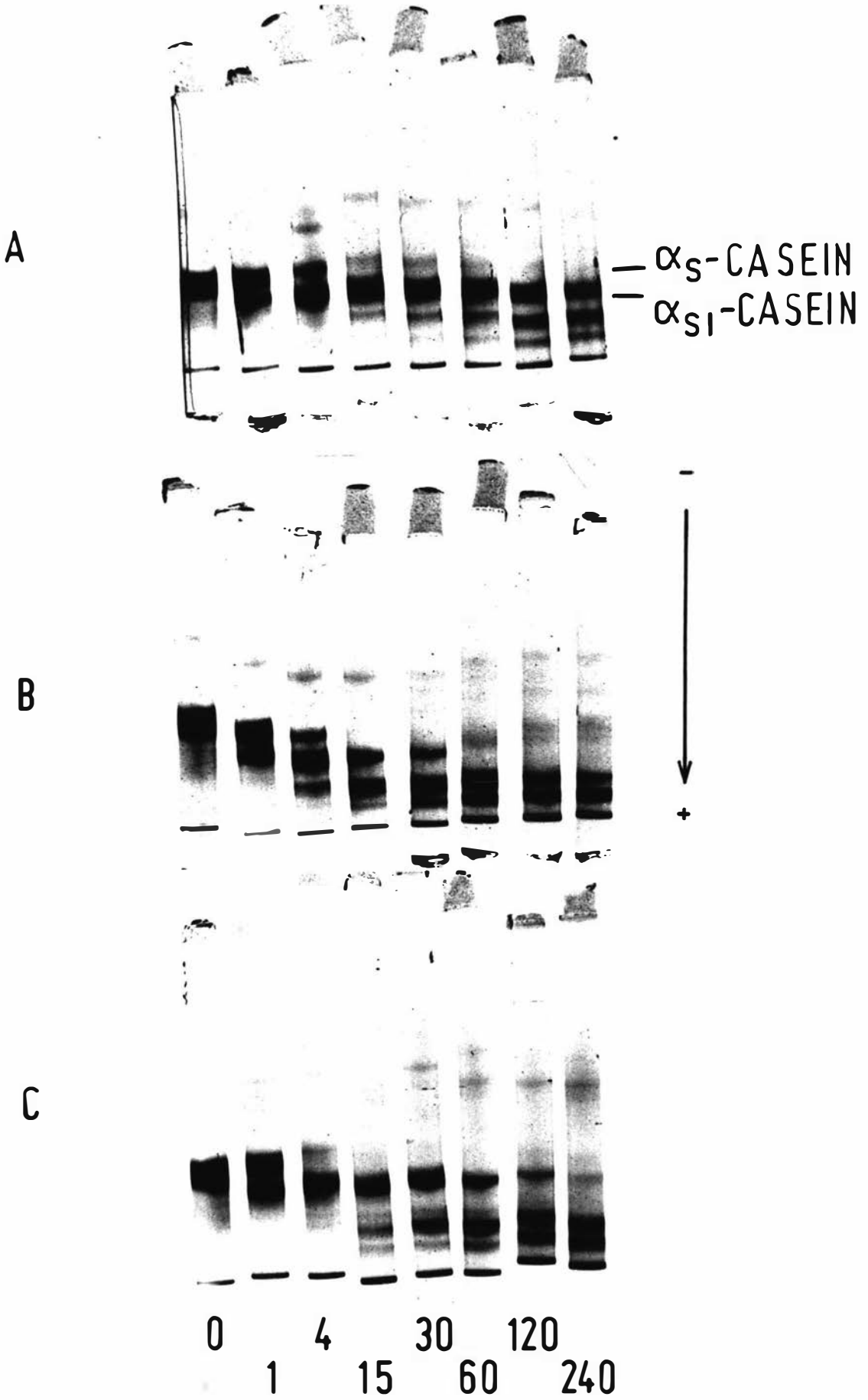

1.6
1.2
0.8
0.4
0
ABSORBANCE (280 NM)
0
15
30
45
FRACTION NUMBER

Figure 18. Alkaline polyacrylamide gel electrophoretograms of α_s-casein hydrolysed for 0, 1, 4, 15, 30, 60, 120 and 240 minutes at pH 6.5 by adult bovine (A), calf (B) and lamb (C) rennets.


A
B
C
α_s-CASEIN
α_{s1}-CASEIN
−
+
0
1
4
15
30
60
120
240

three rennets hydrolysed α_s-casein, in solution at pH 6.5, in the same manner.

β-casein. Electrophoretograms of the hydrolysis products of β-casein produced by adult bovine, calf and lamb rennets are shown in Figure 19. It was found that each rennet rapidly converted β-casein to the peptide called β-I by Creamer, Mills & Richards (1971) (this was confirmed on slab electrophoresis gels). The β-I peptide was further hydrolysed to two more mobile peptides called β-II and β-III, in order of increasing mobility, respectively, by the same workers. Under the conditions used the rate of hydrolysis of β-casein was fastest for adult bovine rennet, then lamb rennet and calf rennet was the slowest. However although adult bovine and lamb rennets hydrolyse β-casein, in solution at 6.5, faster than calf rennet they do so in the same manner and into the same products.

k-casein. Electrophoretograms of the hydrolysis products of k-caseins produced by adult bovine, calf and lamb rennets are shown in Figure 20. Each rennet hydrolysed most of the k-caseins to para-k-caseins which were more mobile on acid polyacrylamide gel electrophoresis (Bingham, 1975). In each case there was one predominant para-k-casein produced and only a trace of a less mobile band in the same region of the gel. Calf rennet hydrolysed k-casein most rapidly and almost completely in 15 minutes. Adult bovine and lamb rennets hydrolysed k-casein at approximately the same rate which was considerably slower than calf rennet. The hydrolysis was not as complete and a considerable proportion of k-casein remained unhydrolysed after 60 minutes reaction time. However adult bovine and lamb rennets hydrolysed k-caseins, in solution at pH 6.5, into para-k-caseins in a similar manner to but at a slower rate and less completely than calf rennet.

Figure 19. Alkaline polyacrylamide gel electrophoretograms of β-casein hydrolysed for 0, 1, 4, 15, 30, 60, 120 and 240 minutes at pH 6.5 by adult bovine (A), calf (B) and lamb (C) rennets.

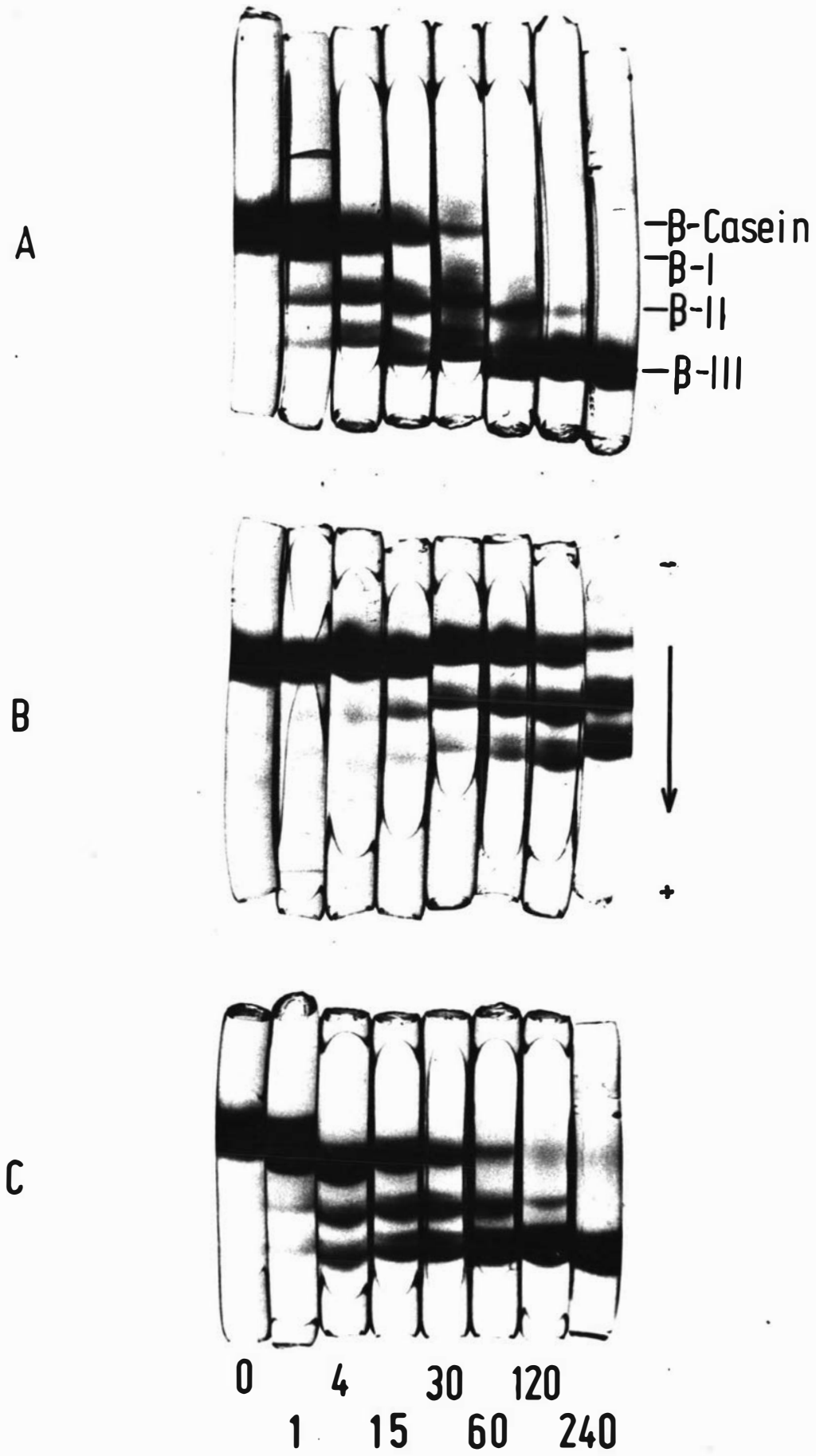
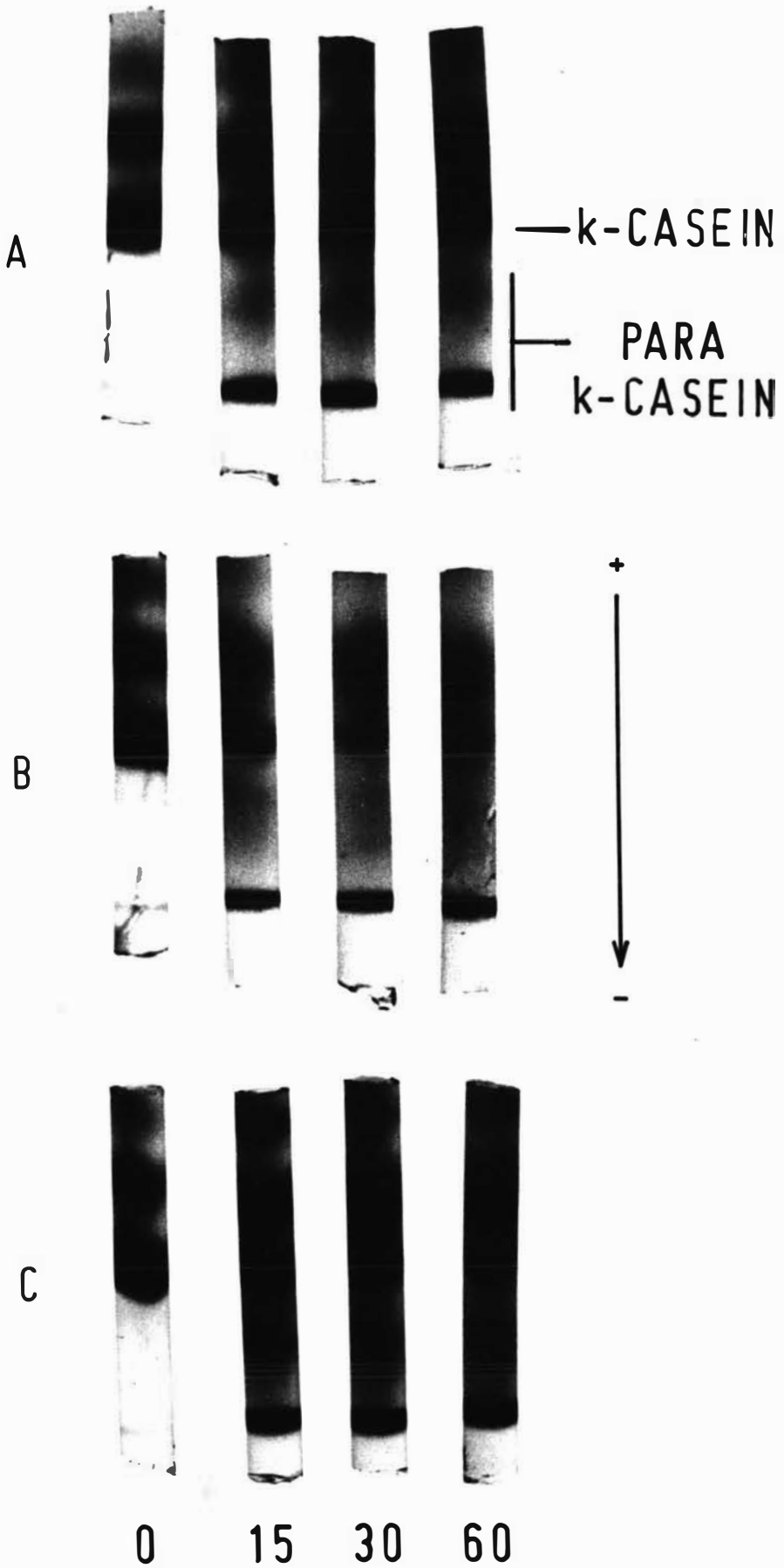

A
β-Casein
β-I
β-II
β-III
B
-
+
C
0
4
30
120
1
15
60
240

Figure 20. Acid polyacrylamide gel electrophoretograms of k-casein hydrolysed for 0, 15, 30 and 60 minutes by adult bovine (A), calf (B) and lamb (C) rennets.


A
k-CASEIN
PARA
k-CASEIN
B
+
-
C
0
15
30
60

Molecular weight determinations

The molecular weight of each purified enzyme from adult bovine and lamb rennets was determined by comparing their elution volumes from a Sephadex G-100 gel chromatography column with the elution volumes of four standard proteins of known molecular weights. The elution volume (Ve) of each standard protein was determined three or four times and this data with the logarithim of the molecular weight (log MW) of each standard protein was used to calculate a regression equation which is;

$$\text{Log MW} = 6.776 - 0.0243\ \text{Ve}$$

with a standard deviation of $\pm$ 0.034.

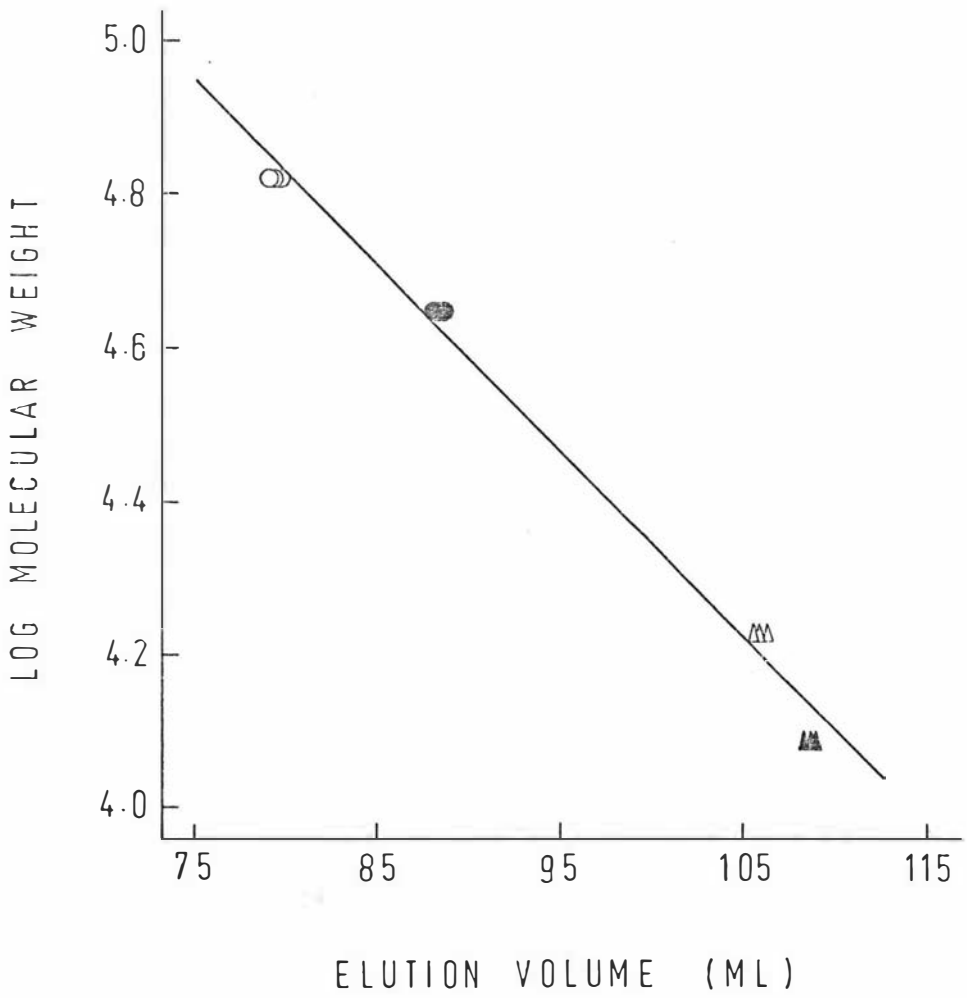
The elution volume data and regression line are shown in Figure 21. The molecular weight of each enzyme was estimated by substituting the average value of three elution volume determinations in the above regression equation and this data is presented in Table III.

Table III. Molecular weight estimations for adult bovine, lamb 1 and lamb 2 enzymes from gel chromatography measurements.

Enzyme	Elution volume (ml)	Standard deviation	Molecular weight	Standard deviation
Adult bovine	94.0	0.00	31,030	2,340
Lamb 1	95.0	0.71	29,340	2,429
Lamb 2	93.2	0.28	32,450	2,547

The estimated molecular weights of the three enzymes were adult bovine 31,030, lamb 1 29,340 and lamb 2 32,450.

Figure 21. The comparison between the logarithim of the molecular weights and the elution volumes from a Sephadex G-100 gel column for bovine serum albumin (○), ovalbumin (●), myoglobin (▵) and cytochrome C (▲).


LOG MOLECULAR WEIGHT
5.0
4.8
4.6
4.4
4.2
4.0
75
85
95
105
115
ELUTION VOLUME (ML)

These values were all of the same order and were similar to values reported for calf rennin 31,000 (Ilie et al., 1966) and porcine pepsin 34,520 (Moravek & Kostka, 1974). Porcine pepsinogen, bovine pepsinogen and calf prorennin have reported molecular weights of 38,944 (Rajogoplan, Moore & Stein, 1966), 38,943 (Chow & Kassell, 1968) and 36,200 (Ilie et al., 1966) respectively. Therefore if the adult bovine, lamb 1 and lamb 2 enzymes are of the rennin or pepsin type it appears that a peptide of molecular weight 4,000 to 9,000 is split from each zymogen upon the activation of these enzymes.

Amino acid composition

The amino acid composition of the adult bovine, lamb 1 and lamb 2 enzymes was determined on a Locarte Mark IV amino acid analyser or spectrophotometrically for tryptophan. The hydrochloric acid hydrolysates of each enzyme were analysed in triplicate and the amino acid compositions, in residues per molecule, calculated from the molar ratios. For methionine, threonine and serine only 24 hour hydrolysis data was used although the latter two were increased by 5% and 10% respectively to compensate for destruction. Valine, isoleucine and leucine were calculated from 72 hour hydrolysis data while the other amino acids were calculated from the average of all nine analyses (Creamer & Richardson, 1974). The amino acid composition of the enzymes, to the nearest whole number of residues per molecule, was calculated using the molecular weights determined by gel chromatography and is shown in Table IV. A molecular weight was calculated for each enzyme from the amino acid composition and is also presented in Table IV.

Table IV. Amino acid composition of adult bovine, lamb 1 and lamb 2 enzymes.

Amino acid	Adult bovine	Lamb 1	Lamb 2
Aspartic acid	31	23	34
Threonine	21	17	22
Serine	37	24	38
Glutamic acid	26	24	27
Proline	11	10	13
Glycine	28	24	29
Alanine	11	11	13
Half cystine	7	11	8
Valine	21	23	22
Methionine	2	5	3
Isoleucine	25	14	26
Leucine	19	19	21
Tyrosine	15	15	16
Phenylalanine	13	13	14
Histidine	1	4	1
Lysine	0	7	0
Arginine	2	4	2
Tryptophan	12	13	9
Number of residues	282	261	298
Molecular weights			
(a) Chromatography	31,030	29,340	32,450
(b) Calculated	30,823	29,345	32,303

The amino acid composition of the adult bovine and lamb 2 enzymes were very similar. They differed by no more than one residue for 12 amino acids and by no more than three residues for any amino acids. When the amino acid composition of these two enzymes was compared with that of bovine pepsinogen (Chow & Kassell, 1968) there

were many similarities allowing for the removal of a peptide upon activation of the pepsinogen to pepsin. The amino acid analysis of porcine pepsin (Moaravek & Kostka, 1974) also had similarities to that of adult bovine and the lamb 2 enzymes. For instance they all contained large numbers of acidic residues and small numbers of basic residues, 68 and 4 respectively in porcine pepsin, 57 and 3 in adult bovine and 61 and 3 in lamb 2 enzymes. These similarities suggest that the adult bovine and lamb 2 enzymes were pepsins and were probably the bovine pepsin (Fox, 1969, b) and the ovine pepsin (O'Leary & Fox, 1973) reported previously.

When the amino acid composition of the lamb 1 enzyme was compared with that of calf rennin (Ilie et al., 1966) and that of lamb rennins A & B (Oruntaeva & Seitov, 1971) it was found that there was a general similarity between the amino acid compositions of all four enzymes. This became particularly apparent when the data reported by the above workers was adjusted for tryptophan, cystine and methionine residues some or all of which they did not report. The basic amino acids in the lamb 1 enzyme totaled 15 which was considerably more than found in either adult bovine or lamb 2 enzymes and was similar to the number found in calf rennin, 17, lamb rennins A & B, 22 and 21 respectively. The similarities to the data reported for calf and lamb rennins plus the relatively large numbers of basic residues, compared to pepsins, suggested that the lamb 1 enzyme is a rennin.

DISCUSSION

Sodium chloride is used in rennet manufacture to dissolve the enzymes, stabilise solutions against bacterial action and act as carrier in rennet powders. It is cheaper than most other commercially available salts, is easy to use and therefore the stability of a rennet in the presence of sodium chloride is of importance in designing manufacturing and handling procedures for rennets.

The adult bovine, calf and lamb rennets used in this study each has a region of maximum pH stability which is enhanced by the addition of sodium chloride. The stability of each rennet is affected by temperature and all are most stable at 10°C or below. Therefore in the commercial processing of adult bovine, calf or lamb rennets sodium chloride solutions between pH 4.3 to 5.5 for adult bovine rennet, pH 5.0 to 6.5 for calf rennet and pH 3.8 to 4.6 for lamb rennet at temperatures of 10°C or less should be used to achieve maximum retention of activity.

Solutions of adult bovine, calf or lamb rennets containing 200 mg/ml of sodium chloride are all more resistant to heat denaturation than solutions containing 17 mg/ml of sodium chloride. However it seems unlikely that heat treatment could be used as a means of controlling bacteria in rennet production as all three rennets are partially inactivated by heat treatments less than that required for milk pasteurisation.

The adult bovine, calf and lamb rennets all retain some activity after a heat treatment of 68°C for 30 minutes in 200 mg/ml sodium chloride solution and curds and whey which have a higher concentration of organic matter than rennet solutions would help protect such labile substances from heat denaturation.

The most severe heat treatment used in cheese manufacture is 52°C for a maximum of 200 minutes for Swiss types of cheese and therefore adult bovine, calf and lamb rennets should not be greatly affected by such heat treatment probably remaining active in cheese manufactured with them.

Lamb rennet appears to have a lower relative activity at pH values above pH 6.55 than either calf or adult bovine rennets when activity is measured in casein solution. If this is confirmed then it would be necessary to use more lamb rennet than calf or adult bovine rennets when the pH of milk was above 6.55. This situation occurs towards the end of a New Zealand dairying season as the solids in the milk increase or during unusual climatic conditions. However when the activities of the rennets are compared by curd tension measurements lamb rennet appears to be less sensitive to pH change than either adult bovine or calf rennet. The disparity between these two methods of activity measurement could be due to the differences in the substrate used for each measurement or the solution used to dilute the rennets. The milk used for curd tension measurements had a solids content of 100 mg/ml while the caseinate solution used for the activity assay contained 30 mg/ml. This difference would not be expected to cause all of the disparity found between the two methods of activity measurement.

Water was used to dilute the rennet for curd tension measurements while a phosphate buffer at pH 6.55 was used to dilute the rennets for activity measurements in casein solution. Lamb rennet at pH 6.55 and 30°C in a buffer of 0.04 ionic strength lost 80% of its activity in 96 hours.

It would therefore seem that some of the disparity found between the two activity measurements may be due to the instability of lamb rennet under the conditions of the assay. This may also have been part of the reason for the observation that lamb rennet has a lower optimum temperature than either adult bovine or calf rennets. At pH 6.55 and 30°C in a buffer of 0.04 ionic strength adult bovine rennet lost 30% of its activity in 96 hours while calf rennet lost only 8% of its activity under similar conditions. Lamb rennet was quite heat stable at its most stable pH of 4.1 but the pH used in the activity assay in casein solutions, 6.55, was well removed from its stable region. The most stable pHs of adult bovine, (4.7) and calf rennet (5.4) are much closer to pH 6.55 and so they should be more stable at pH 6.55. Lamb rennet may therefore appear less active at higher temperatures as a greater proportion of it is denatured before it can hydrolyse k-casein in solution.

A cheesemaker uses his judgement of coagulum firmness to decide when a vat should be cut and obtains a firmer coagulum by;

(i) lowering the pH of the milk by allowing the starter bacteria to develop acid or by adding more starter.
(ii) adding calcium ions to the milk.
(iii) extending the setting time.
(iv) using more rennet. (this increases costs and is not generally practiced).

The adult bovine and lamb rennets used in this study respond to treatments simulating each of the above in a manner similar to the calf rennet controls. Therefore a cheesemaker can use adult bovine and lamb rennets in the same way as calf rennet to obtain the desired coagulum.

A satisfactory calf rennet substitute must have a high milk coagulating to proteolytic activity ratio (Fox, 1969, b) and ideally should also hydrolyse the individual casein fractions in the same manner as calf rennet. The adult bovine and lamb rennets used in this study are both slightly more proteolytic on whole casein in solution than calf rennet for the activities used in cheesemaking. However neither of these two rennets is as proteolytic as porcine pepsin (Fox, 1969,b) which has been used commercially for cheesemaking. The hydrolysis products from the three main casein fractions in solution produced by adult bovine and lamb rennets are similar to those produced by calf rennet. The rates of hydrolysis of α_s- and k-caseins, in solution, are faster for calf rennet than either adult bovine or lamb rennets. However β-casein, in solution, is hydrolysed more rapidly by adult bovine and lamb rennets than by calf rennet. Therefore it appears that the adult bovine and lamb rennets are suitable alternatives to calf rennet from a consideration of their proteolytic actions on whole casein and the three main casein fractions. The slower rate and less complete hydrolysis of k-casein, in solution, partially explains the necessity of using more active solutions of adult bovine and lamb rennets in cheese manufacture.

The amino acid composition of the enzymes isolated from adult bovine and lamb rennets indicates that the adult bovine rennet contained only one enzyme with milk coagulating activity and that it was a pepsin. Some bovine pepsin is present in most calf rennets depending largely on the age of the calves at slaughter and their diet prior to slaughter. There was no rennin present in the adult bovine rennet but this is as expected as the rennet was extracted from the abomasums of cattle slaughtered at over one year of age, and their diet would not have included milk since they were calves. Lamb rennet contained two

enzymes with milk coagulating activity, one pepsin and one rennin. It is of interest to find 22% of the milk coagulating activity of the lamb rennet in the form of a rennin enzyme as the rennet was obtained from the abomasums of lambs slaughtered at three to six months of age whose diet would still have included milk. Rennet obtained from calves of a similar age and diet will also have rennin present but probably not in such a large proportion. No attempt was made to separate any of these enzymes into possible components.

CHAPTER V

CHEESE MANUFACTURE

REVIEW OF LITERATURE

Cheese manufacture in New Zealand

The main type of cheese manufactured in New Zealand is cheddar which accounts for approximately 80% of the 100,000 tons manufactured annually. Milk used for cheese manufacture is from predominantly Jersey cows which produce milk with a higher fat content (national average 4.7%) than milk used for cheese manufacture in other countries. A survey of the manufacture of cheddar cheese in New Zealand was prepared by Robertson (1970) and unless otherwise quoted the following discussion is based on that report.

The milk used for cheese manufacture in New Zealand must be pasteurized at 70°C for at least 15 seconds and may be standardised by the removal of fat to produce a cheese containing 52 to 55% fat in the water free substance (Dolby, 1971). The milk is standardised either by diverting some of the milk through a separator for the time necessary to remove a calculated quantity of fat during the filling of each vat (Dolby, 1971) or by diverting a portion of the pasteurized milk through a separator continuously to remove the required quantity of fat (Gill es, 1971). In both cases the skim milk from the separator is returned to the milk before it reaches the cheese vat.

Virtually all the cheddar cheese manufactured in New Zealand is made using single strain starters of *Streptococcus cremoris* and *Streptococcus lactis*.

The starters are maintained, usually in laboratories close to each factory, in skim milk and mother cultures prepared for daily innoculations into water sealed bulk starter vessels. Different strains of bacteriophage unrelated starters are used in rotations of three or more fills of milk and strict hygiene is practiced to reduce the possibility of bacteriophage build up and subsequent attack on starters causing slow acid development and poor quality cheese.

The flavour of cheddar cheese is largely dependent on the starters used and a postulated theory (Lowrie & Lawrence, 1972) suggests that both the starter and rennet proteases cause a breakdown of casein to peptides, some of which are bitter. Some starters always produce bitter cheese with calf rennet but not with a protease of fungal origin (Lawrence, Creamer, Gill es & Martley, 1972) suggesting that each starter strain should be used with each rennet type to determine combinations that produce good flavoured cheese. The bitterness often associated with cheddar cheese made with single strain starters is affected by the starter population reached during cheesemaking and can be controlled by the cooking temperature used for each strain (Lowrie, Lawrence & Pearce, 1972) or by the addition of bacteriophage to the cheese milk (Lowrie, Lawrence & Peberdy, 1974). Martley & Lawrence (1972) suggested that to make good flavoured cheddar cheese a starter must have the following characteristics;

(i) poor survival in cheese matured at 13°C.
(ii) a low rate of cell division at the cooking temperature.
(iii) low proteolytic activity.
(iv) high acid phosphatase activity.

A typical manufacturing scheme for cheddar cheese is described and commences when the starter (1.5 to 2.5%) is injected into the pasteurized milk, which has been cooled to 31 to 32°C, during vat filling.

When a vat is full rennet is added and stirred into the milk. After setting for 30 to 40 minutes the coagulum is cut into 1 cm cubes and stirring commenced. A few minutes later heating begins, slowly at first, and then more rapidly to achieve a temperature of 37 to 40°C in not less than 30 minutes. Approximately two hours later agitation stops, the curd allowed to settle and the whey drained off. Mechanical agitation is used to keep the curd particles free and to assist whey drainage. By the time the curd is dry the acidity of the fresh whey running from the curd is 0.18 to 0.20% lactic acid. The curd fuses into blocks and is cheddared until an acidity of 0.55 to 0.65 % lactic acid is obtained, usually about two hours after running off the whey. The cheddared curd is milled into fingers before salt addition followed by mixing to ensure uniform salt distribution and whey drainage. The curd is then hooped and pressed overnight to form 18 kg blocks of cheese. Next day the cheese are wrapped, to maintain moisture and prevent mold contamination etc., placed in a carton and stored at 13°C for 14 days. Cheese from each vat is then graded by the Ministry of Agriculture and Fisheries and transfered to a store at 6°C until required for shipment to a market.

Calf rennet is the only coagulant used for cheese manufacture in New Zealand although trials have been carried out with a number of other rennets (Robertson & Gill es, 1969; Clarke, 1974). In their trials Robertson & Gill es (1969) compared Meito fungal rennet with calf rennet and found that provided calcium was added to the milk to achieve a similar coagulum to calf rennet the cheese manufacturing proce dures were the same for both. This was true for a variety of coagulants, of microbial and animal origin, although Clarke (1974) achieved similar ccagulums to calf rennet by adjusting the quantity of coagulant added to the milk.

METHODS

Cheese manufacture

Cheddar cheese was manufactured from pasteurized (72°C for 15 seconds) commercial whole milk, in the New Zealand Dairy Research Institute's Processing Hall, by a small scale (320 l) simulation of the conventional commercial methods (Robertson, 1970). The quantity of adult bovine, calf, lamb or mixture (50/50 calf-adult bovine rennets) rennets was the same (18 ml/100 l) for each vat although the activity of each coagulant had been adjusted to achieve the same curd tension as calf rennet under similar conditions. Cheese was manufactured four times during the 1973/74 dairying season on consecutive days with a different pair of starters (ML8, AM1; P2, AM2) on each of the two days. Cheese was made on two occasions with all four rennets and on two occasions without the mixture rennet. All of the vats were set at 32°C and cooked to 37.8°C in 30 minutes. The coagulum was cut with 1 cm knives and the titrateable acidities at running, drying, milling and salting as well as the number of dry stirs and salt addition were all kept constant, as far as possible, between vats on any one day. Two 18 kg rindless cheese were made from each vat and stored at 13°C for two weeks after manufacture. One cheese from each vat remained at 13°C while the other was stored at 6°C and both were kept for up to 12 months. All cheese were analysed at 14 days for salt, moisture and fat while the pH was measured the day after manufacture.

Cheese flavour and body assesment

Cheddar cheese, manufactured with each rennet was evaluated organoleptically 3 and 6 months, after the date of manufacture, for flavour, body and various

faults (see Appendix I). A team of four to eight experienced graders, drawn from a panel of nine, was used for each evaluation which was as described by Lowrie, Lawrence & Peberdy (1974). All the cheese made on the same day was presented to the graders at any one evaluation session and the individual vats were identified by random numbers. A control cheese which had a good flavour was included in each grading session as a reference. Cheese flavour was scored on a five point scale with one representing poor and five very good flavour.

Casein degradation in cheese

Cheddar cheese was manufactured with each rennet and stored for up to twelve months at 6°C. The degradation of the casein components of the cheese was followed from electrophoretograms of each cheese sampled at one, three, six, nine and twelve months after manufacture. Samples of each cheese were stored frozen until all were available for alkaline polyacrylamide gel electrophoresis which was by the method of Creamer (1970).

RESULTS

Cheese manufacture

On each occasion that cheese was manufactured the analyses showed the cheese to be typical of New Zealand cheddar and that differences between the cheese from each vat were small. A typical day was the four vats of cheese manufactured on 24.1.74 using 2% of a 1:2 mixture of P2 and AM2 starters. Acidities were 0.15% lactic acid at setting, 0.095% at cutting, 0.125% at running, 0.14 to 0.145% at drying, 0.49 to 0.52% at milling and 0.68 to 0.71% at salting. Total make time from renneting to salting was uniform at 5 hours 20 minutes. Cheese pH at one day was 5.08 to 5.10 and at 14 days moisture was 33.0 to 33.8%, salt 1.52 to 1.62%, moisture in the non-fatty substance 53.2 to 54.5% and salt in the moisture 4.54 to 4.91%. These analyses show that the cheeses made with each rennet were of similar composition 14 days after manufacture and demonstrated that any differences between the cheeses in other characteristics were not due to differences in composition.

Cheese flavour and body assesment

The frequency of the flavour scores of cheese made with adult bovine, lamb or mixture rennets being higher or lower than the score for the corresponding cheese made with calf rennet was recorded and is shown in Table V.

Appraisal of Table V suggests that cheese made with adult bovine, lamb or mixture rennets was preferred approximately as often as cheese made with calf rennet. Therefore these data were tested against the null hypotheses (Ho) that the cheese made with calf rennet was preferred in half of the comparisons, ie Ho (Pc = 0.5)

Pc = the probability that cheese manufactured with calf rennet was preferred to the alternative.

Table V. The frequency of the difference between flavour scores of cheese made with adult bovine (B), lamb (L) or mixture (M) rennets compared with the corresponding cheese made with calf (C) rennet.

Date of manufacture	Age at evaluation (months)	Number of comparisons	Flavour scores C>B	B>C	C>L	L>C	C>M	M>C
20.9.73	3	10	1	1	0	1		
	6	12	5	2	0	2		
21.9.73	3	14	5	1	5	2		
	6	8	0	3	1	2		
3.12.73	3	10	1	2	0	3		
	6	10	1	2	0	3		
4.12.73	3	10	3	1	3	1		
	6	8	1	2	0	2		
23.1.74	3	13	0	1	0	0	2	1
	6	10	1	0	2	1	1	1
24.1.74	3	14	3	2	2	1	2	0
	6	10	3	1	4	1	5	2
11.3.74	3	13	2	3	1	4	2	4
	6	10	4	2	1	1	4	1
12.3.74	3	15	5	0	7	0	0	0
	6	10	3	2	2	2	1	1
Totals		177	38	25	28	26		
		95					17	10

The data, Table V, shows that in the majority of the comparisons there was no difference in the flavour scores between the two cheese considered and half of this number was added to the frequency of calf rennet cheese having a higher flavour score than the cheese made with the alternative rennet, (American Society for Testing and Materials, 1968). As the number of comparisons was large data was considered to be from a normal distribution and was tested against Ho (Pc = 0.5) by the formula;

$$\mathcal{U} = \frac{(\hat{p} - p) - 1/2n}{\sqrt{pq/n}}$$

Where $\mathcal{U}$ = normal variable for $n \geq 30$
n = number of observations
$\hat{p}$ = observed proportion
p = null hypotheses of probability
$q = 1 - p$
(Moore, Shirley & Edwards, 1972).

Table VI. Normal variable values testing the hypotheses that cheese made with calf rennet was preferred in half of the comparisons with cheese made with adult bovine, lamb or mixture rennets.

Rennet	Number of comparisons	$C^a > X^b$ Uncorrected	$C^a > X^b$ Corrected	Normal variable	Significance
Adult bovine	177	38	95	0.89	NS[c]
Lamb	177	28	89.5	0.08	NS
Mixture	95	17	51	0.63	NS

a = cheese made with calf rennet
b = cheese made with rennet shown in the left hand column.
c = not significant at the 5% level.

From Table VI it can be seen that in all cases there was no significant difference between the null hypotheses and the observed data and hence the null hypotheses can be accepted. Therefore there was no significant difference in the flavour of the cheese made with calf rennet compared individually with the cheese made with adult bovine, lamb or mixture rennets.

Cheeses were also examined for the flavour characteristics of bitterness, astringency, fruitiness, sharpness and other flavours. Scores for these characteristics were alloted to each cheese at each grading and the average score for cheese made with adult bovine, calf, lamb or mixture rennets were calculated and are shown in Table VII.

Table VII. The average scores of flavour characteristics for cheese made with adult bovine, calf, lamb or mixture rennets.

Characteristic	Rennet			
	Adult bovine	Calf	Lamb	Mixture
	(average score)			
Bitterness	1.40	1.42	1.39	1.42
Astringency	1.54	1.42	1.54	1.29
Fruitiness	1.50	1.63	1.50	1.59
Sharpness	1.23	1.24	1.26	1.21
Other flavours	1.96	1.66	1.97	2.15

Data shown in Table VII indicates that the flavour characteristics considered were, on average, virtually absent from all the cheese (a score of 2 indicates that the flavour was possibly present). "Other flavours" were possibly present in cheese made from adult bovine, lamb or mixture rennets but there was no agreement between graders as to any one "other flavour" for the cheese made with any one rennet.

The body of each cheese was scored by each grader using a number to indicate the type of cheese body as shown below;

1. sticky, pasty, glutinous, weak.
2. smooth.
3. pleasant.
4. grainy, lumpy.
5. dry, sandy, furry.

The frequency that each body score was alloted to cheese made with each rennet were totalled and the percentage of judgements alloted to each score for each rennet calculated. This data is shown in Table VIII.

Table VIII. The percentage of judgements alloted to each body score of cheese manufactured with adult bovine, calf, lamb or mixture rennets.

Body score	Rennet			
	Adult bovine	Calf	Lamb	Mixture
		(%)		
1	0.0	1.1	0.0	0.0
2	15.9	15.3	12.5	28.7
3	71.6	76.2	77.3	57.5
4	12.5	7.4	10.2	13.8
5	0.0	0.0	0.0	0.0

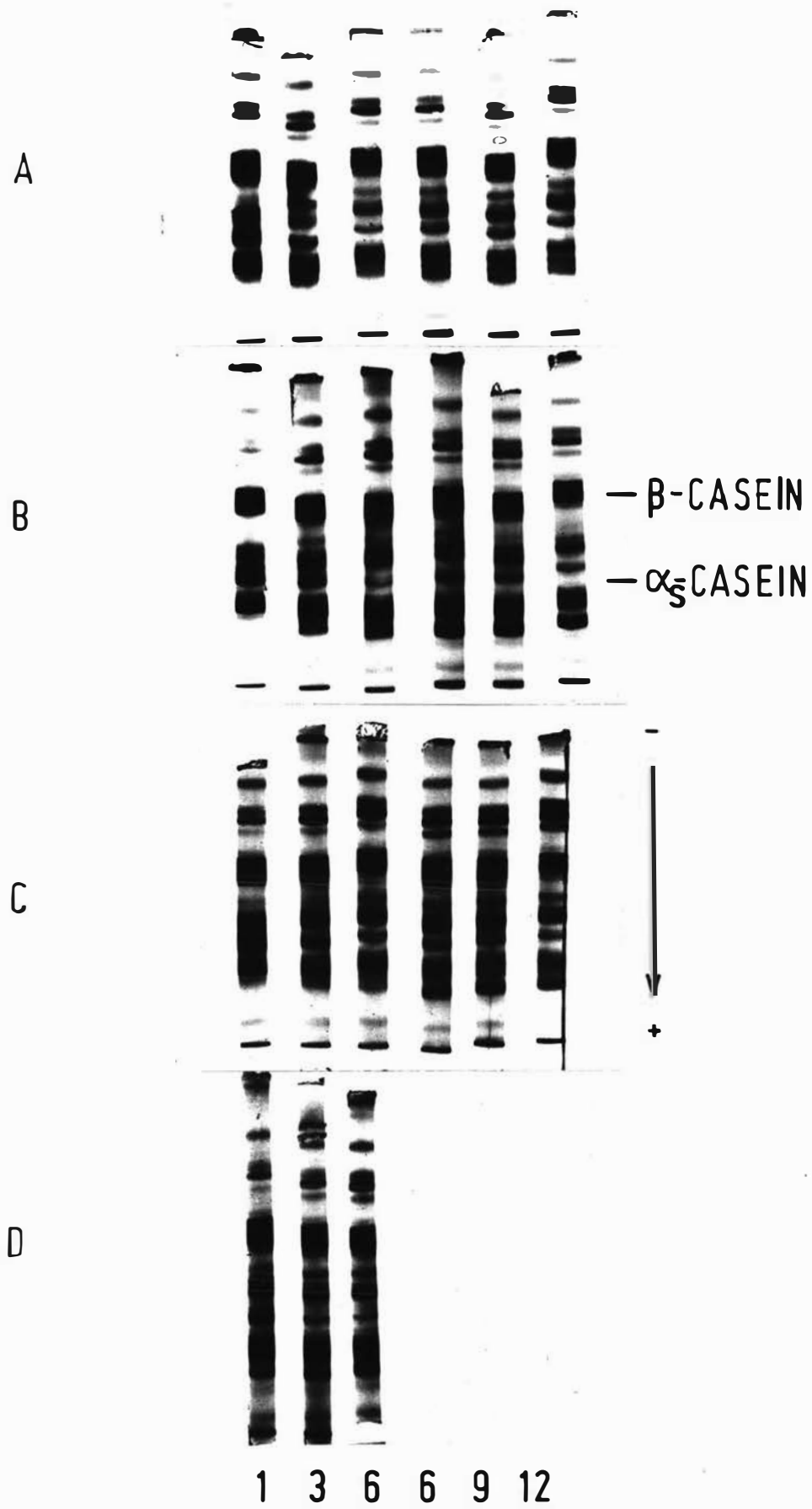
Body scores of 2 or 3 accounted for at least 86% of the judgements of the cheese made with each rennet indicating that the cheese body was usually smooth or pleasant. Differences in the proportion of body scores other than 2 or 3 were small an in no case was there agreement between the majority of graders that any one cheese was other than smooth or pleasant.

Casein degradation in cheese

The degradation of the casein in the cheese manufactured with adult bovine, calf, lamb or mixture rennets was determined from electrophoretograms of cheese at one, three, six, nine and twelve months after manufacture. The oldest cheese (manufactured 20.9.73) made with each rennet was sampled after six, nine and twelve months storage at $6^{o}C$ while the youngest cheese (manufactured 12.3.74) was sampled after one, three and six months

storage at 6°C. All samples from the two cheese made with each rennet were electrophoresed on alkaline polyacrylamide gels. The photographs of these gels, shown in Figure 22, demonstrate that the four rennets produce cheese with indistinguishable electrophoretic patterns. There were the same number of bands from each cheese although the intensity of some varied slightly. The β-casein was largely unhydrolysed as was found in cheddar cheese made with calf rennet (Creamer, 1970). The α_s-casein was hydrolysed almost completely to several bands of peptides. At least four bands were apparent in the area between the origin and the β -casein with another two bands between the β-casein and the α_s-caseins. One further band, more mobile than α_s-casein, and probably the α_{s1}-I peptide (Fox & Guiney, 1973), was present but in turn was hydrolysed as the cheese aged. The casein degradation in these cheese appeared similar to that already reported for cheddar types (Creamer, 1970) and was indistinguishable between the various rennets used to manufacture the cheese.

Figure 22. Alkaline polyacrylamide gel electrophoretograms of cheese, after one, three, six, nine and twelve months storage at $6^{o}C$, manufactured with adult bovine (A), calf (B), lamb (C) and a 50/50 mixture of calf-adult bovine (D) rennets.


A
B
C
D
— β-CASEIN
— α_{s_1}-CASEIN
−
+
1 3 6 6 9 12

DISCUSSION

Cheddar cheese was manufactured with adult bovine, lamb and mixture rennets and compared at all stages with cheese made at the same time from the same milk under the same conditions with calf rennet. The activity of the milk coagulating enzymes was adjusted in each alternative coagulant to ensure a similar coagulum in the same setting time as that produced by calf rennet. This was achieved and from a cheese manufacturing point of view there was no difference in the manufacture of any vat of cheese regardless of the coagulant. Cutting of the coagulum produced varying remarks from the cheesemaker such as, curd fractured more easily, not as smooth or grainy appearance but there was no consistant remark attributed to any one coagulant. Calf rennet received as many such comments as any of the alternative rennets.

Acid production was not affected in any way by the coagulant used and the acidities at each stage throughout the manufacture on any one day were as even as could be expected between three or four vats of cheese. This was confirmed as the pH of one day old cheese was also very even between vats manufactured on the same day. There was no apparent difference in the appearance of the curd in the vat and the rate of moisture expulsion from the curd as it was not found necessary to make variations in manufacture between vats. The weight of curd from each vat was measured each day, to calculate salt requirements, and the weights were invariably within 500 g of each other suggesting that there were no differences in yield, although much larger trials would be necessary to obtain reliable information on yield. The cheeses removed from the hoops the day after manufacture were identical and there were no differences detected in such things as gas formation at any stage during

twelve months storage. A cheesemaker could not detect any difference in the manufacture of cheese using adult bovine, lamb or mixture rennets instead of calf rennet.

Cheese manufactured with these three alternative coagulants was compared for flavour and body characteristics with cheese made with calf rennet after three and six months storage at both 6 and 13°C. There was no overall preference for cheese made with calf rennet compared with cheese made with any of the alternative coagulants or vice versa. Closer examination of this data showed that there was no real preference for cheese made with any coagulant for different pairs of starters or at three months as compared to six months storage or storage at 6°C compared to 13°C and therefore the overall picture was true for these other variables also.

A comparison between cheese made using the alternative rennets and calf rennet for flavour characteristics showed that in most cases there was no difference. For some characteristics the average score for calf rennet was slightly higher than the alternatives and for some other characteristics slightly lower. The only possible differences were in "other flavours" but graders were not in agreement in the type of "other flavour".

The body scores alloted each cheese after three and six months storage showed a very similar pattern, particularly between cheese made with adult bovine, calf and lamb rennets. However cheese was made with mixture rennet on only two occasions, both in the second half of the dairying season, and a true comparison with body scores would be achieved only if cheeses made on the same days were compared. Even so the only difference between the mixture rennet and the other three was the proportion of cheese bodies called smooth rather than pleasant.

The degradation of casein in cheese made with adult bovine, calf, lamb or mixture rennets was evaluated from alkaline polyacrylamide electrophoretograms of cheese after various lengths of storage at 6^{0}C. The electrophoretograms showed that all rennets produced the same number of bands of casein degradation products in cheese as was expected from information obtained on the hydrolysis of individual casein fractions in solution. The intensity of some of the bands varied between coagulants but again this was expected as the rate of hydrolysis of the casein fractions in solution varied slightly as did the proteolytic activity of each rennet on whole casein. However each coagulant produced cheese with electrophoretograms typical of cheddar cheese.

CHAPTER VI

CONCLUSIONS

The study of any enzyme system requires a reliable method to assay the enzyme activity, and a survey of the methods available to assay milk coagulating enzymes indicated the need for a better method. With this in mind perchloric acid was compared with trichloroacetic acid as a precipitant for casein-rennet reaction mixtures and found to be very similar in all respects. Therefore perchloric acid was able to be used to quench casein-rennet reactions so that a direct spectrophotometric measurement of the soluble products released could be obtained. This was the basis of an assay developed to measure milk coagulating enzyme activity. The method had many advantages over most reported methods. A paper describing this assay was published (Clarke & Richards, 1973).

The large number of cattle and lambs killed in New Zealand each year is an obvious source of raw material for rennet extractions. Hence extracts of milk coagulating enzymes were prepared from the abomasums of these two species by small scale simulation of commercial methods used to extract calf rennet. The properties of these two coagulants, as they relate to the extraction and handling in rennet manufacture, were found to be similar in some respects to calf rennet. All three rennets were most stable in sodium chloride solutions, within specific pH limits for each coagulant, at temperatures below $10^{\circ}C$. However both adult bovine and lamb rennets were more resistant to heat denaturation than calf rennet but heat treatment is not recommended during processing.

Cheese manufacturing properties of adult bovine and lamb rennets were similar in most respects to those

properties of calf rennet. The activity of lamb rennet appeared to be lower than that of either adult bovine or calf rennets at high pH and temperatures above 30°C when measured in casein solutions but was of similar activity to the other two rennets at high pH in milk. Both adult bovine and lamb rennets appeared to coagulate milk in a similar manner to calf rennet although both coagulants appeared more sensitive than calf rennet to the calcium concentration of the milk. Less adult bovine or lamb rennet would be required if the calcium concentration of the milk was maintained above a level of approximately 38 mM calcium.

The proteolysis products of both adult bovine and lamb rennets were similar to those of calf rennet both in solutions of casein fractions and cheese manufactured with each rennet. The rate of proteolysis varied slightly in both media but the difference in the cheese body was not detectable after 6 months storage at 6 or 13°C.

Milk coagulating enzymes were readily isolated from the crude extracts of both adult bovine and lamb rennets. There was a pepsin present in each extract and the lamb rennet contained a rennin as well. The ion exchange chromatography method used to remove the mucoproteins from these rennets suggested that a similar method would also apply to calf rennet. Successful trials led to the development and operation of a commercial scale plant to remove mucoproteins from crude solutions of calf rennet. This development has solved a problem that has concerned rennet manufacturers for many years. The first report of a commercial method for the removal of mucoproteins from rennet solutions was published in 1937 by van der Berg and van der Scheer, but it did not remove all the mucoproteins.

Cheese manufacture was not affected in any way by the use of adult bovine or lamb rennets instead of calf rennet. The cheese produced by these three rennets was not significantly different in either body or flavour after storage at 6 or 13°C for 6 months.

Both adult bovine and lamb rennet proved to be acceptable alternatives to calf rennet for the manufacture of cheddar cheese. The source of raw material of these two alternative coagulants appears more certain in the future than the source of calf rennet. However the acceptance of adult bovine and lamb rennets by cheese manufacturers will depend upon further successful cheese manufacturing trials on a commercial scale. Legislation in each country will also need to be considered by any cheese manufacturer wishing to change the rennet used to manufacture cheese. The supply of calf rennet and other coagulants will also affect any decision to change rennets as of course will the cost of each rennet. It was apparent from the small scale extraction of both adult bovine and lamb rennets that a larger quantity of abomasum tissue was required to obtain similar activity of milk coagulating enzymes than for calf rennet. Therefore the cost of abomasums from cattle or lambs may reflect only their value for other uses, such as pet food, rather than their value as an enzyme source. There is a challenge to develop an extraction method that will enable the sale of the abomasum tissue after enzyme extraction to reduce the raw material costs.

APPENDIX I

Cheese flavour assessment

Please examine the cheese in terms of the following flavour characteristics. These will be bitterness, astringency, fruitiness and sharpness. Other flavours that might occur are rancid (fat breakdown), sour, sulphide, butter, salt, malt and musty. The cheese will also be given scores for texture and "cheesiness" or cheddar flavour.

The flavours will be scored 1-5 as follows:

1 - that particular flavour absent
2 - flavour possibly present, perhaps largely masked by other flavours
3 - definitely present
4 - quite strongly present, predominant flavour
5 - strongly flavoured

Note Astringency and bitterness usually occur as after-tastes.

Body to be scored as follows:

1 - sticky, pasty, glutenous, weak
2 - smooth
3 - pleasant
4 - grainy, lumpy
5 - dry, sandy, furry

Cheesiness to be assessed in the range 1-5.
Neglect the effect of defect flavours, if possible.

From New Zealand Dairy Research Institute.

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