THE ACTION OF RENNIN ON β-CASEIN

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ABSTRACT

A study was made of the action of the enzyme rennin on β-casein. Hydrolysis of β-casein initially at a single sensitive bond under controlled conditions of temperature, pH and relative enzyme and substrate concentrations, formed the basis of the investigation. Information on the hydrolysis of this sensitive bond was gained from the isolation of a small peptide produced and from a study of the effect of several parameters on the rate of hydrolysis. Evidence obtained from electrophoresis and gel filtration allowed the assumption that attack on the sensitive bond resulted in a macropeptide and a small peptide of molecular weight about 2000. The small peptide was isolated and partially characterised. As a result it appears that the small peptide is derived from the C-terminal end of the β-casein molecule.

A polyacrylamide electrophoresis technique was used to study the effect of ionic strength and calcium ions on the rate of hydrolysis and the rate of appearance and disappearance of

degradation products at 10°, 25° and 37°C. It was found that an increase in ionic strength retarded the reaction and the addition of calcium ions at a constant ionic strength further retarded the reaction. Also, the rate of appearance and disappearance of degradation products was found to increase with increasing temperature. A development of the polyacrylamide technique into a quantitative one enabled the determination of the Michaelis constant at pH 6.50 and 37°C for the rennin hydrolysis of β-casein as 9.59 g/l. This technique was also used to study the rate of hydrolysis at pH 6.12, 6.50 and 6.94 where an optimum rate occurred at pH 6.50.

Finally, assuming that the small peptide is derived from the C-terminal end of the β-casein molecule and allowing for the sequential degradation elucidated by the temperature studies, alternative courses of rennin degradation of the β-casein molecule have been proposed.

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INTRODUCTION

(a) General

Milk contains most of the ingredients required for a balanced diet. The average composition of bovine milk is given in Table 1[1].

TABLE 1

Butterfat	4.7%
Whey protein	0.9%
Caseins	2.8%
Lactose	4.6%
Mineral	0.7%

One form in which much of the nutritional value of milk may be preserved is cheese, where some 80% of the protein, 95% of the fat and smaller amounts of the mineral are precipitated by the combined action of rennet and an acid producing bacterial culture. Many of the final qualities and characteristics of the cheese are determined by the way in which the milk proteins, and in particular the caseins, are broken down or hydrolysed into smaller peptide fragments.

(b) Caseins of bovine milk

Until the 1920's casein was believed to be a single protein. However in 1939 Mellander[2] observed

the resolution of acid precipitated casein into three peaks on moving boundary electrophoresis which he called α-, β- and γ-casein in order of decreasing mobility at pH 7.0. It is now known that casein also contains a number of other components. In 1956 Waugh and Von Hippel[3] showed that α-casein could be further divided into two components which they called α_s- and $\varkappa$-casein. The α_s-casein fraction has since been found to be further subdivisible into α_{s1}-, α_{s2}- and α_{s3}-caseins in order of decreasing mobility[4]. The existence of genetic variants of α_{s1}-, β-, and $\varkappa$-caseins has been shown. Thompson et al.[5], using starch gel electrophoresis described the genetic variants α_{s1}-casein A, B and C in order of decreasing mobility. Grosclaude et al.[6] have described α_{s1}-casein D which has a mobility on starch gel electrophoresis intermediate between the A and B variants. Aschaffenburg[7] demonstrated the occurrence of three genetic variants of β-casein designated as β-casein A, B and C in order of decreasing mobility on paper electrophoresis. The β-casein A variant has since been shown to

have three distinct genetic types[8] observable on polyacrylamide electrophoresis at acid pH. A suggested nomenclature for these three types is β-casein A^1, A^2 and A^3. In the case of κ-casein, genetic polymorphism was demonstrated by gel electrophoresis after reduction of the intermolecular disulphide bonds. Independently three workers[9,10,11] demonstrated the presence of two variants of κ-casein, which are called κ-casein A and B. The relative mobilities of the casein variants on disc polyacrylamide electrophoresis at pH 8.9 are shown (Fig. 1).

In this thesis definitions given by the American Dairy Science Association (ADSA) Committee on Milk Protein Nomenclature, Classification and Methodology will be followed[4]. Alpha-casein is defined as "a fraction of whole casein containing α_s- and κ-caseins, soluble in 6.6 M urea, but insoluble in 4.6 M urea. $Alpha_s$-casein is the fraction insoluble in 0.40 M $CaCl_2$ at pH 7.0 and at 0-4°C and stabilized by κ-casein in the presence of Ca^{++}". Kappa-casein is "that fraction soluble in 0.40 M $CaCl_2$

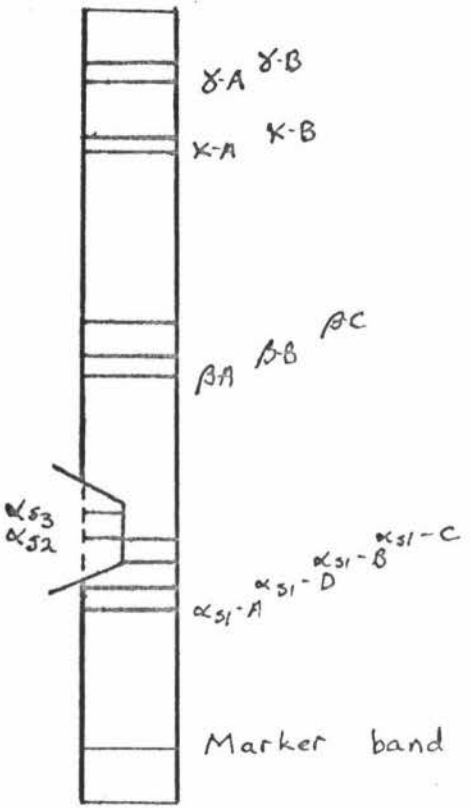


Fig. 1. Diagram showing the relative mobilities of the caseins of bovine milk on disc PAE at pH 8.9.

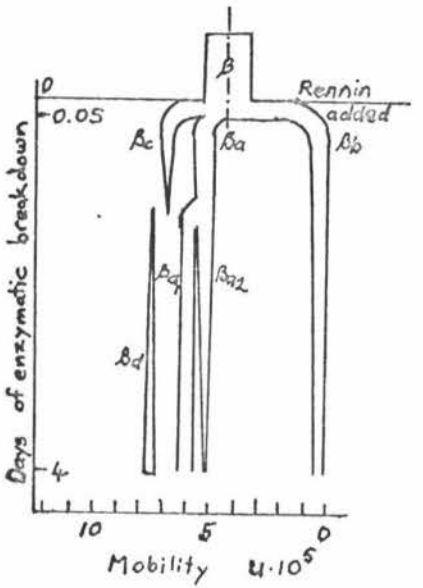


Fig. 2. Diagram of the course of the degradation of β-casein by rennin at pH 5.10 and 17°C. Reproduced from the work of Linqvist and Storgards[36].

at pH 7.0 and 0-4°C and is the principal casein affected by rennin in the primary phase of rennin action; an insoluble para-κ-casein and a soluble glycomacropeptide being formed". Beta-casein is defined as "a fraction of whole casein soluble in 4.6 M urea at pH 4.6 and is precipitated with Ca^{++} at 35°C, but not at 4°C, and possesses ultracentrifugal association-dissociation properties which are pronounced at 8.5°C".

The caseins, phosphate, citrate and metal ions exist in milk as spherical aggregates or micelles having a diameter between 40 and 300 nm. Many models of micelle structure have been proposed but the more recent ones are based on the concept that an insoluble core of aggregated α_{s1}- and β-casein is coated with κ-casein or a κ-/α_{s1}-complex which prevents the α_{s1}- and β-casein from aggregating further and forming a precipitate[12,13,14].

(c) Rennin and its action in cheesemaking

Rennin is the predominant active enzyme of rennet, an extract from the fourth stomach of newly born calves. Commercial rennet, as

obtained, from the New Zealand Co-op. Rennet Co. Ltd., has more than 95% of its enzyme activity due to rennin[15]. A quantitative measure of rennet activity presents some difficulties because of its narrow specificity and because a suitable standard substrate is not readily available. However calibration of rennet solutions within one laboratory is possible by using standard milk powders. A standard rennet is usually made available by the rennet manufacturing company. In the present work crystalline rennin was used as a standard.

The basic step in cheesemaking or in the production of rennet casein is the introduction of rennet into milk which rapidly forms a clot or gel. Rennin action has been considered to consist of three distinct but overlapping phases. In fact the three phases can only be distinguished from each other by the order in which they finally cease. When an optimum amount of rennin for the observation of physical change is added to milk a period is observed before a clot or coagulum forms. This period is denoted as the

primary phase. The clot synereses (shrinks with the exudation of moisture) and may be readily separated from the liquid or whey. This is denoted as the secondary phase. After a further period of time the protein clot breaks down and becomes less rigid. This is denoted as the tertiary phase.

It is now established that in the primary phase rennin preferentially attacks a phenylalanyl-methionine bond[16,17,18,19] in κ-casein with great facility. The production of para-κ-casein and a glycomacropeptide (GMP) by this attack destroys κ-casein's micelle protecting property[20,21,22]. The GMP is split from the C-terminal end of the κ-casein molecule and has methionine as N-terminal[18,19].

The secondary phase comprises many different steps during which the degree of dispersion of the casein micelles is changed. These are disaggregation, reaggregation and coagulation, and syneresis of the coagulum formed. The changes before and during coagulation have been demonstrated with a variety of techniques including electron

microscopy[23,24,25,26] and viscometry[27,28] (from which the most complete description has been developed). Syneresis also appears to proceed stepwise[29,30,31] beginning with the removal of protein bound water from the micelles during the hydrolysis of κ-casein. The next stage involves formation of hydrogen bonds between the casein aggregates. Bonds are established between more and more of the polar groups resulting in the visible shrinking of the coagulum.

The relatively non-specific proteolytic breakdown which is so apparent in the tertiary phase proceeds simultaneously with the primary phase. Other bonds of κ-casein and the other caseins are attacked by rennin more slowly than the sensitive κ-casein bond[32]. The final stage of the tertiary phase is reached when there are no further rennin sensitive bonds to be hydrolysed.

(d) Rennin proteolysis of β-casein

Reports on the resistance of β-casein to rennin action have been diverse because of the disregard of the relative enzyme and substrate concentrations and reaction time, choice of

conditions for precipitating the products and the techniques by which the process was followed. Early reports have also proved to be misleading. Cherbuliez and Baudet[33] in 1950 and Alais et al.[32] in 1953 reported that rennin action on sodium caseinate solutions resulted in attack on α-casein alone. Cherbuliez and Baudet followed the reaction by electrophoresis while Alais et al. followed it by the estimation of non-protein nitrogen (NPN) soluble in 2 or 12% trichloroacetic acid (TCA). These reports were made before the discovery of χ-casein and the realisation that the α-casein fraction contained both α_{s1}- and χ-casein and it seems likely that they were only observing the initial attack on χ-casein and the relative concentrations of reactants were too low to observe a non-specific attack.

Others have reported that rennin action on β-casein shows only a slow general proteolysis[34]. In this case a measure of proteolysis was obtained from electrophoresis of a 12% TCA soluble fraction. Cerbulis et al.[35] showed that the pH 4.7 soluble fraction was a more accurate measure of proteolysis

and in fact identified seven products on free boundary electrophoresis. A significant contribution to the elucidation of the rennin degradation of β-casein was made by Lindqvist and Storgards[36] in 1960. They attempted to chart the primary course of the degradation of β-casein by crystalline rennin. Their conclusion was that the degradation follows distinctly different paths depending on pH. Under their conditions, however, they did not observe any one bond with greater sensitivity, but only a primary cleavage giving three components. They found that over a period of time there was such a profusion of products with such widely varying electrophoretic properties that the elucidations of the various paths of degradation under different conditions was difficult, and that it was not possible to give any generalised degradation curves covering the whole pH range. A diagrammatic representation of the course of degradation, as shown by moving boundary electrophoresis curves for pH 5.10, is shown (Fig. 2). Lindvist and Storgards noted that corresponding diagrams are somewhat similar at higher pH values and during the early stages

at low pH values. These authors also report that NPN formation is reasonably independent of pH and is therefore no indication of the course of proteolysis of β-casein.

Little further useful work was done on the degradation of β-casein until 1968. However, Lahav and Babad[37], in 1964 showed that the action of rennin on α_{s1}-, κ- and β-caseins gave rise to calcium precipitable and soluble fractions; a specific fraction for each of the caseins.

In 1968 Ledford et al.[38] examined the products of commercial rennet proteolysis of purified fractions of α_{s1}- and β-caseins. Fractions soluble in 5% TCA were subjected to electrophoresis on polyacrylamide gels (PAE) and absorbancy at 280 nm was measured. They conclude that rennin preferentially degrades α_{s1}-casein. Increase in absorbance was measured as a function of pH (5.5-8.5) and time. The conclusion that α_{s1}-casein was preferentially degraded was made on the assumption that 280 nm absorbing material is equally available for release from both α_{s1}- and β-caseins. They

showed that α_{s1}- and β-caseins in solution are degraded more rapidly than when in the micellar form (both at pH 5.6). It should be noted, however, that there are necessarily other differences between these systems e.g. the milk sample contains calcium ions and is at a higher ionic strength. In addition they found, by electrophoresis, that the α_s-casein bands disappeared more rapidly than the β-casein bands. In contrast examination of the rennet degradation of carefully purified casein fractions by Lawrence and Creamer[39] using PAE showed that at pH 6.50 and 37°C the rates of degradation of α_{s1}- and β-caseins, to the primary products, are very similar.

Very little has been reported on the influence of parameters other than pH on the proteolysis of β-casein. Bakri in 1968[40] examined the effect of $CaCl_2$ and NaCl on this reaction by measuring the turbidity of the reaction solution. Bakri reported that the activity of rennin action on β-casein measured as initial velocity, reached a peak at 8×10^{-3}M Ca^{++} on 0.666 gm/l β-casein, inhibition occurring

at Ca^{++} concentrations above $8x10^{-3}M$. He also concludes that NaCl totally inhibits rennin action on β-casein. These conclusions are not justified as the turbidometric technique is not always a reliable guide to proteolysis. For example Bakri reported that the addition of increasing amounts of α_s-casein to χ-casein increasingly inhibited rennin action. It has since been conclusively shown by Lawrence and Creamer[39] using PAE that χ-casein proteolysis continues at the same rate but that aggregation of the para-χ-casein is being inhibited.

(e) Present work

It was felt that much of the previous work on casein proteolysis was of limited value because no account was taken of the non-specific nature of NPN determinations and because turbidity measurements are open to misinterpretation. In addition confusion has resulted from the comparison of protein behaviour in milk and in solution. It was believed that a useful contribution could be made by examining in greater detail the course of degradation of one of the caseins. Of the

two major caseins, α_{s1}- and β-casein, β-casein was selected for study for practical considerations. In the first place β-casein is relatively easy to purify by the chromatography of urea-fractionated whole casein. Secondly the generation of the first major product of β-casein degradation appears to proceed almost to completion before further breakdown commences[38,39]; this does not appear to be so with α_{s1}-casein where a number of products appear simultaneously. Finally, because of the lower mobility of β-casein there is good resolution of β-casein degradation products on PAE with a relatively short electrophoresis time[39].

It should be possible to carry out kinetic measurements on the β-casein degradation reaction. Disc PAE of the quenched reaction mixture would be the most suitable method of following the production and further degradation of the dye binding peptides. If disc PAE can be developed as a quantitative method it should be possible to determine the rate of hydrolysis of the most sensitive bonds of β-casein. Examination of the initial peptides may give a clue to the nature of the rennin sensitive bonds.

II.

EXPERIMENTAL

(a) Treatment of reagents including rennin and rennet

Solvents and reagents were purified as in Purification of Laboratory Chemicals by Perrin, Armarego and Perrin*.

2,4 Dinitro-1-fluorobenzene - redistilled at a pressure of approximately 5 mm of mercury.
Diethyl ether - washed with an equal volume of distilled water, then concentrated H_2SO_4 then water again. Dried with $CaCl_2$ and redistilled.
Methanol - refluxed with $CaSO_4$ for 1 h then redistilled.
2-Chloro-ethanol-dried with, then distilled from $CaSO_4$ in the presence of a little Na_2CO_3.
Toluene - redistilled.
Pyridine - distilled from KOH using a short reflux column.
Chloroform-twice washed with equal volumes of distilled water, refluxed for 1 h over $CaCl_2$ and then distilled.
Glacial acetic acid - analytical reagent grade.
Rennet. — All commercial rennet used came from the same stock and was obtained from the New Zealand

* Published by Pergamon Press, London, 1966.

Co-op. Rennet Co. Ltd., Bridge Street, Eltham. Rennin. - Crystalline rennin was obtained from Pentex Inc., Kaukakee, Illinois 60901 U.S.A.

(b) Preparation of β-casein

Whole casein was extracted from skim milk obtained from a single cow, basically by the method of Waugh et al.[41] for first cycle casein. Calcium chloride was not added to the milk at pH 7.0 and 37°C. The skim milk was centrifuged at 37°C for 90 min at 19,000 rpm in a Beckman L2-65 ultracentrifuge using a Type 19 rotor. The pellets obtained from centrifuging were disintegrated with a Polytron (Kinematica GmbH, Lucerne, Switzerland) and dissolved in 9 M urea solution. The whole casein thus obtained was then fractionated by a modification of the urea fractionation procedure of Hipp et al.[42]. The pH was adjusted to 4.7 with 1 N HCl and the solution was diluted to 3.3 M urea. The precipitate contained most of the α_s- and χ-caseins while the supernatant contained the β- and γ-caseins. Dialysis of the supernatant (800 ml), containing β- and

γ-caseins, against distilled water (5 l) at 6°C caused extensive precipitation. The water was changed every 3 h for 12 h. The precipitate was dissolved in 300 ml of 9 M urea solution. Pure β-casein was isolated from this solution by DEAE- cellulose - urea chromatography in the presence of 2-mercaptoethanol as described by Thompson[43]. A salt gradient was used to elute a column of Whatman DE52 ion exchange cellulose manufactured by W. & R. Balston Ltd., England. The exchanger was prepared in a buffer solution of 0.01 M imidazole neutralised to pH 7.0 with HCl and poured into an 8x15 cm column by the procedure recommended by the manufacturers of the exchanger. A sample of 100 ml of the crude β-casein containing approximately 5 g of protein in 9 M urea was adjusted to pH 7.0 with 0.1 M HCl and 0.01 M imidazole. The sample was placed on the cellulose column together with two drops of 2-mercaptoethanol. Fifty ml of buffer solution was used to wash the last of the sample onto the column. The elution gradient consisted of 3 l of 0.03 M NaCl in 4.5 M urea buffered to pH 7.0

with 0.01 M imidazole -HCl buffer. The gradient was run at 220 ml/h by means of a Sigmamotor finger pump (Sigma Motor Inc., North Main Street, Middleport, N.Y. 14105, U.S.A.). The column was coupled to a fraction collector via a 2 mm path length flow cell in a Zeiss PMQII spectrophotometer, the 280 nm absorbance being recorded on a Sargent SRL logarithmic record. Material eluted at the peak (Fig. 3) was electrophoresed by disc electrophoresis. The tubes containing the purest fractions were retained, bulked, dialysed against distilled water and stored at -20°C.

Beta-casein concentrations were measured spectrophotometrically on a Zeiss PMQ II spectrophotometer. The optical densities (O.D's) of the β-casein solutions were measured in a 1 cm pathlength quartz cell at 280 nm and 320 nm. Solvent O.D's at 280 nm and 320 nm were measured in the same cell. The two β-casein O.D's were then corrected for solvent absorption and the following formula was used, to correct for

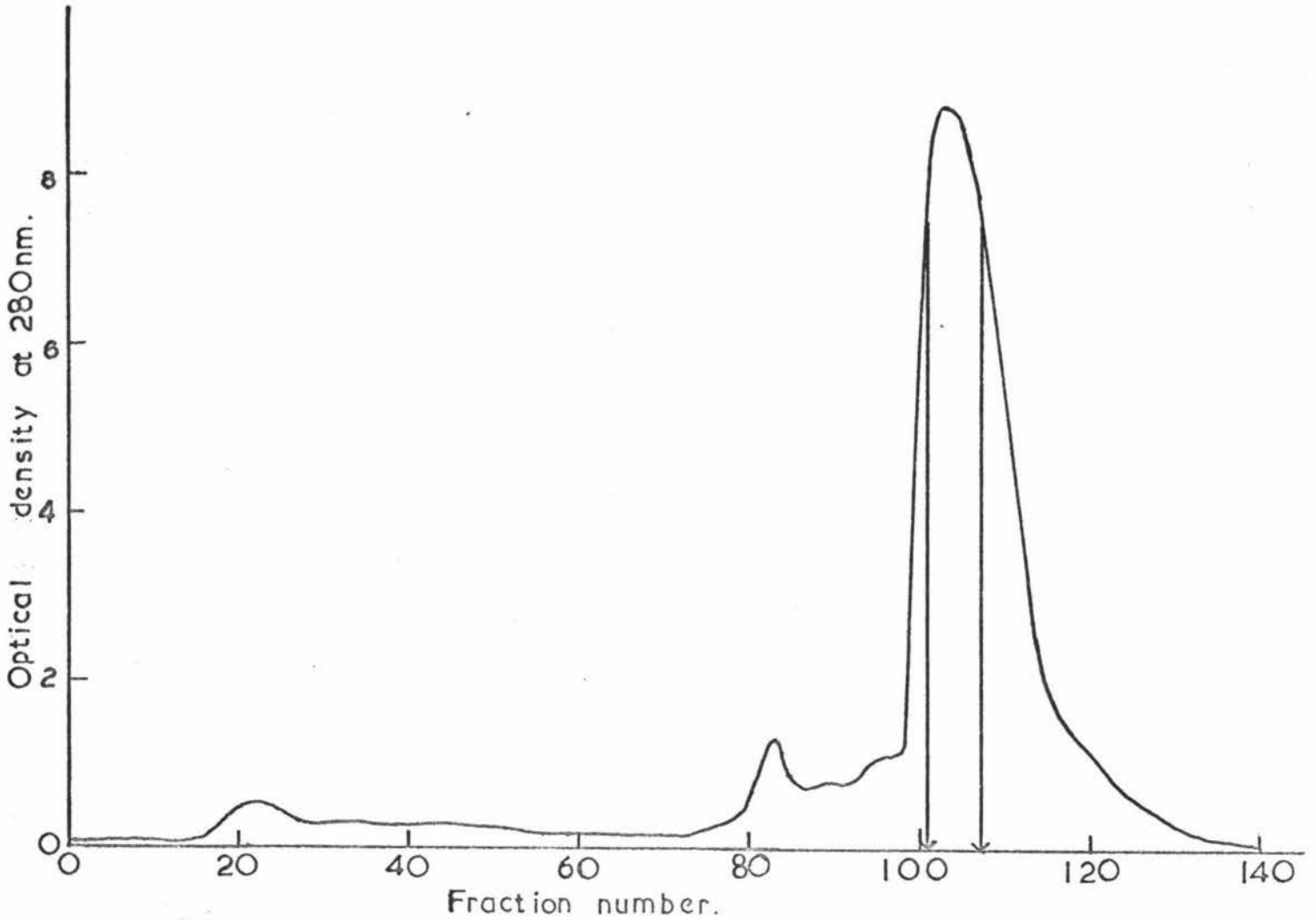


Fig. 3. An elution pattern obtained from DEAE-cellulose chromatography of crude β-casein. The eluting buffer was 0.01 M imidazole-HCl at pH 7.0 in 4.5 M urea. A NaCl gradient of 0-0.3 M NaCl was used. The 8x15 cm column was eluted at a rate of 220 ml/h and 25 ml fractions were collected. The area

turbidity,

$$\% \beta\text{-casein (w/v)} = \frac{\text{O.D.}_{280} - 1.7\ \text{O.D.}_{320}}{4.66}$$

where 4.66 is the extinction coefficient of β-casein[44].

(c) Rennet standardisation

The procedure adopted for measuring rennet strength by coagulation time was that described by Dolby[45].

The milk substrate was prepared by adding 12 gm of low heat skim milk powder to 100 ml of 0.01 M $CaCl_2$, and then held at 30°C for 1 h. A 25 ml aliquot of the substrate was pipetted into a wide mouth bottle. One ml of the rennet solution was accurately made up to 100 ml with distilled water. One ml of the enzyme solution was added, the bottle shaken and then revolved at 8 rpm at an angle of 30° in a water bath at 30°C. Time was taken from the beginning of enzyme addition until the first sign of coagulation.

(d) Polyacrylamide disc electrophoresis

Polyacrylamide disc electrophoresis was performed essentially by the method of Davis[46] using gels containing 5.5 M urea. The 12 tube apparatus used had the features shown (Fig. 4). The procedure producing the gels requires five stock solutions, mixed according to Davis to give three working solutions.

TABLE 2

Stock solutions

A		B	
1M HCl	48 ml	1M HCl	48 ml
TRIS	36.6 g	TRIS	5.98 g
TEMED	0.23 ml	TEMED	0.46 ml
Water to pH 8.9	100 ml	Water to	100 ml
C		**D**	
Acrylamide	28.0 g	Acrylamide	10 g
BIS	0.735 g	BIS	2.5 g
Water to	100 ml	Water to	100 ml
E			
Riboflavin	4 mg		
Water to	100 ml		

TRIS tris (hydroxymethyl) amino methane

TEMED N,N,N',N' - tetramethylethylenediamine

BIS NN' - methylene - bis - acrylamide

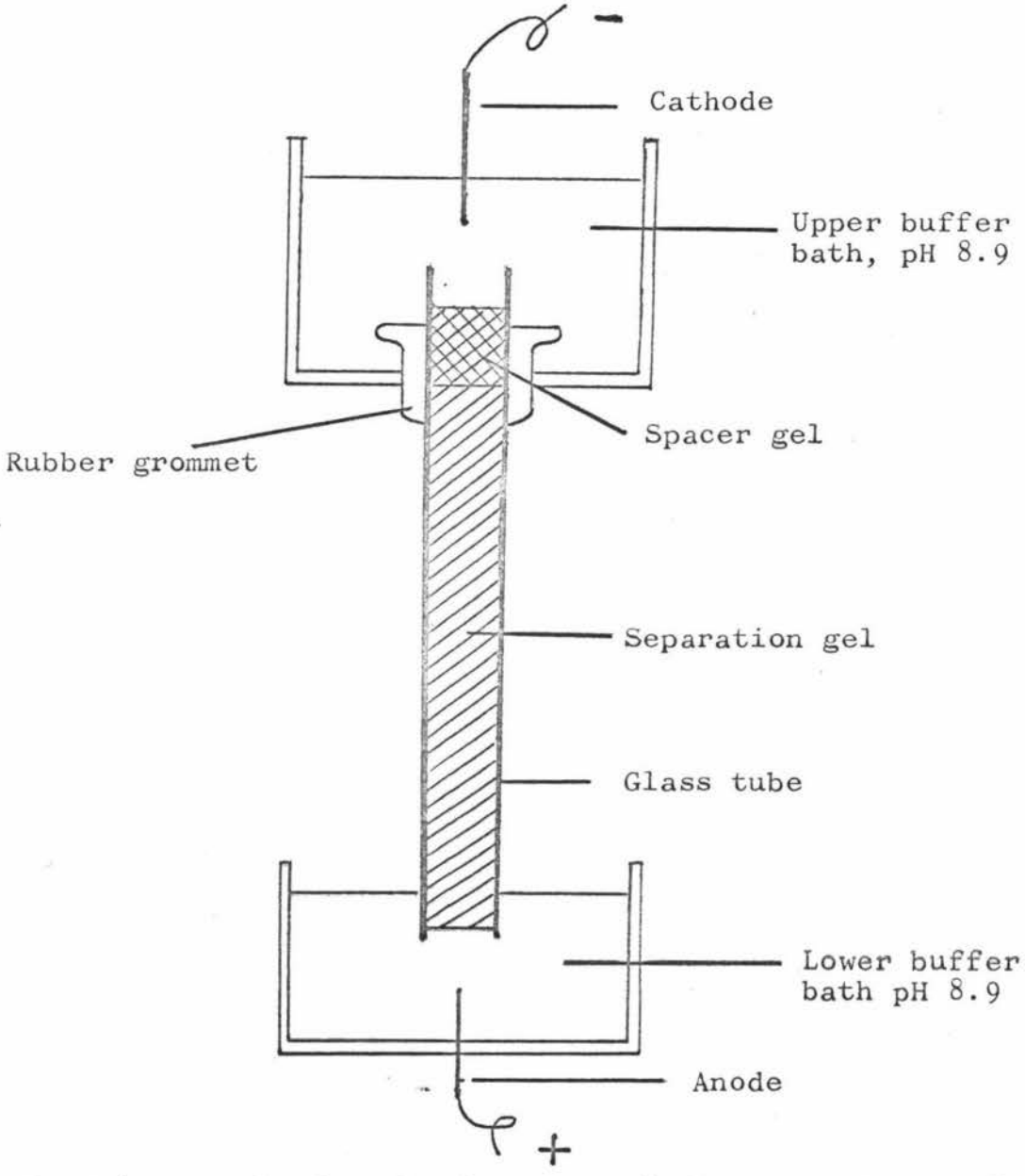


Fig. 4. Diagram showing the features of the apparatus used for disc PAE.

TABLE 3

Working solutions

Small - pore Solution 1 1 Part A 2 parts C 1 part 9 M urea	Small - pore Solution 2 Ammonium persulphate 0.14 g to 100 ml 9 M urea
Large - pore solution 1 part B 2 parts D 1 part E 60 parts 9 M urea	Buffer solution 3 g TRIS 14.4 g glycine in 1 l of water

The separation gel is made by mixing equal parts of small pore 1 and 2. The separation gel is thermosetting and after pouring into the tube requires about 30 min to gel. The spacer or large pore gel is made on top of this. It is photosetting and requires about 10 min in sunlight to gel. Samples were applied to each tube and buffer added without mixing or loss of sample. A voltage of 60 volts giving 1.25 mA

per tube was applied until the thin disc of bromophenol blue (previously mixed into the buffer) was about 1 mm into the separation gel. The voltage was then increased to 120 volts giving 2.67 mA per tube and run until the marker disc was 5 mm from the bottom of the tube. Total electrophoresis time was about 3 h. The gels, on removal from the tubes, were placed in a fixative stain of 7% acetic acid (v/v) and 0.2% amido black (w/v) and left for at least 4 h. Unbound dye was removed by electrophoresis in 3% acetic acid.

(e) Isolation of degradation products

Commercial rennet (0.70 ml, undiluted) was added to 175 ml of 1.5% β-casein at pH 6.5, 37°C and negligible ionic strength. The enzyme was inactivated after 45 min by rapidly heating to, and maintaining at 70°C for 10 min. Disc electrophoresis showed that at this time about 60% of the β-casein had been degraded to a single visible product.

A column of Sephadex G25 fine (molecular weight fractionating range 1,000-5,000) obtained from Pharmacia Fine Chemicals, Sweden, was

prepared by suspending 19 g of Sephadex G25 in 2 l of 0.01 M imidazole-HCl buffer, pH 7.0 and 0.05 M in KCl. After swelling for 3 h the resultant gel was poured into a column, 2x33 cm, in one pour. A 5 ml sample of the above hydrolysate was eluted from the column with 100 ml of 0.01 M imidazole-HCl buffer, pH 7.0, at a rate of 20 ml/h. The volume of buffer required to elute all products from the column was determined by eluting a tyrosine sample. One ml fractions from the β-casein hydrolysate were collected and absorption was measured at 280 nm. One elution peak only was observed.

The 'ninhydrin' analysis of Moore and Stein[47], preceded by alkaline hydrolysis[48], was performed on each of the above 1.0 ml fractions. Alkaline hydrolysis consisted of adding 1.0 ml of 2.5 M NaOH to 0.3 ml of sample and placing the unstoppered tubes in an open water bath at 90°C for 2.5 h, after which 1.0 ml of 30% (v/v) acetic acid was added with thorough shaking. To carry out the ninhydrin analysis 1.0 ml of alkaline hydrolysate and 1.0 ml of ninhydrin solution were shaken together briefly and heated for 15 min (accurately timed) in a covered boiling water bath.

The ninhydrin solution was made according to Moore and Stein by mixing 2 g ninhydrin and 0.3 g hydrindantin in 75 ml of 2-methoxy-ethanol plus 25 ml of pH 5.5 acetate buffer. The cooled tubes were thoroughly shaken and the absorbance measured at 570 nm (Fig. 5). By eluting a sample of urea solution from the Sephadex G25 column a ninhydrin positive peak was obtained in the same position as the smaller peak from the β-casein hydrolysate.

The 1.5% β-casein hydrolysate was lyophilized and made up to a concentration of 10%. Three ml were applied to the Sephadex G25 column and eluted with distilled water, which was found to successfully replace the imidazole-HCl buffer. The column was eluted at 25 ml/h and 3.0 ml fractions were collected. Absorption measurements were made at 280 nm (Fig. 6).

(f) Molecular weight determination

Sedimentation velocity studies of β-casein and an hydrolysate were made using a Beckman Model E Analytical Ultracentrifuge. Two single sector cells with a sector angle of 4^{o} were chosen. One cell was filled with 0.5% β-casein (ionic

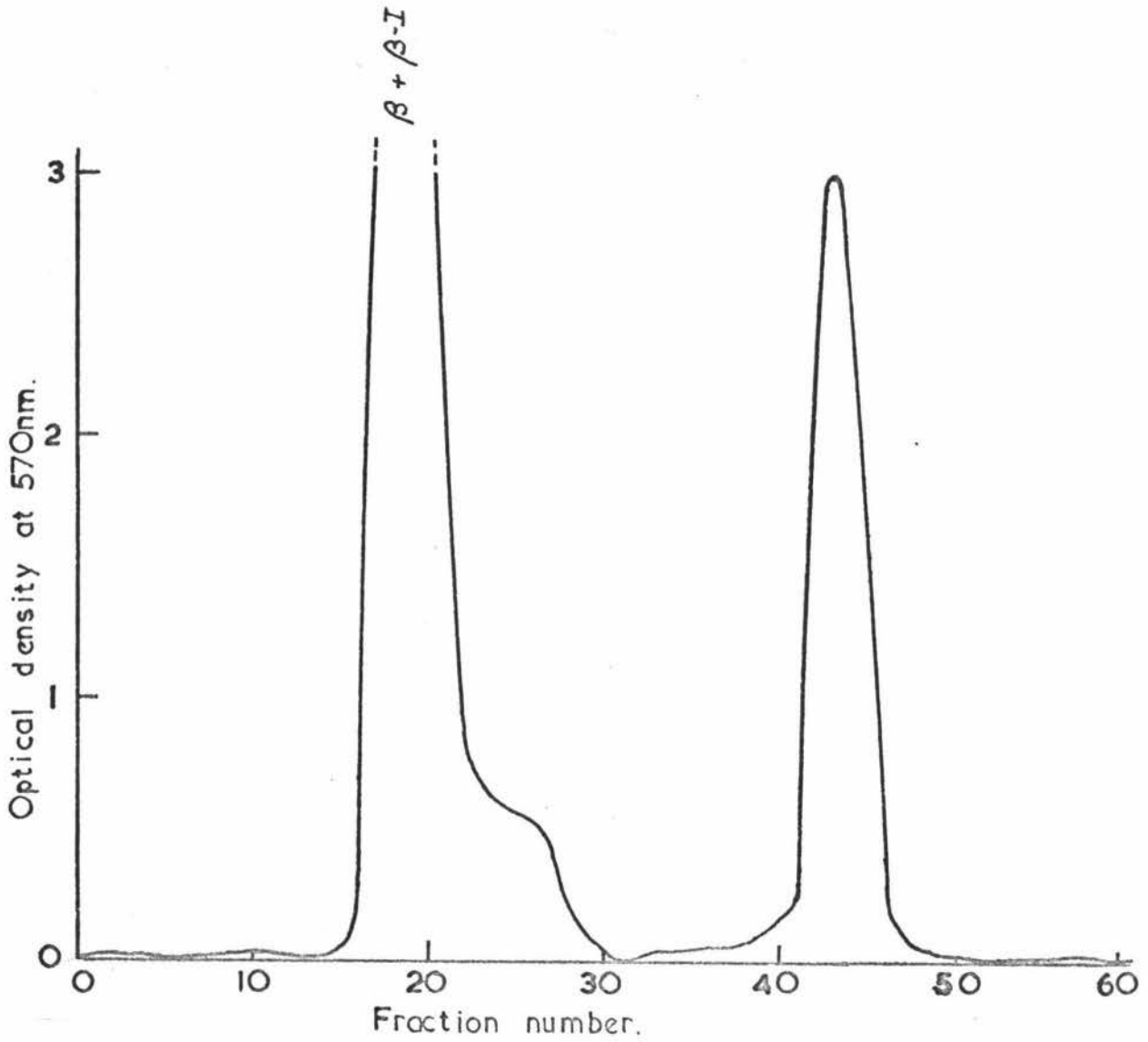


Fig. 5. Analysis of fractions eluted from a sephadex G25 column, by alkaline hydrolysis followed by incubation with ninhydrin.

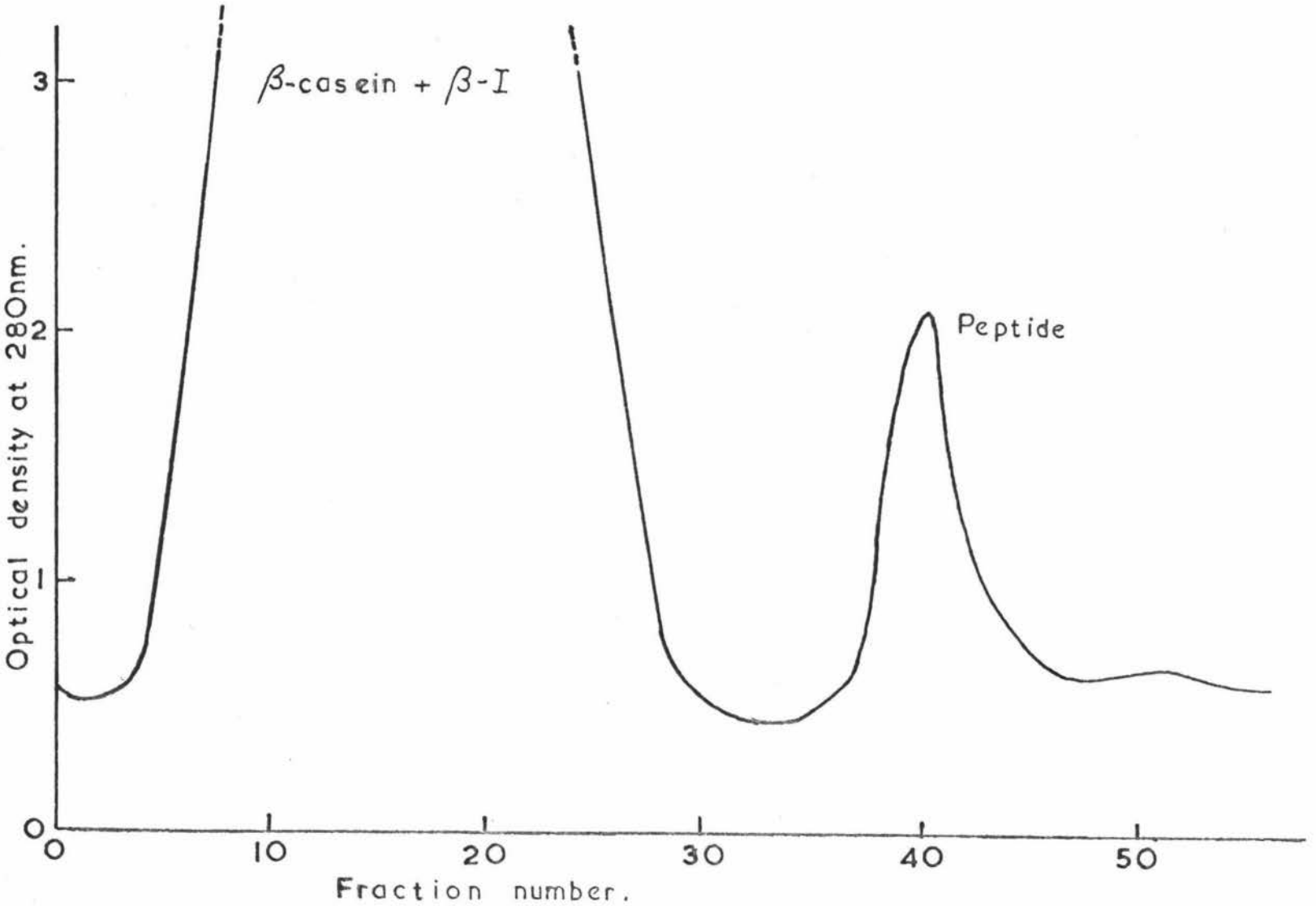


Fig. 6. Elution pattern obtained by eluting β-casein hydrolysate from Sephadex G25.

strength 0.1, pH 7.0). The hydrolysate was obtained by reacting 5 ml of 0.5% β-casein at pH 6.0 and negligible ionic strength with 0.1 ml of 50% (v/v) commercial rennet at 37°C. The enzyme, in a 1.0 ml sample extracted from the reaction mixture after 9 min, was inactivated by rapidly heating to 70°C and holding there for 10 min. The rotor and two cells were held at 6°C overnight. Measurements were made at 60,000 rpm and at a constant temperature of 5.75°C. A Schlieren optical system which gave a visible pattern of refractive index gradient was used (Plate 1). Photographs were taken every 9.6 min and had a magnification of 2.192. Measurements on the photographs were made with a travelling microscope (Beck Instruments, London).

(g) Ultraviolet spectra of the peptide and β-casein

The ultraviolet spectra of the peptide and also unhydrolysed β-casein at pH 6 and 12 were determined using a Beckman DB double beam spectrophotometer. The β-casein was at a concentration of 1.55% and had been exhaustively dialysed against distilled water. The peptide

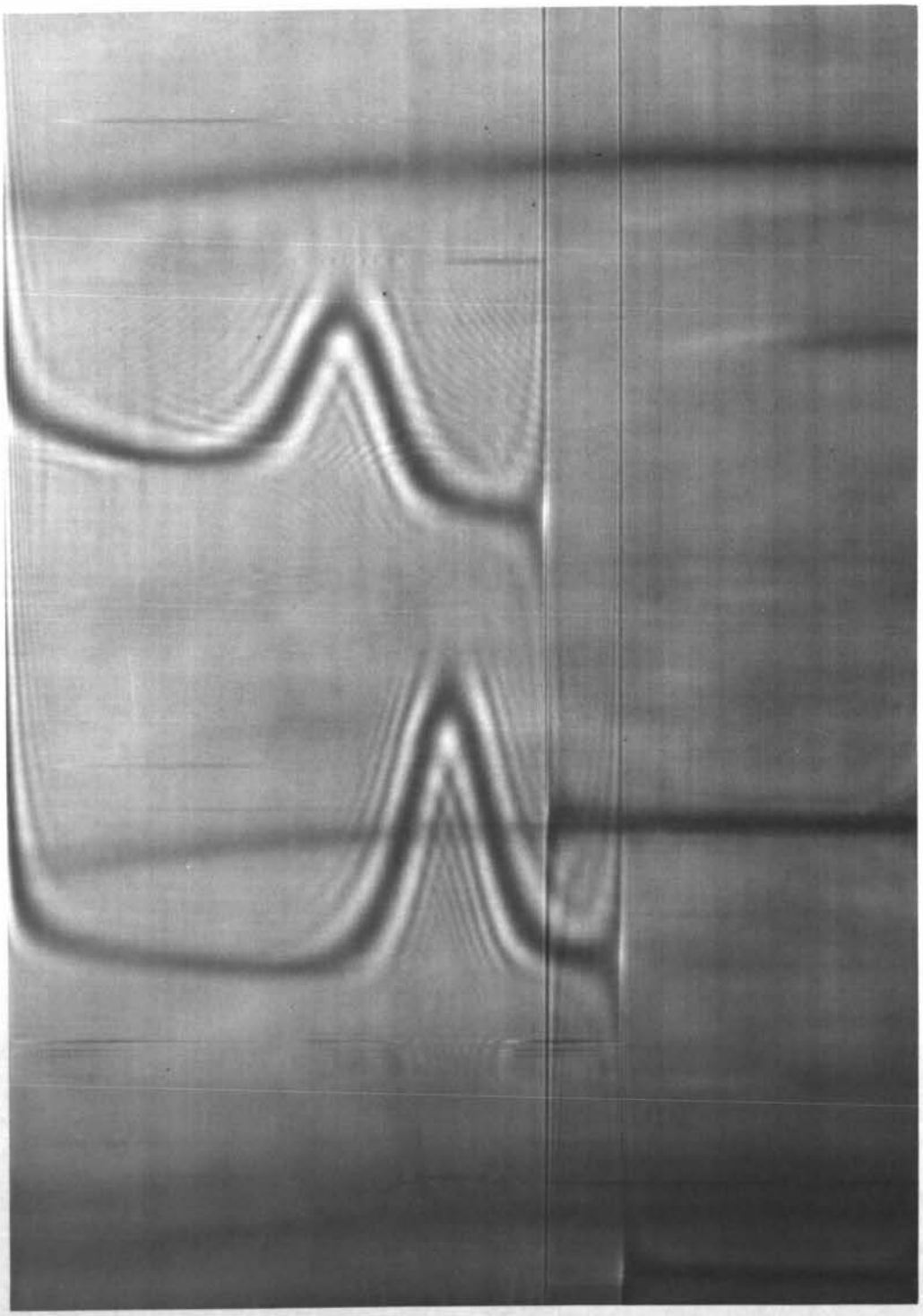


Plate 1. Refractive index gradient as shown by Schlieren optics during sedimentation velocity run.

solution was obtained from the hydrolysis of β-casein and purified by gel filtration. Its concentration was approximately 0.6%.

(h) Amino acid analysis

The amino acid analysis of the small peptide, as isolated from the 10% β-casein hydrolysate, was carried out by J. Dingle of the Massey University Poultry Research Centre on a Beckman 120 C Amino Acid Analyser.

(i) N-Terminal amino acid determination

The dinitrophenylation procedure was basically that of Levy and Li[49].

The peptide, before dinitrophenylation, was passed through the Sephadex G25 column to remove remaining salt and urea. The peptide (1.24 mg, about 0.5 μmol) was dissolved in 3 ml of 0.05 M aqueous KCl. The pH was continually adjusted to 8.0, using 0.05 M KOH and a Radiometer Titrator Type TTT II b, after the addition of 2,4-dinitrofluorobenzene (DNFB). The solution was continually stirred in the dark at 40°C and progress of the reaction was followed on a Radiometer Type SBR2c Titragraph. The

dinitrophenylation was considered to have ceased when the consumption of alkali had become constant. The solution was then extracted three times with ether. The aqueous solution was acidified and the DNP derivative isolated by elution with water from a Sephadex G25 column. The resulting yellow fractions were pooled and evaporated to dryness on a boiling water bath. The DNP-peptide was hydrolysed in a sealed tube for 22 h at 110°C with 1 ml of 5.5 M HCl. The hydrolysate was diluted until it was 1 M with respect to HCl and then extracted five times with purified diethyl ether. These extracts were washed three times with 0.1 M HCl. Thin layer chromatography on silica gel G of the pooled acid soluble fraction and the 0.1 M HCl washings in an n-propylalcohol-33% ammonia (70+30) solvent system showed only ϵ-DNP lysine to be present.

Thin layer chromatography on silica gel G in two solvent systems was employed to analyse the ether soluble fraction. Plates coated with silica gel G were air dried at room temperature overnight then run in purified methanol alone

until the solvent front reached the top of the plates. The top 2 cm was scraped from the plate. The plates were once more air dried until all traces of methanol had gone.

The first solvent system to be used was the "Toluene-system" of Biserte and Osteux[50]. Toluene, pyridine, 2-chloroethanol and 0.8 N ammonia solution were mixed in the proportion of 100 : 30 : 60 : 60. Two layers formed, the lower one being used for pretreatment of the thin layer, the upper one as the solvent for chromatography. Pretreatment was carried out by placing the thin layer plate in the vapour of the pretreatment solvent overnight.

Chromatography of the ether soluble fraction was carried out against a number of standard DNP amino acids (Plate 2). The ether soluble fraction was evaporated down and the residue redissolved in 0.5 ml ether. One μl of standard DNP amino acids was applied and 5 μl of unknown. The unknown had the R_f value of DNP phenylalanine. As confirmation a second solvent system was chosen and three of the DNP

acids on either side of the unknown in the first solvent system were run. This second system consisted of chloroform, methanol and glacial acetic acid in the proportion 95 : 5 : 1. In this system the unknown did not move from the base line (Plate 3).

Photographs of the thin layer plates were made on Agfa film BH111 by placing the plates face down on the film and exposing to a U.V. lamp.

(j) Kinetics of β-casein hydrolysis

All proteolyses of β-casein in this section were carried out in the same way.

A reaction vessel containing β-casein solution was equilibrated in a constant temperature water bath. Commercial rennet was diluted to the required concentration (v/v) and added to the substrate by means of a blow-out pipette thus ensuring rapid addition. The solution was mixed by briefly shaking and time was taken from the instant of enzyme addition. At preselected time intervals a 0.5 ml sample was drawn from the reaction vessel and added to a measured

quantity of 8 M urea solution containing 2-mercaptoethanol. Small accurately measured volumes of quenched samples and β-casein standards were electrophoresed in a random order in duplicate. In a number of the later runs the β-casein standards were omitted (j and k of Table 4). The resulting gels, after staining and destaining were preserved in 3% acetic acid in 1x10 cm test tubes. Each gel was scanned quantitatively at 700 nm using a Joyce Chromoscan. The gel, in its tube, was then rotated 90° and a second scan was made. Normally the peak areas or peak heights of the two scans on each of the duplicates were averaged (i.e. the four scans). A standard curve was drawn from the β-casein standard samples. The concentration of β-casein in the hydrolysates could then be estimated from the dye absorption using the standard curve.

(k) Effect of temperature

Proteolysis of 0.10% (w/v) β-casein, at negligible ionic strength, was carried out at 10°C, 25°C and 37°C. The pH of the β-casein solutions was adjusted to 6.50 at each temperature

by adding 0.01 M HCl or 0.01 M imidazole dropwise. The pH meter was recalibrated at each temperature. All the reaction conditions are set out in a,b,c of table 4. Casein concentrations were estimated as described previously.

(1) Effect of pH, substrate concentration and ionic strength

Beta-casein solutions used in experiments described in d-k of table 4 were prepared similarly. Two litre samples of buffer solution at the required pH's, temperatures and ion levels were prepared for d,e,f,j and k. In d,e,j and k 10 ml of 0.10% β-casein solution, and in f, 20 ml of 0.20%. β-casein solution, were dialysed against the appropriate buffer for 4 h. The 0.20% β-casein solution was then diluted with its dialysate to give the required concentrations (g,h,i). The β-casein in the solutions was hydrolysed using the method outlined earlier and the concentration of β-casein in the hydrolysates was estimated by quantitative disc electrophoresis.

	4.5 ml β-casein. Conc. % (w/v)	0.1 ml rennet. Conc. % (v/v)	Temperature °C	pH	Ionic Strength, μ					Inactivation, 8 M urea in 0.1% 2-mercaptoethanol, ml.
					μ_{HCl}	μ_{KCl}	μ_{Na_3cit}	μ_{CaCl_2}	μ_{Total}	
a	0.10	100	10	6.50	-	-	-	-	-	4.5
b	"	"	25	"	-	-	-	-	-	"
c	"	"	37	"	-	-	-	-	-	"
d	0.10	2	37	6.12	0.01	0.03	-	-	0.04	9.5
e	"	"	"	6.94	0.01	0.03	-	-	"	"
f	0.194	2	37	6.49	0.01	0.03	-	-	0.04	19.5
g	0.10	"	"	6.50	"	"	-	-	"	9.5
h	0.052	"	"	6.48	"	"	-	-	"	4.5
i	0.025	"	"	6.48	"	"	-	-	"	2.0
j	0.10	2	37	6.48	0.01	0.025	0.005	-	0.04	9.5
k	0.10	"	"	6.49	"	0.02	-	0.01	0.04	"
l	0.10	0.5	37	6.50	-	-	-	-	-	9.5

TABLE 4

III.

RESULTS AND DISCUSSION

Introduction

Preliminary work on the hydrolysis of β-casein with rennet and disc electrophoresis of the degradation products showed that initially β-casein was degraded to a single observable product. More slowly two other products appear whilst the first product disappears. These products have been called β-I, β-II and β-III in order of appearance (and incidently in order of increasing mobility at pH 8.9). It was assumed that a single small peptide would be released when β-casein was converted into β-I. In the first section results from the examination of the nature of the small peptide split off in the initial reaction will be presented and discussed. The second section will deal with results concerning the influence of various parameters (pH, temperature, calcium, etc) on the rate and course of the reaction.

Section I

(a) Molecular weight of small peptide

The similarity in size between β-casein and

β-I was shown by disc electrophoresis on polyacrylamide of 5%, 7%, 11% and 14% acrylamide. There appeared to be no appreciable difference in the ratio of distance moved by β-casein to that moved by β-I and therefore it was assumed that there was very little difference in their size.

Sedimentation velocity studies were made on a β-casein solution and on a β-casein hydrolysate solution, (shown by disc electrophoresis to contain about 80% of β-I) as a means of estimating the size of the small peptide produced by this first degradation step.

The velocity of sedimenting molecules per unit centrifugal field or sedimentation coefficient was calculated using the following equation,

$$s = \frac{2.303}{\omega^2} \frac{d\ (\log_{10} X)}{dt} \quad - - - (1)$$

where s is the sedimentation coefficient,

X is the position of the maximum ordinate of the refractive index gradient curve in mm,

t is time in seconds and

ω is the angular velocity in radians per second.

Data obtained was plotted as log X versus t, (Fig. 7);

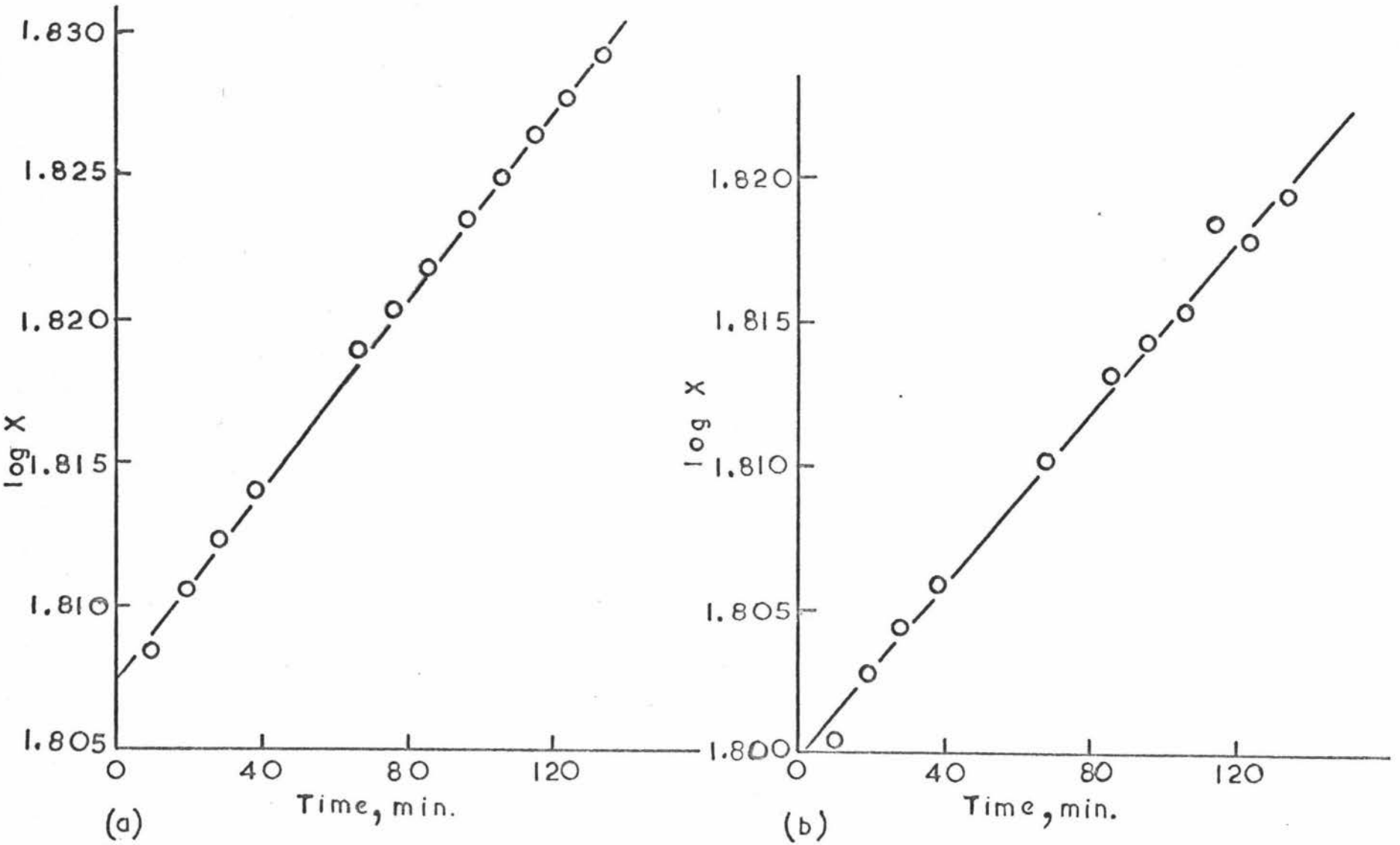


Fig. 7. Graphs showing the variation of log X with time in the determination of the sedimentation coefficient of (a) β-casein and (b) β-casein hydrolysate.

the slope of this line was determined and s calculated.

s- β-casein = 1.59 S

s- β-I = 1.46 S

Now $s = M \frac{(1 - \bar{v}\rho)}{Nf}$ - - - (2)

where M is the molecular weight of solute,

$\bar{v}$ is the partial specific volume of the solute,

ρ is the density of solution,

N is Avogadros number and

f is a frictional factor, dependent on the size and shape of the sedimenting particle.

The factor $\frac{(1 - \bar{v}\rho)}{Nf}$ in equation (2) for the purpose of this estimation was taken as the same for β-casein and β-I. Hence the equation

$$\frac{\text{Molecular wt. } \beta\text{-I}}{\text{Molecular wt. } \beta\text{-casein}} = \frac{\text{S- } \beta\text{-I}}{\text{S- } \beta\text{-casein}}$$

follows. The molecular weight of the β-casein monomer as obtained from the literature[51] is 25,000. Hence the molecular weight of β-I is approximately 23,000 and the peptide molecular weight about 2,000.

From previous investigations[52,53] it is known that β-casein shows a strong tendency to associate on rising temperature. At 4°C the protein exists in solution as a monomer whereas at 8.5°C in a 0.5%

solution, pH 7.5 and ionic strength of 0.2,. Payens and van Markwijk[51] calculated an apparent molecular weight of about 110,000. These conditions are not too dissimilar from those used in the current study and hence at 5.75°C some degree of association can be expected. The value of 1.59S for the sedimentation coefficient of β-casein, however, is in good agreement with that of 1.50S obtained for β-casein at 4°C by previous authors[52,53] and would suggest that the degree of association is small. Error not only arises from the association of β-casein but also from the possible association of β-I with itself or with β-casein as the solution is an hydrolysate in which 80% of the β-casein had been hydrolysed. Another source of error is the possible differences between β-casein and β-I in their respective $\bar{v}$ values. Although the results obtained contain some error the procedure adopted was effective in determining the approximate size of the small peptide.

(b) Isolation of the small peptide

Gel filtration was chosen as a possible means of isolating the small peptide. Inactivation of the

enzyme by addition of concentrated urea solution would necessitate dialysis, with the possible loss of the small peptide, to eliminate the urea. Consequently, inactivation of the enzyme was carried out by heating the reaction mixture to 70°C.

As a result of sedimentation velocity studies, Sephadex G25 fine was chosen as having a suitable molecular weight fractionating range for isolating the small peptide which had an approximate molecular weight of 2000. Absorbance at 280 nm of the fractions collected from gel filtration of the 1.5% β-casein hydrolysate produced one peak only, shown by disc electrophoresis to contain β-casein and β-I. It was assumed that either there was insufficient peptide available or there was no 280 nm absorbing chromophores to be found in the small peptide. A 'ninhydrin' analysis was decided upon as an alternative to u.v. absorption. By this method a second peak was located on the lower molecular weight side of the major peak (Fig. 5). Urea was found to give a ninhydrin positive peak in this position. Residual urea could possibly be present as the result of incomplete dialysis of the β-casein after ion exchange chromatography. The

small peak could then be urea alone, the small peptide or urea obscuring a peptide peak. The 'ninhydrin' method was abandoned for further concentration of the hydrolysate. The 1.5% hydrolysate was concentrated to 10% and again passed through the Sephadex G25 column. Ultraviolet absorption at 280 nm of the fractions obtained this time produced two peaks, the smaller of which was assumed to be the small peptide (Fig. 6).

(c) Ultraviolet spectra

The ultraviolet absorption spectrum of β-casein between 320 nm and 230 nm shows a very strong absorption below 260 nm due to the peptide bond and a second absorption peak between 260 nm and 290 nm. This second peak is due to the absorptivity of the transition $\pi \rightarrow \pi^*$ in the aromatic amino acids tryptophan, tyrosine and phenylalanine. In this region the maximum absorptivity of tryptophan is about four times greater than phenylalanine. At alkaline pH tryptophan still absorbs twice as much as ionized tyrosine. The increase in absorptivity with the ionization of the phenolic hydrogen of tyrosine is also accompanied by a long-wave shift.

Ultraviolet spectra of β-casein at both acid and alkaline pH give a long-wave shift of 15 nm (Fig. 8). Ultraviolet spectra of the small peptide, however, did not show this (Fig. 9), In fact the spectra did not show a substantial peak at all between 260 nm and 290 nm but merely a shoulder. None of the features of this shoulder were observed to move with changing pH. The amino acid composition of β-casein as determined by de Koning[54] shows nine residues of phenylalanine, four or five residues of tyrosine and one residue of tryptophan. Because the absorption of the peptide is very much reduced it may be assumed that there is no tryptophan present and that u.v. absorption in the region 270 nm to 290 nm comes from phenylalanine alone.

(d) Amino acid analysis

The results from the amino acid analysis of the small peptide isolated from the β-casein hydrolysate are tabulated (Table 5). Beta-casein B has five residues per molecular weight of 24,100 of arginine[54] one of which is N-terminal[55]. There is no arginine present in the peptide so it can be assumed that the peptide is derived from the C-terminal end

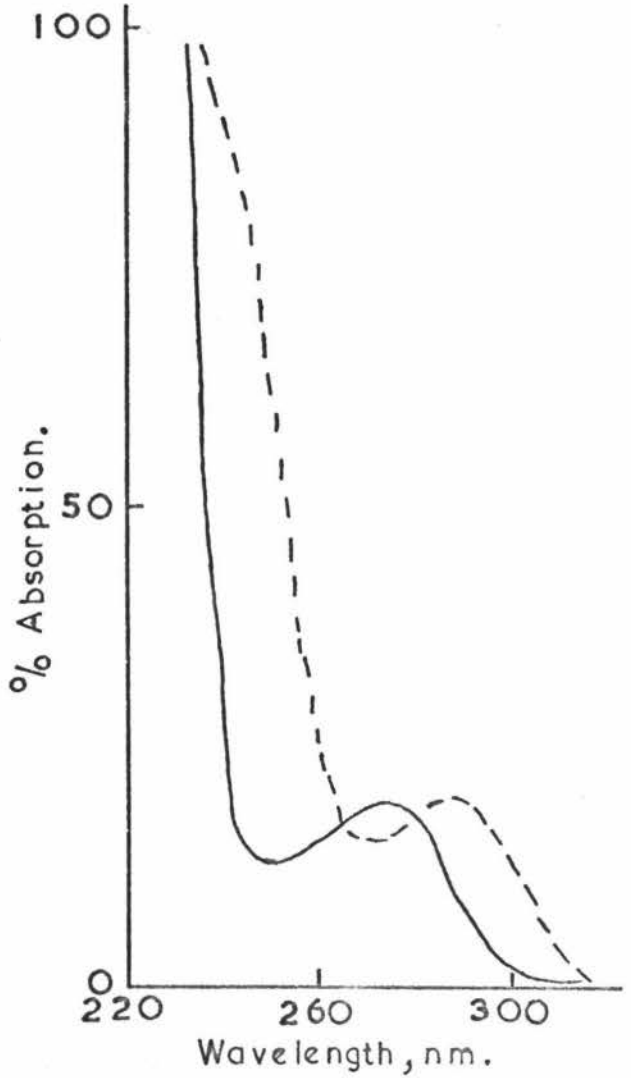


Fig. 8. Ultraviolet spectra of β-casein.
—— acid pH
- - - alkaline pH

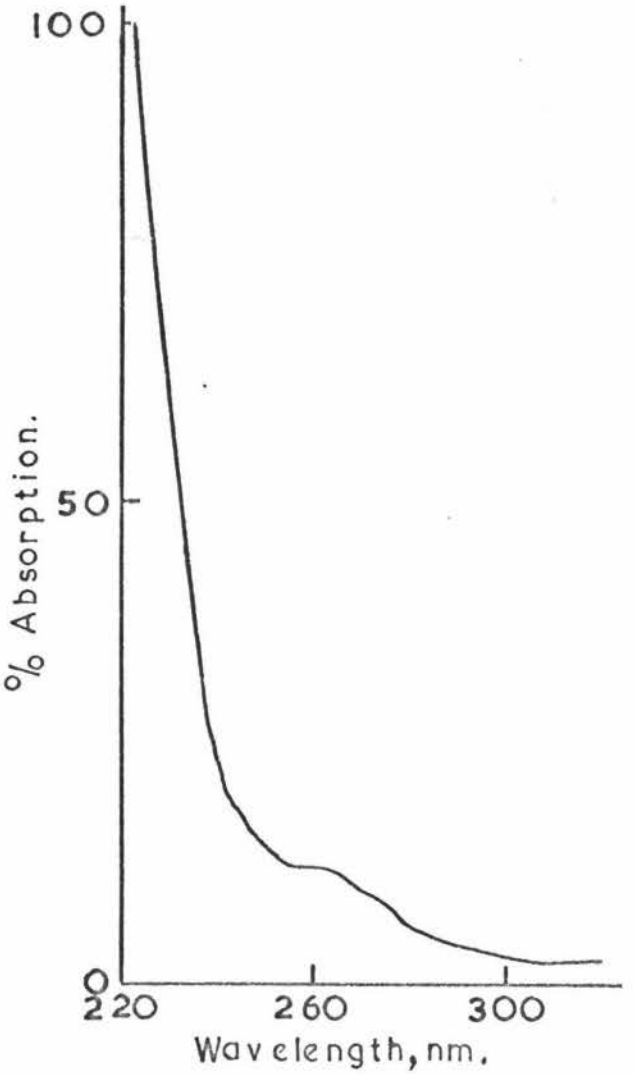


Fig. 9. Ultraviolet spectrum of small peptide.
Identical for acid and alkaline pH.

TABLE 5

Number of amino acid residues per mole of peptide	
Aspartic acid	2.10
Threonine	1.87
Serine	3.68
Glutamic acid	3.35
Proline	2.64
Glycine	4.57
Alanine	2.74
Valine	1.54
Isoleucine	1.00
Leucine	3.25
Phenylalanine	1.09
Lysine	1.02

of the β-casein molecule. There is no tyrosine present and only one residue of phenylalanine. These results are consistent with the ultraviolet absorption spectrum. Beta-casein contains eleven residues of lysine and five of arginine and it is probable that these residues are largely responsible for the binding with amido black to produce the blue discs on polyacrylamide gels. The peptide contains

one lysine only. This may in part be the reason why the peptide cannot be located on disc electrophoresis.

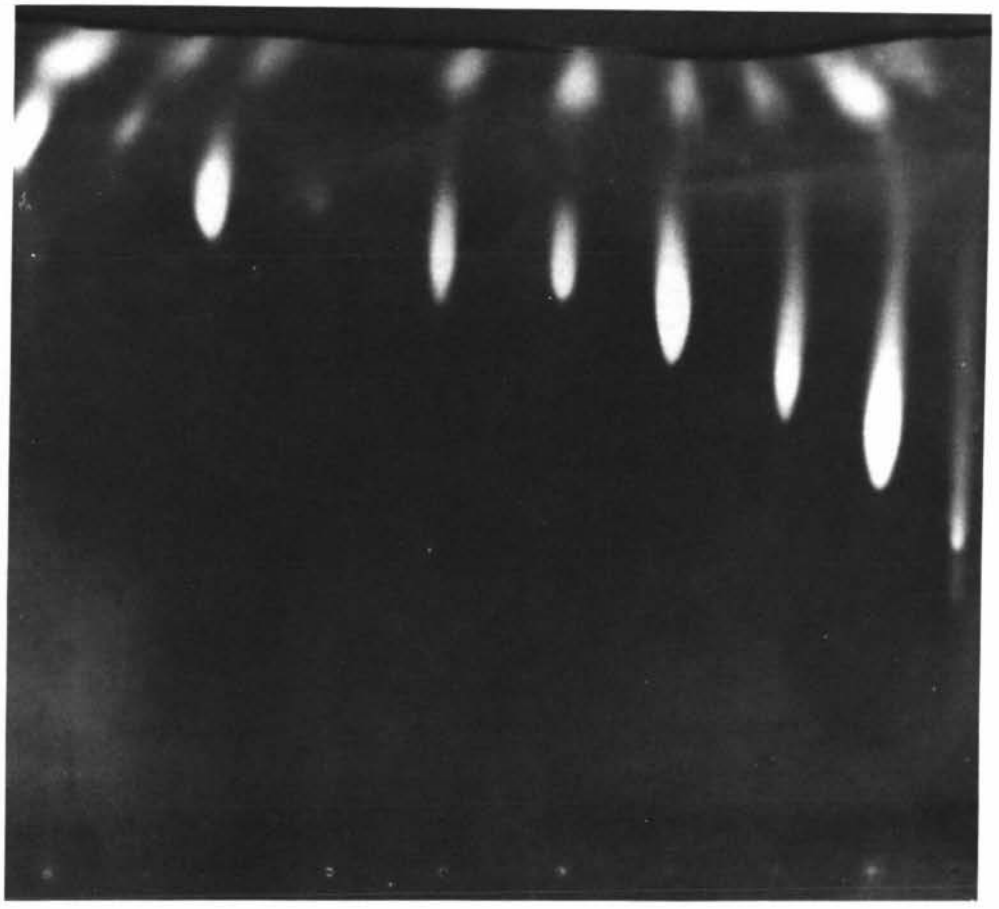
The C-terminal amino acid sequence of β-casein as determined by enzymatic hydrolysis with carboxypeptidase A[55], is either isoleucine - valine - isoleucine or isoleucine - isoleucine - valine. Although the current investigation shows only one isoleucine residue present in the C-terminal peptide, it is well known that this sequence of amino acids would be quite resistant to acid hydrolysis[58].

The charts produced during the amino acid analysis show a few unidentified peaks, possibly due to urea or partially hydrolysed material.

(e) N-Terminal amino acid analysis

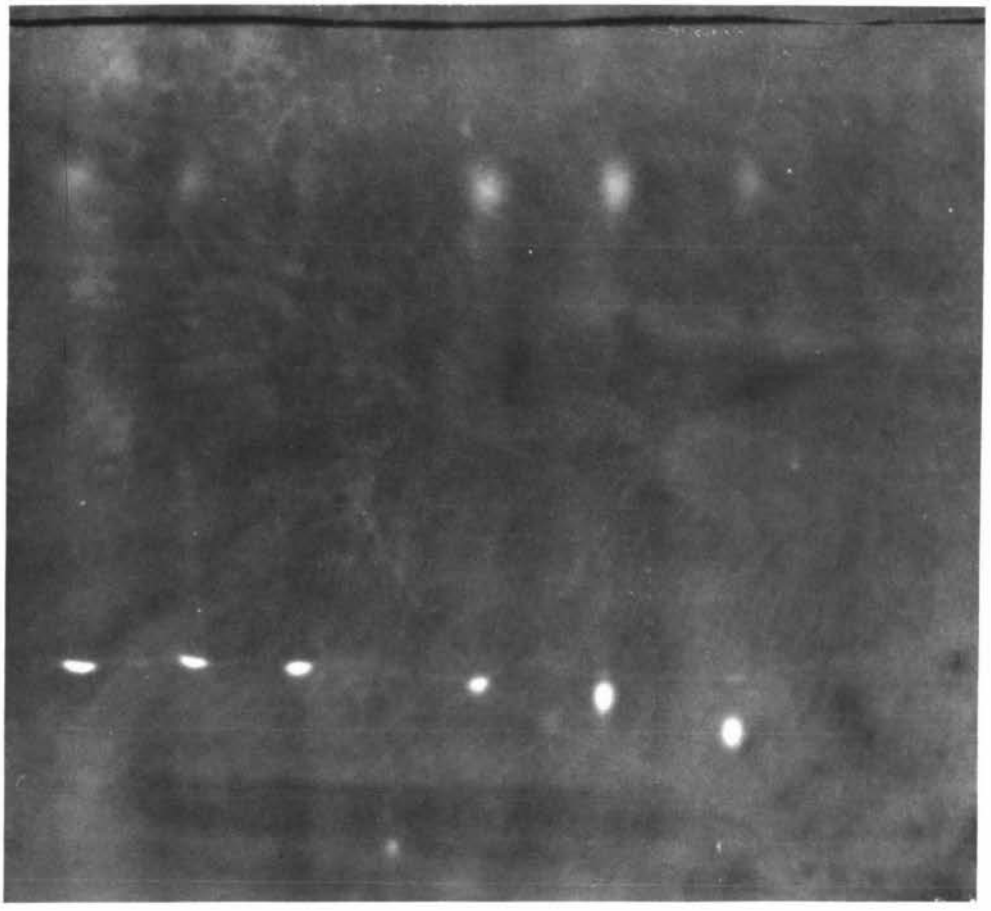
The products obtained from acid hydrolysis of the DNP peptide were separated into an acid soluble fraction and an ether soluble fraction. The only DNP-derivative found by thin layer chromatography of the acid soluble fraction was ε-DNP-lysine which could not have been N-terminal. The ether soluble fraction, chromatographed using

the 'toluene' system gave a single spot which appeared to have an Rf value similar to that of DNP-phenylalanine (Plate 2). To confirm that it was DNP-phenylalanine, this and five other DNP amino acids with closely similar Rf values were chromatographed with the unknown using a second solvent system (Plate 3). As the unknown did not move from the base line in the second solvent system it could not have been the same compound as one of the standards. This unusual result was confirmed not only by running duplicate thin layer chromatographs but by repeating the complete experiment starting at the dinitrophenylation of the small peptide. One explanation of these results would be that the bond attaching the N-terminal amino acid to the small peptide is particularly stable towards acid hydrolysis. It could have been stabilised by the addition of the DNP group. After dinitrophenylation and acid hydrolysis a DNP-peptide of two or more amino acids might remain. If it is assumed that the specificity of rennin is as shown[56,57] on the B- chain of oxidised insulin (Fig. 10) and that κ-casein



1 2 3 4 5 6 7 8 9 10

Plate 2. Unknown and standard DNP-amino acids in the 'toluene' system. 1. di-DNP-tyrosine, 2.di-DNP-lysine, 3. DNP-phenyl-alanine, 4. unknown, 5. DNP-leucine, 6. DNP-tryptophan, 7. DNP-isoleucine, 8. DNP-methionine, 9. DNP-valine, 10. DNP-alanine. The white spots just below the solvent front are 2,4-dinitroaniline.



1 2 3 4 5 6 7

Plate 3. Unknown and standard DNP amino acids in chloroform-methanol-glacial acetic acid solvent system.

1. DNP-isoleucine, 2. DNP-leucine, 3. DNP-phenyl-alanine, 4. unknown, 5. di-DNP-lysine, 6. di-DNP-tyrosine, 7. DNP-tryptophan.

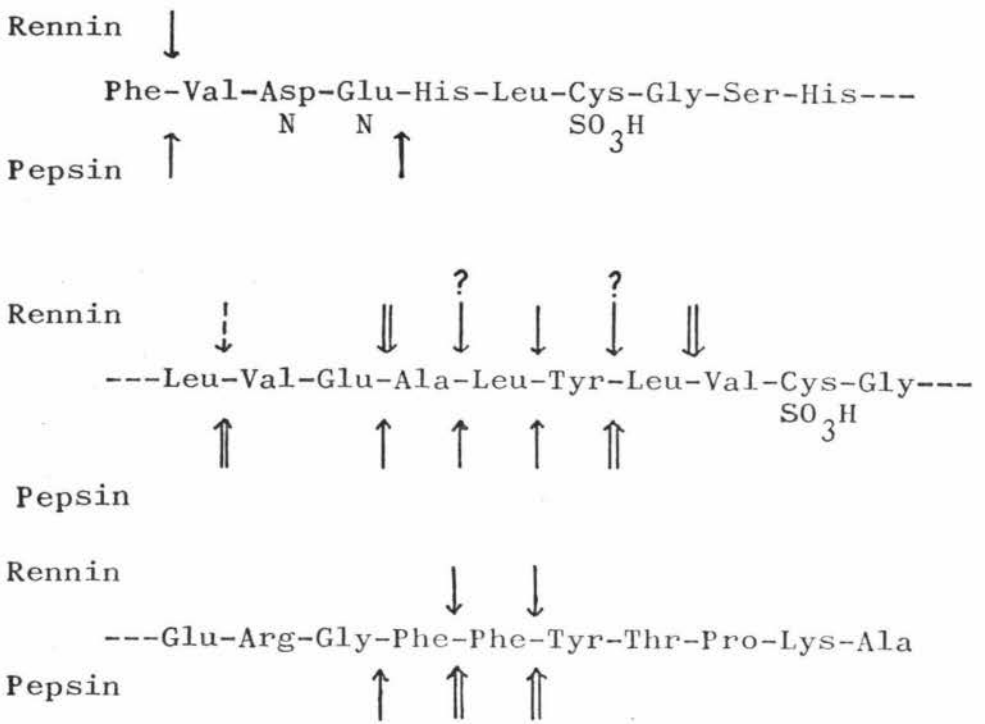


Fig. 10. Diagram showing the specificity of rennin and pepsin on the B chain of oxidised insulin[37].
⇑bonds split rapidly, ↑ other sites of action, ⇕bonds split slowly. ? indicates that some of the spots on paper electrophoresis followed by ascending chromatography possibly contained a mixture of two peptides.

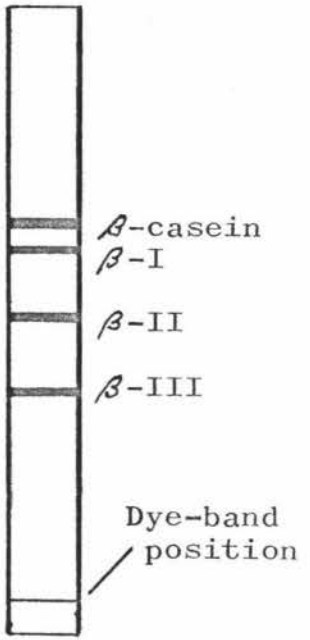


Fig. 13. Disc PAE showing relative mobilities of the major degradation products of rennin on β-casein.

is cleaved at the phenylalanyl-methionine · ··· bond,[16,17,18,19] then the possible N-terminal amino acids of the small peptide would be tyrosine, leucine, phenylalanine, methionine or valine. When account is taken of the amino acid analysis and the known tendency of valine and leucine to form peptide bonds stable to acid hydrolysis[58] then the N-terminal amino acid may well be either leucine or valine.

(f) Summary

Examination of the amino acid analysis showed that the number of residues are in some cases sufficiently different from integral that experimental error alone may be ruled out as a likely cause. This type of result would be expected to arise from either a mixed peptide sample or the incomplete acid hydrolysis of the peptide. The conclusion that the peptide sample analysed probably contained only one peptide follows from the presence of only one spot and hence only a single N-terminal amino acid on thin layer chromatography during the N-terminal amino acid determination. Incomplete acid hydrolysis is more likely as unknown

compounds were shown to be present during the amino acid analysis. If this is the case then the possible acid resistant bond found after dinitrophenylating the peptide is peculiar to β-casein rather than as a result of dinitrophenylating.

The limit to lower molecular weight resolution on Sephadex G25 is about 1000, therefore, as urea and the small peptide are eluted together or very close to one another it appears that the molecular weight of the small peptide would be about 1500. This result presents a problem as to the correct molecular weight of the small peptide. On the basis of the amino acid analysis a molecular weight of about 3,000 was calculated whereas by sedimentation velocity studies, (previously mentioned as having questionable accuracy,) and gel filtration the molecular weight would appear to be about 1500 to 2,000. It is assumed, then, that the amino acid analysis is correct as far as the absence of methionine, tyrosine, histidine and arginine is concerned but the percentages of the present

amino acids could be incorrect. The u.v. spectrum also indicated that neither tyrosine nor tryptophan were present.

Section II

(a) Quantitative disc electrophoresis

Good reproducibility on disc electrophoresis can be obtained if care is taken with variables such as electrophoresis time, buffer pH and stock solutions. If gels from more than one electrophoretic run are to be compared then all runs must be made with the same buffer solution and the same stock solutions.

As little as 10^{-6} g of β-casein can be detected by disc electrophoresis. The dye-binding capacities of α_s- and χ-caseins are greater than that of β-casein by 15% and 8% respectively at the particular amido black concentration of destaining[59]. Alpha$_s$- and χ-casein would therefore be detectable to less than 10^{-6} g but when 100 μl of 0.2% β-casein solution was electrophoresed a single sharp disc appeared, showing that

β-casein constituted more than 99% of protein present.

There are two points which need to be mentioned if the results obtained in the kinetic investigations are to be taken as valid. Firstly, the assumption made when setting up a standard curve is that Beer's Law holds and disc intensity is proportional to the concentration of the protein although in this case particulate matter is being measured. Secondly the optical properties of the 10 cm x 1 cm glass tubes were considered to be consistent from tube to tube. Nevertheless, a reasonably good straight line was obtained when dye absorbance was plotted against protein concentration (Fig. 11).

When investigating the effects of pH, added cations and varying substrate concentrations, peak heights only were measured as β-casein alone was being studied and the ratio of peak height to peak area did not alter. Investigations into the effects of temperature, however, were quantified by

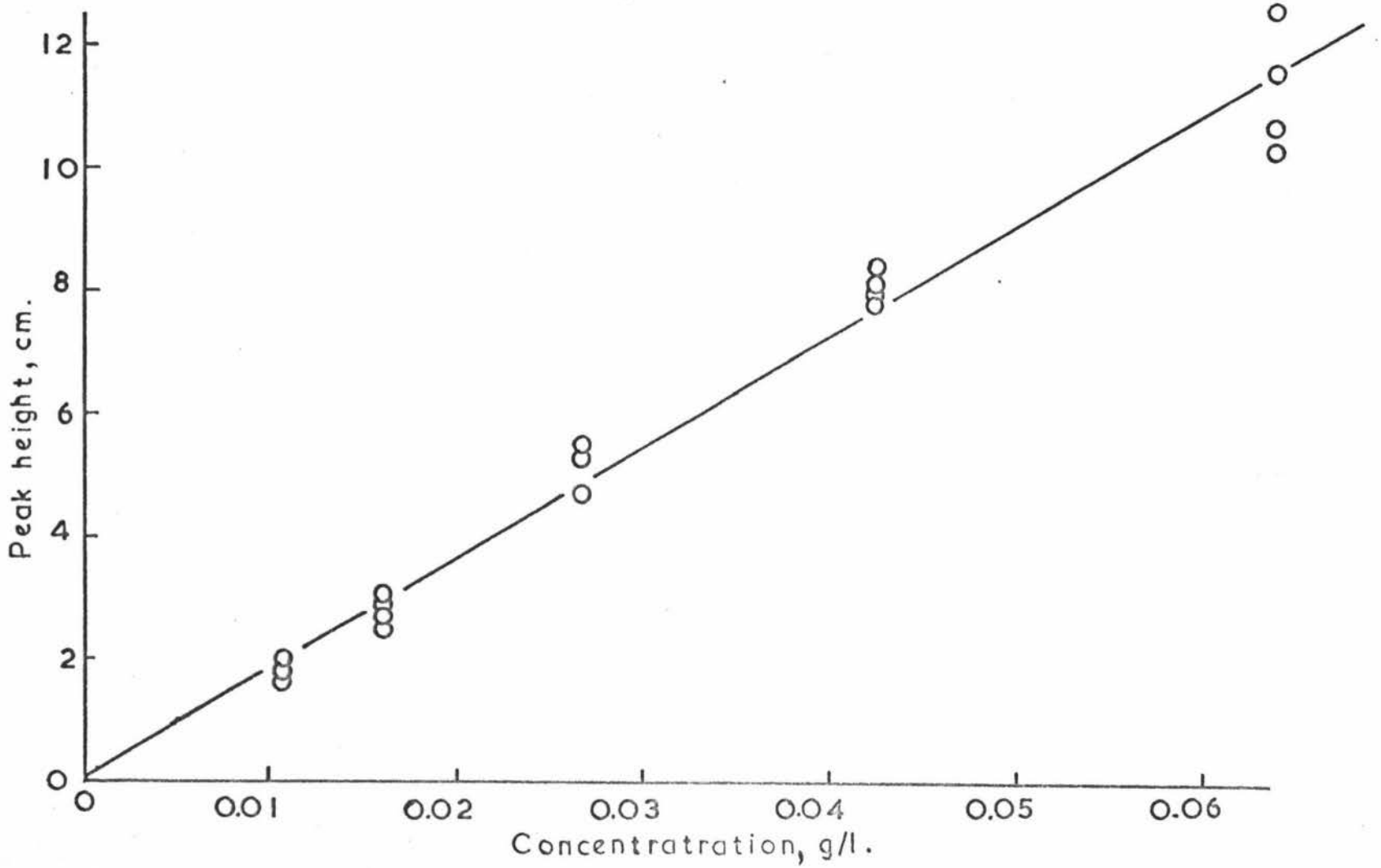


Fig. 11. A typical standard curve of peak height of densitometer trace vs. conc. for β-casein solutions of known concentration.

measuring peak areas. These were calculated as peak height times width at half height. The calculation of peak areas was necessary as β-II and β-III gave rise to more diffuse discs. A typical scan is shown (Fig. 12).

(b) Effect of temperature

When following the complete degradation of β-casein by disc electrophoresis three visible products occur. These have been named β-I, β-II and β-III in order of increasing mobility. The positions they appear in after a 3 h electrophoretic run at pH 8.9 are shown (Fig. 13). Only the large stainable products are observed but this nevertheless does provide a means of following the reaction. Uptake of dye is measured for each product and although the number of overall stainable groups may be decreasing from step to step, comparison between the uptake of dye for the same product at each temperature is permissible. A typical set of gels obtained in this experiment are shown (Plate 4).

At all three temperatures the degradation of

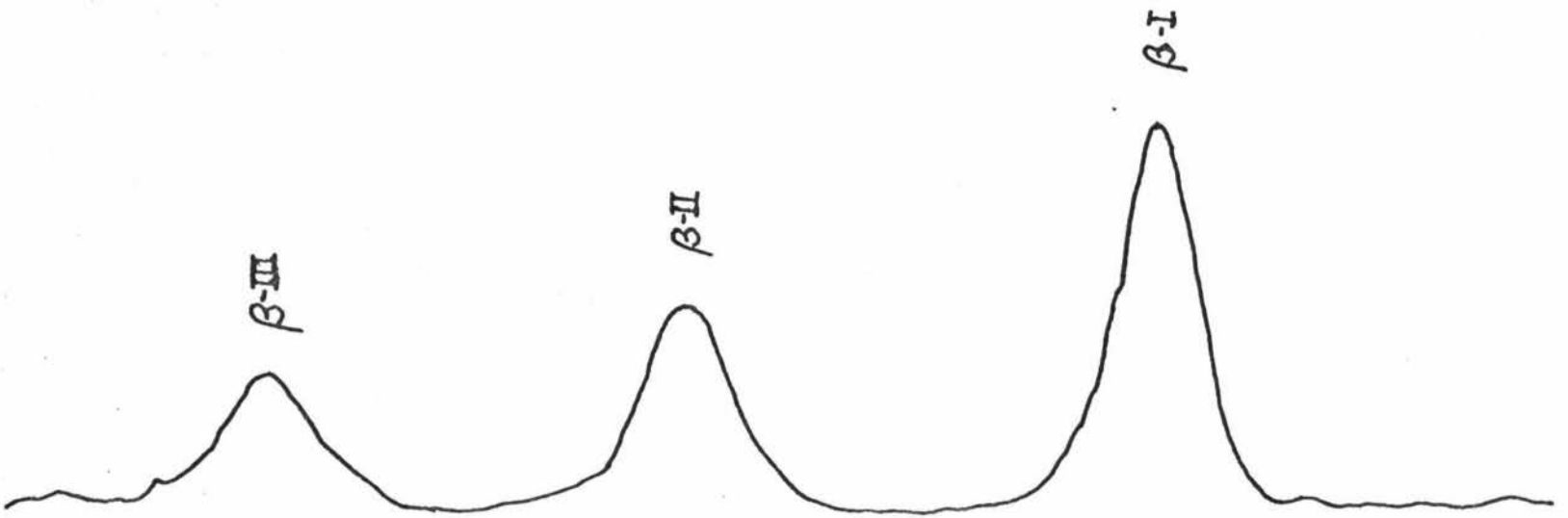


Fig. 12. A typical densitometer trace of a polyacrylamide gel from which peak heights or areas were measured.

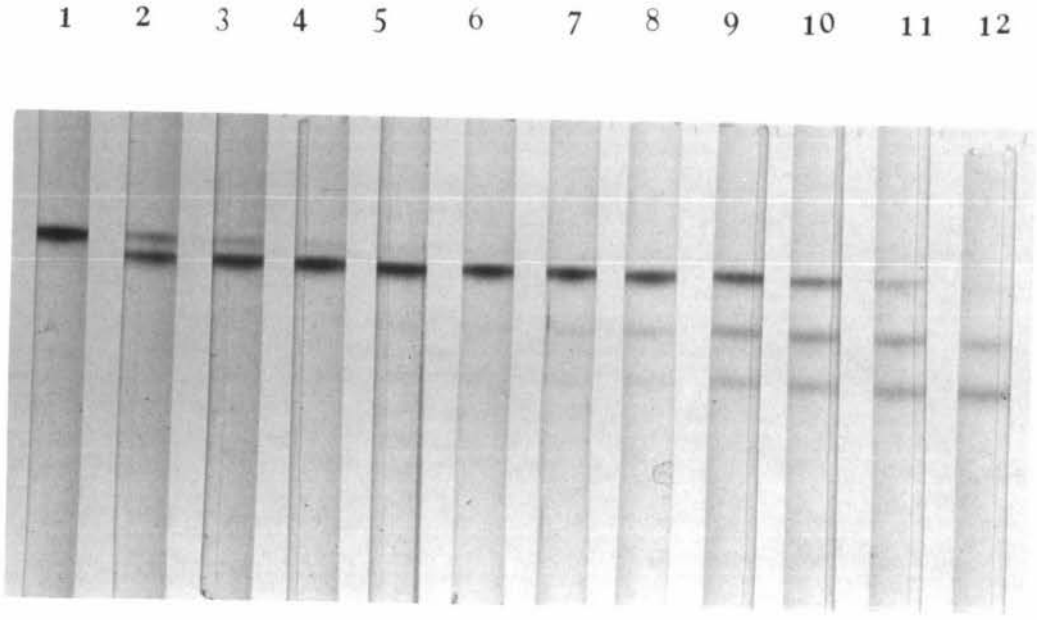


Plate 4. A set of polyacrylamide gels showing the degradation of β-casein by rennin at 10°C and pH 6.50. The experimental specifications were as in a, of Table 4.

β-casein into β-I is complete before the appearance of β-II and β-III (Fig. 14, 15, 16). This step may then be treated, without interference, in terms of the Michaelis-Menten theory of enzyme plus substrate giving an enzyme-substrate complex and the dissociation of the complex to give the products and free enzyme. The course of degradation remains the same for the three temperatures, the rate however increasing with temperature. Table 6 shows the decrease in half-life with increase in temperature.

TABLE 6

	Temperature		
	10°C	25°C	37°C
β-casein	2.8 min	<1.25 min	<1.25 min
β-I	10 h	0.933 h	0.85 h
β-II	>48 h	7.2 h	4.8 h

It is notable that the difference in rate between 10°C and 25°C is much greater than the difference in rate between 25°C and 37°C although there is not much difference in temperature interval, 15° and 12°. Much is known of the effect of temperature on the association of β-casein[51,52,53].

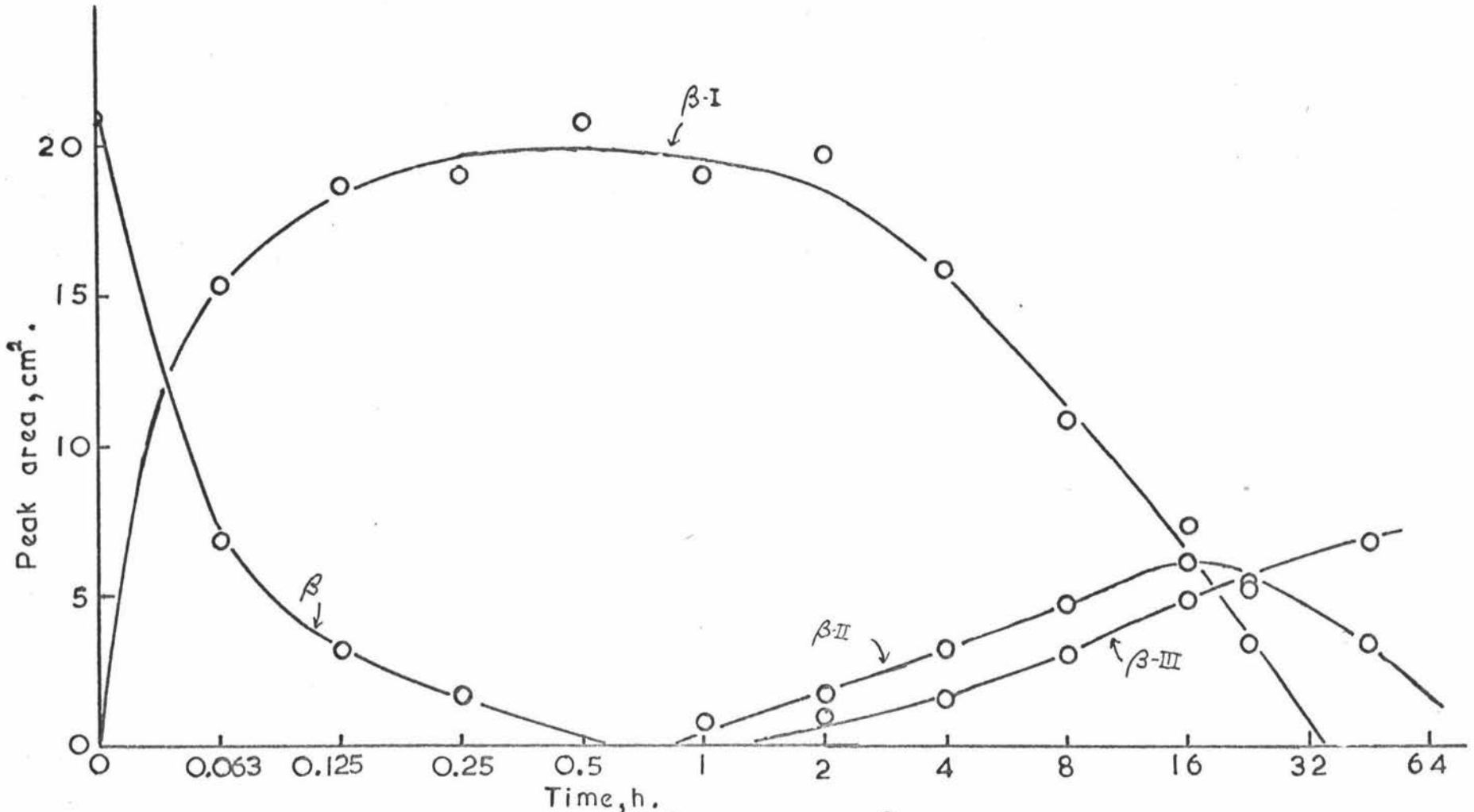


Fig. 14. The rennin proteolysis of β-casein at 10°C and pH 6.5. Diagram shows the generation and depletion of the products as shown on disc PAE.

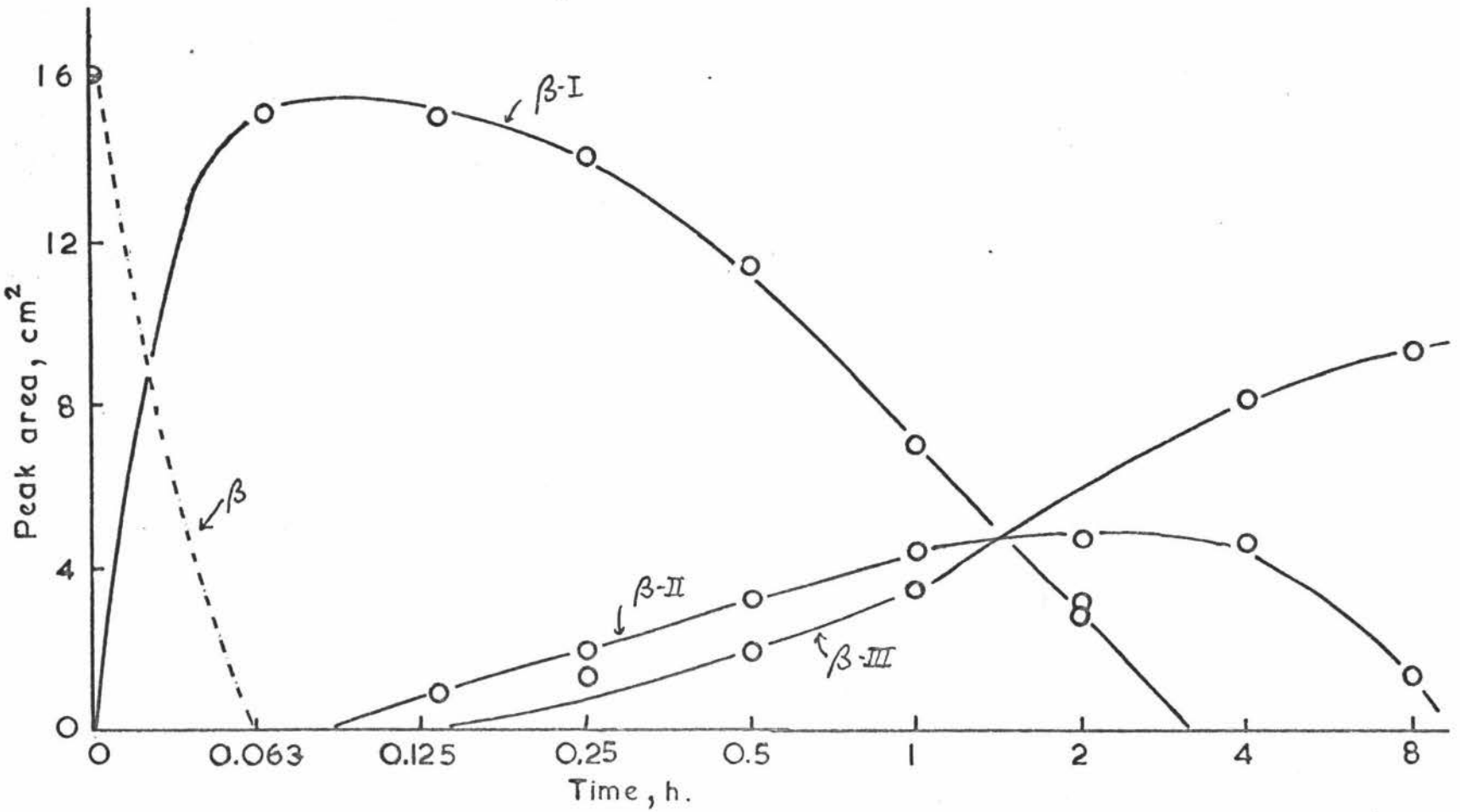


Fig. 15. Proteolysis at 25°C and pH 6.5.

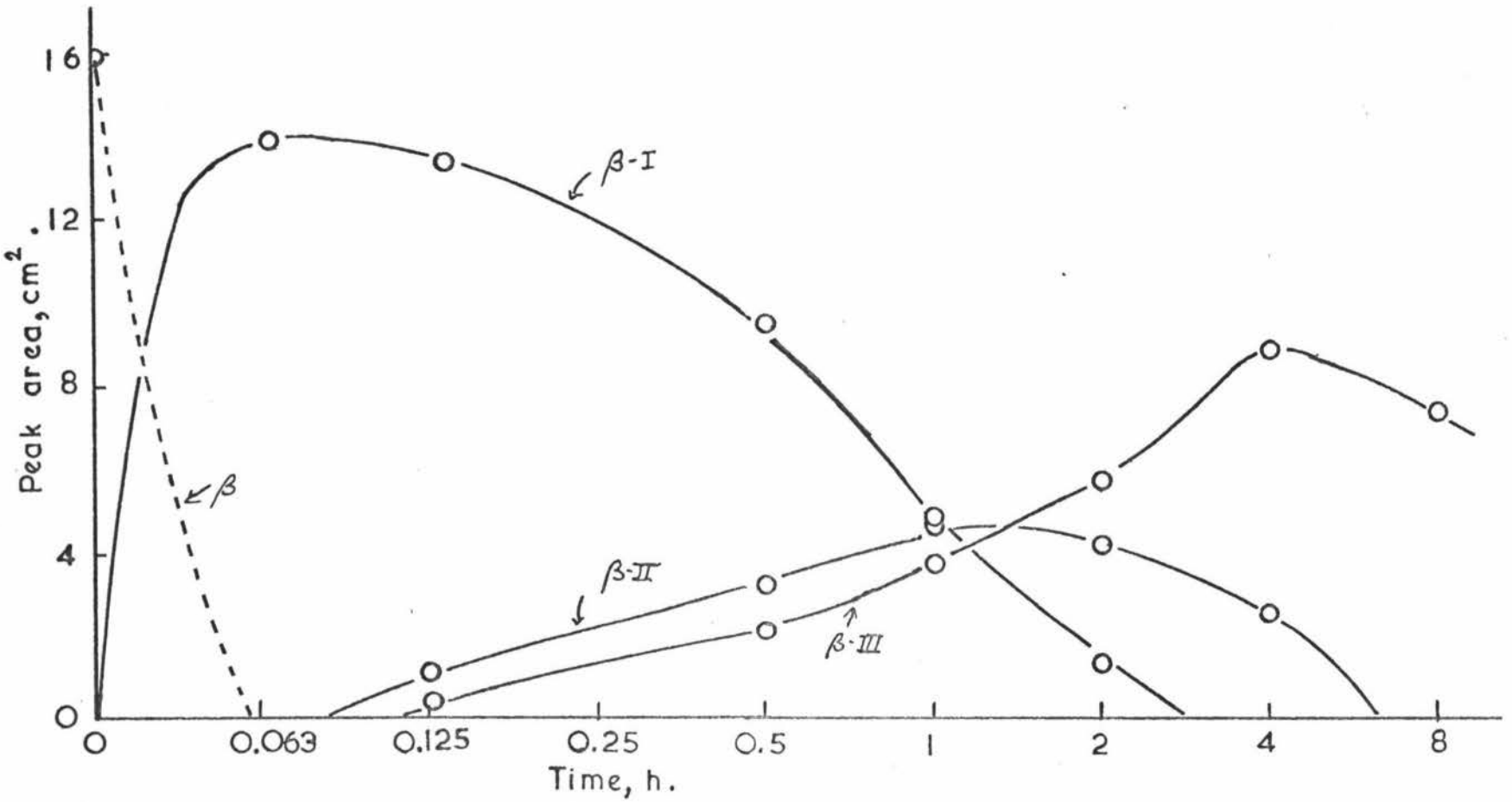


Fig. 16. Proteolysis at 37°C and pH 6.5.

In a recent investigation reported by Garnier[60] β-casein is shown to undergo a conformational change from State 1 at 5°C to State 2 at 40°C, the temperature at half transition being 23°C. State 2 is the more folded and compact. Denatured randomly coiled proteins are more susceptible to proteolysis. This then could explain the observed difference in rates, over the two temperature intervals.

(c) Effect of pH

Results obtained by hydrolysing β-casein as described in g of Table 4 have been included as a typical set (Table 7). The standard curve used was $y = 1973.10959\ x - 0.17817$ where y is the peak height in cm and x is the β-casein concentration in g/l.

Although a limited pH range was chosen, it would appear that, within experimental error and under the conditions of the experiment, hydrolysis of the most sensitive bond gave a maximum rate at pH 6.50 (Fig. 17). Ledford et al.[38] who measured total β-casein degradation by determining

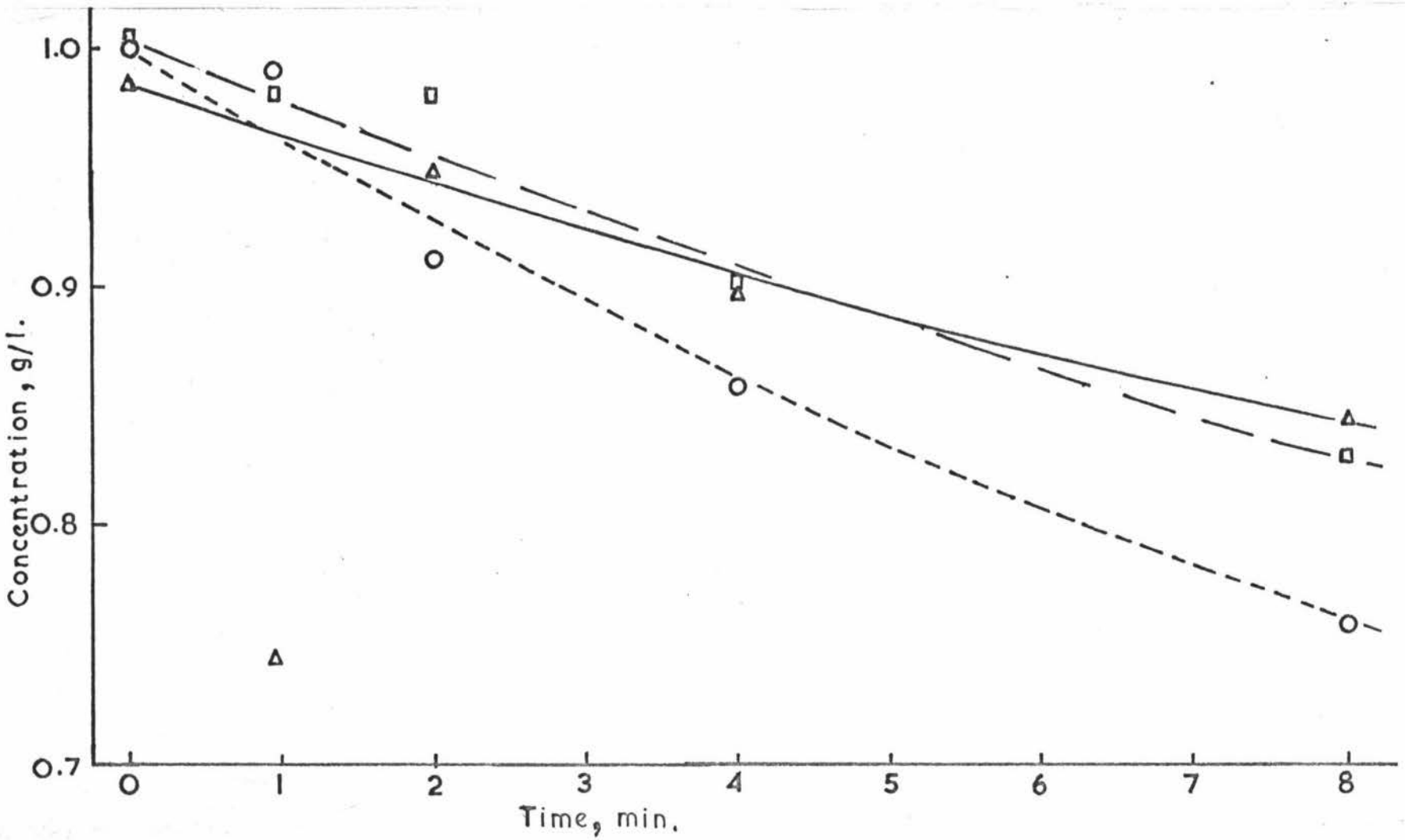


Fig. 17. Diagram showing the variation in the rate of rennin proteolysis of β-casein with pH at 37°C and an ionic strength of 0.04.
---O--pH 6.50,— —□—pH 6.12,—Δ— pH 6.94.

TABLE 7

Time from rennin addition (min)	Peak height of duplicates (cm)	Rotated 90°	Mean peak height (cm)	1/20 dilution in urea solution (g/l)	Concentration of β-casein (g/l)
0	9.3 9.9	10.0 9.5	9.7	0.0501	1.002
1	10.0 9.4	10.0 9.0	9.6	0.0496	0.992
2	9.8 7.3	10.1 7.9	8.8	0.0455	0.910
4	8.4 8.4	7.7 8.6	8.3	0.0430	0.860
8	7.6 6.6	7.6 7.4	7.3	0.0379	0.758

increases in absorbancy at 280 nm of supernatants obtained by precipitating with 5% TCA, showed that there was an increase in absorbancy from pH 7.0 to pH 5.5. This result means that total degradation increases from pH 7.0 to pH 5.5. In the current work only the rate of the first step was measured at changing pH and there is the possibility, then, that with decrease in pH, from 6.5, the rates of the second and subsequent steps increase while

that of the first step decreases. In this way the amount of 5% TCA soluble material could increase from pH 6.5 to 6.0.

(d) Effect of added cations

It became obvious in preliminary experiments that the ionic strength of the β-casein solution was an important factor in the rate of rennin hydrolysis of β-casein to β-I; to obtain equal rates of hydrolysis it was found necessary to increase the concentration of rennet from 0.5% for the low ionic strength solution (1 in Table 4) to 2.0% for the 0.04 ionic strength solution (g in Table 4).

Three experimental runs were conducted in order to test the hypothesis put forward by Bakri in which he says that the true substrate is a β-casein-divalent cation complex and the enzyme is not active on β-casein in the absence of the added divalent cation. For the first run the β-casein solution was unaltered (0.03 ionic strength derived from KCl). For the second run some of the KCl was replaced by $CaCl_2$, but the ionic strength maintained constant. For the

third run, some of the KCl was replaced by tri-sodium citrate (Table 4). The purpose of the citrate was to chelate any residual divalent cations possibly present in the β-casein or the rennet solutions. It was found that the citrate had a negligible effect on the rate of reaction but that calcium depressed the rate considerably (Fig. 18). It is most probable that there was a negligible quantity of multivalent cations in the normal solutions, and that citrate effects can be ascribed solely to ionic strength. The total cation concentration is less in the citrate containing solution and it follows that the concentration of a monovalent cation is relatively unimportant. Any alternative explanation of this result would necessarily be much more complex.

The inhibiting effect of increasing ionic strength on the hydrolysis of β-casein can be explained in terms of the Michaelis-Menten theory. At pH 6.5 both rennin and β-casein have an overall negative charge as their isoelectric points are at pH 4.6. Increasing

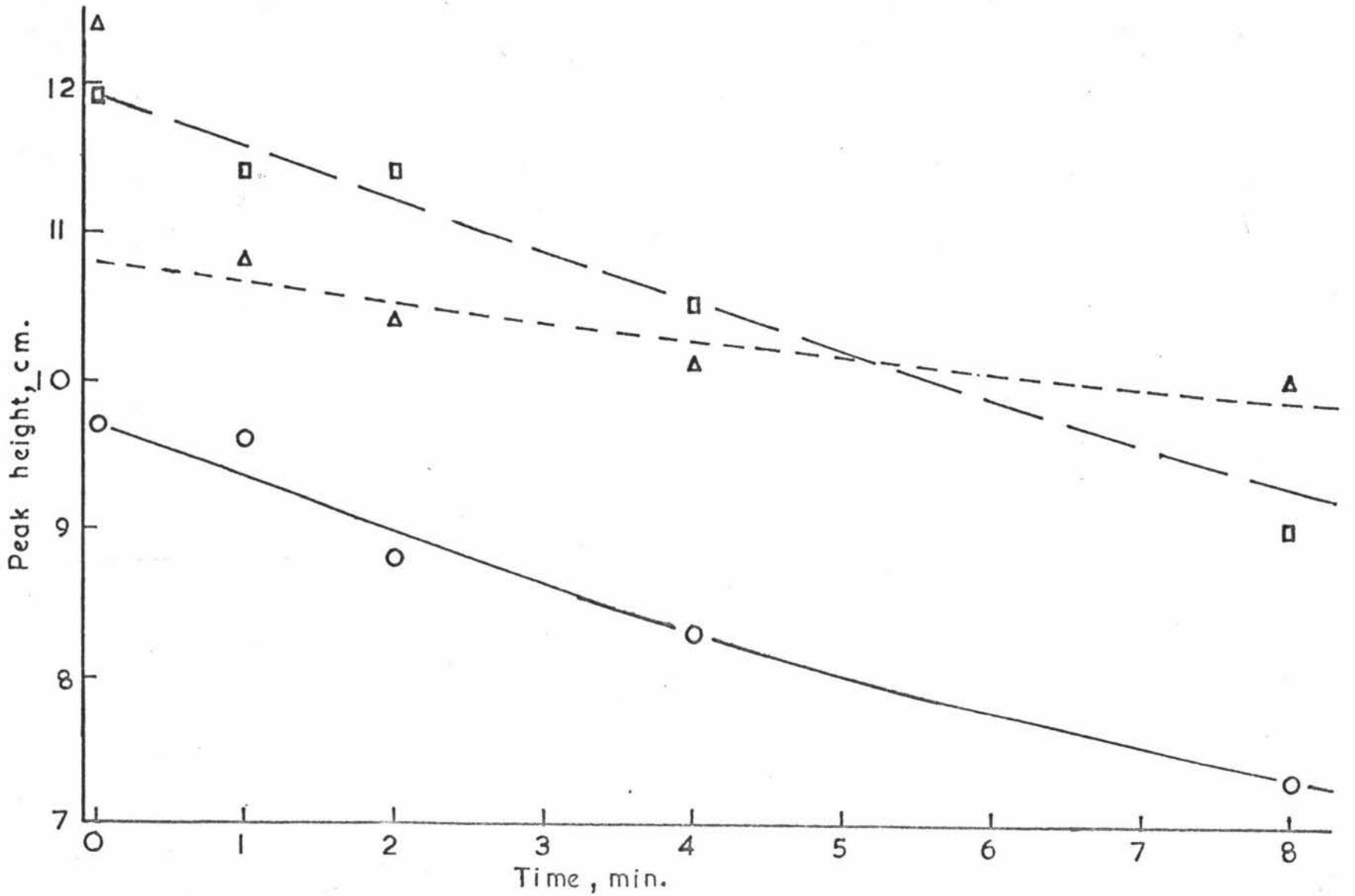


Fig. 18. Variation in the rate of rennin proteolysis of β-casein with change in cation at 37°C and pH 6.5. The ionic strength was constant at 0.04. ---Δ-- 3.7×10^{-3}M $CaCl_2$, — —□— 8×10^{-4}M sodium citrate, ——O—— standard.

the ionic strength will produce increased electrostatic shielding of the two like charges. It is probable then that an enzyme-substrate complex of greater stability results and consequently the rate of dissociation of the complex to the free enzyme and products would decrease.

The extra inhibition caused by added calcium ions has a probable explanation in the formation of an enzyme-metal-substrate complex. This type of complex suggests competition between the metal-substrate complex, and the metal ion for the active centre of the enzyme. As an alternative suggestion the addition of calcium ions to β-casein might result in a structural change of the substrate in which the rennin sensitive bond becomes less accessible.

(e) <u>The Michaelis constant</u>

The basis of the Michaelis-Menten theory is

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

where E - enzyme

S - substrate

ES - enzyme - substrate complex

P - products

k_1, k_{-1}, k_2 are rate constants.

From this reaction scheme the following equation is derived.

$$v = \frac{V [S]}{K_m + [S]}$$

v is the velocity of the reaction

V is max. velocity

$Km = \frac{k_{-1} + k_2}{k_1}$ and is the Michaelis constant.

The technique used did not lend itself very well to determining initial velocities using the tangent to the curve at zero time (Fig. 19). Instead the average velocity over the first four minutes was taken as the initial velocity. A value of 9.59 g/l was determined for K_m which was the intercept on the $\frac{1}{S}$ axis of the Lineweaver-Burk plot ($\frac{1}{v}$ versus $\frac{1}{S}$) (Fig. 20). The maximum velocity, V, was calculated from the intercept at the $\frac{1}{v}$ axis and had a value 5.69×10^{-4} g/l.s. The Lineweaver-Burk plot is a reasonable approximation to a straight line and hence supports

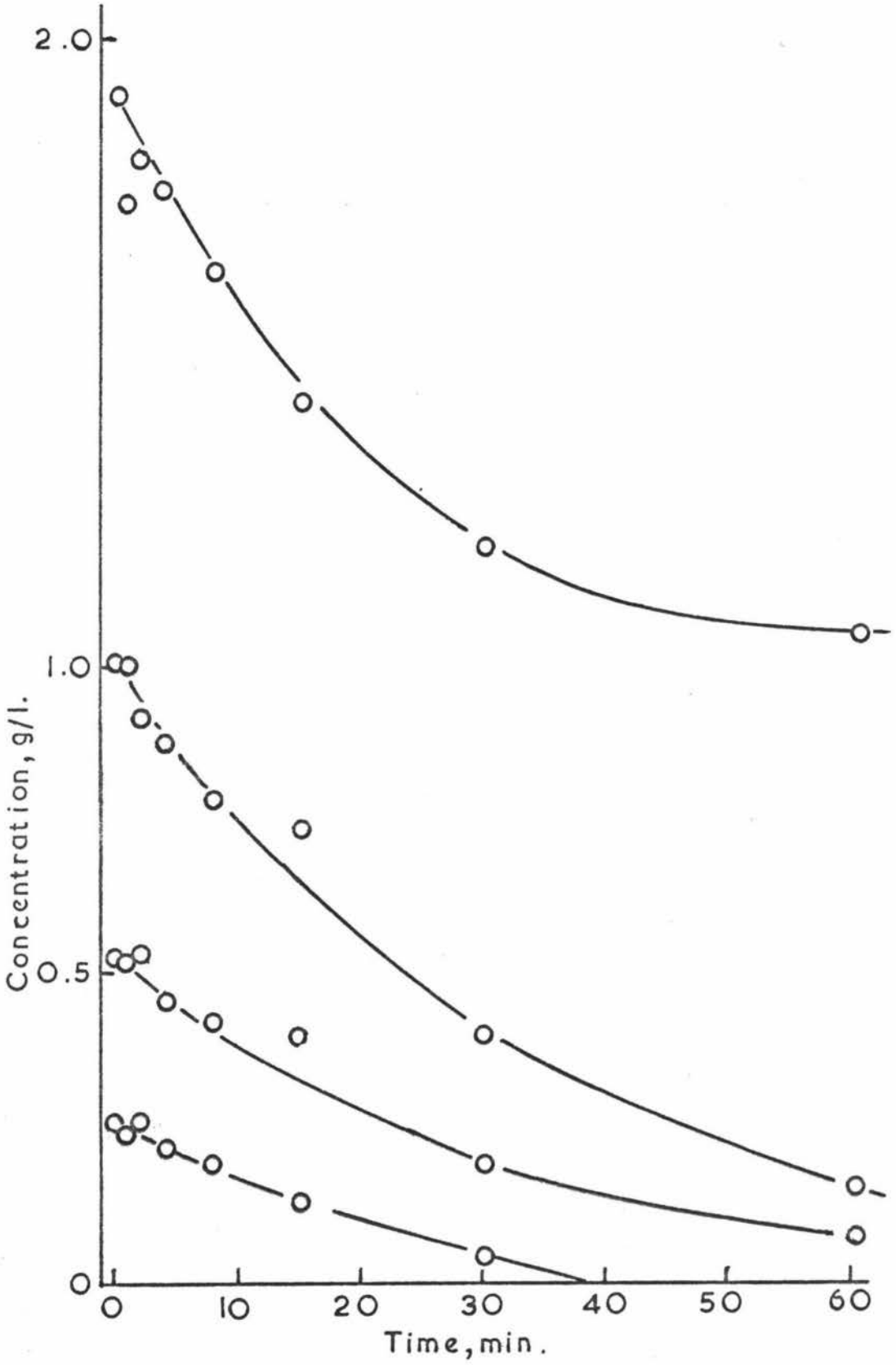


Fig. 19. Diagram showing the effect of substrate concentration on the rate of rennin proteolysis of β-casein (2% rennet, 37°C, pH 6.5, ionic strength 0.04).

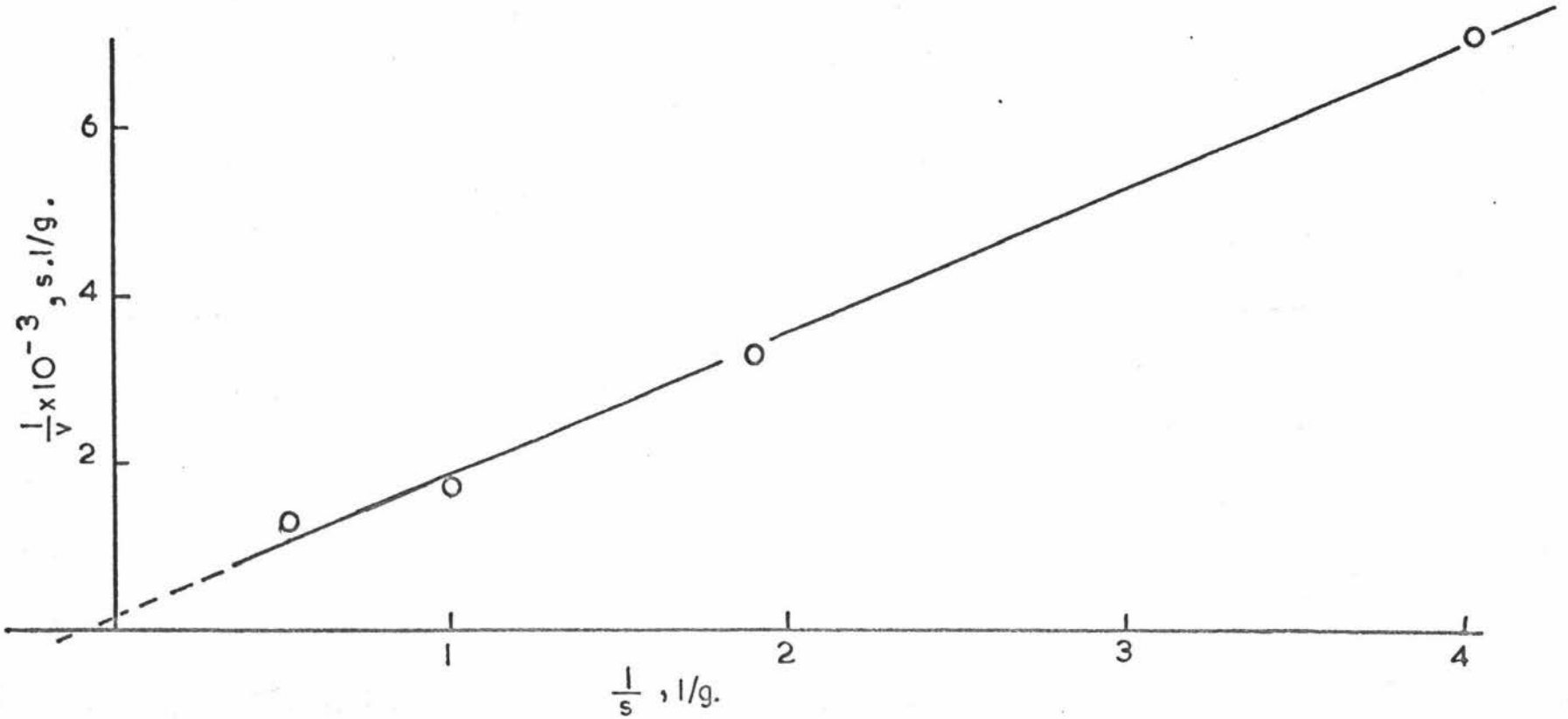


Fig. 20. Lineweaver-Burk Plot.
Rennin proteolysis of β-casein at 37°C, pH 6.5 and ionic strength of 0.04.

the assumption that the degradation from β-casein to β-I is in fact the first step of the degradation.

The velocity constant k_2 describes the rate of product formation from ES, the active intermediate. The velocity of formation of product from the active intermediate is then $V = k_2[ES]$. At the start of the reaction the substrate concentration is high. If all the enzyme is present as ES, then the velocity will be maximal and $V = k_2[E]$. Now if concentrations of both enzyme and product are quoted in moles/l k_2 will be the maximum turnover number of the enzyme. As the molarity of the rennin in the rennet solution is not known, an approximation may be made. Data obtained by comparing milk clotting ability of rennet and pure rennin powder, showed that 41 mg/ml of rennin in 0.072 M NaCl had the same milk clotting activity as undiluted commercial rennet (1 ml of 1% (v/v) rennet clotted the milk in 251 s as compared with 250 s for 1 ml of 0.041% rennin (w/v) in 0.72 M NaCl (w/v)). Assuming the molecular weight of rennin is 40,000[61] then the reaction mixture is approximately 4×10^{-7} M rennin. The maximum velocity as determined

(Fig. 20) is 5.69×10^{-4} g/l.s or 2.28×10^{-8} moles/l.s assuming a molecular weight of 25,000 for β-casein. Then k_2 is 6×10^{-2} s^{-1}.

Garnier[62] studied the hydrolysis of κ-casein by rennin under similar conditions using a titrimetric method. He determined a value of 36 s^{-1} for k_2 and 1.55 g/l for K_m. The rate of first bond hydrolysis of β-casein would appear then to be of the order of 10^{-3} times that of κ-casein, a factor not very different from that reported by Waugh[63].

IV

CONCLUSIONS AND SUGGESTED DEVELOPMENTS

Suggested locations of the rennin sensitive bonds of β-casein can be diagrammatically represented (Fig. 21). It has now been shown that β-I contains the N-terminal end of the β-casein molecule and that the degradation of β-casein to β-I is apparently complete before further degradation occurs. Also, β-II appears before β-III hence β-II plus β-III could not make up β-I. On this basis there are only four alternative representations which can be suggested. Bond I would be broken very rapidly whilst bonds II and III would be broken at comparable rates. Only those pieces indicated as β-I, β-II, β-III and β^1 should appear on disc electrophoresis. The other pieces are small peptides and either diffuse too rapidly or have too few stainable groups to be located on disc electrophoresis. In alternatives (a) and (d) it is possible that β^1 has the same electrophoretic properties as β-II but it is more likely that four discs would appear as degradation products on disc electrophoresis.

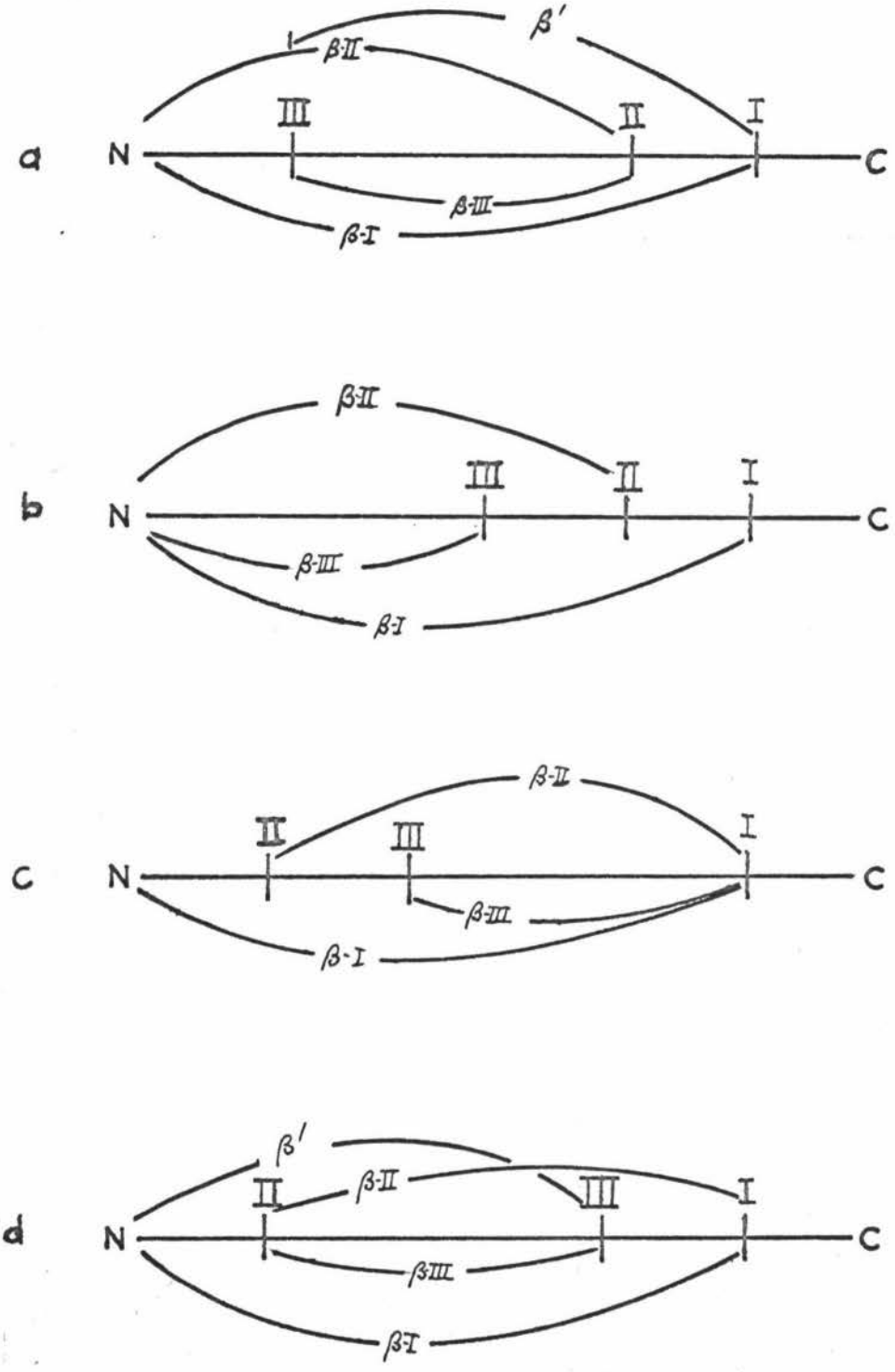


Fig. 21. Diagrammatic representation of the possible modes of degradation of β-casein by rennin.

The most probable bond sequence then is (b) or (c) and from the present work it is not possible to differentiate between them. By isolating β-I, β-II and β-III and determining their C-terminal and N-terminal amino acids and by comparing the results, a final choice of the alternatives could be made. There is, however, one point which should be made. It was mentioned previously that the diffuse nature of β-III on disc electrophoresis could be the result of diffusion of β-III during electrophoresis and staining. Alternatively rennin might cleave adjacent or close peptide bonds producing two peptides with an amino acid difference of one or two but with similar charges.

There is little obvious similarity between the results obtained by Lindqvist and Storgards[36] and those obtained in the present work. This is primarily due to the differences in the techniques used. Lindqvist and Storgards used moving boundary electrophoresis which, although more products can be seen, has a poorer resolution

than PAE. The only product which resembles any in the present work is the one they designate β_c. This could be the product named β-I in the present work as it has a slightly greater mobility than β-casein, appears early in the degradation at all pH's from 2.40 to 6.10 and is degraded relatively rapidly. The difference in time scales used for these two pieces of work adds further to the difficulty of comparison. Because hydrolysis of the most sensitive bond is much faster than the following ones a time scale in which the intervals are doubled seems more appropriate than the linear time scale used by Lindqvist and Storgards. As a consequence of the present work it is concluded that the disc electrophoresis technique in combination with the doubling interval time scale for sampling would be highly successful applied to the rennin degradation of other caseins or even to other protein-enzyme systems. A system of particular interest would be the degradation of β-casein with pepsin because of the almost identical specificity of pepsin and rennin as shown by the

hydrolysis of the B chain of oxidised insulin[56,57].

It is suggested that future investigations might be directed towards determining whether the effect of addition of calcium ions to the β-casein-rennin system could be explained in terms of a substrate-metal-enzyme complex or in terms of a conformational change. Conformational changes could be investigated using techniques such as light scattering, depolarisation of fluorescence or viscosity.

It is obvious by now that the proteolysis of β-casein by rennin is complex and that a complete understanding of it is not possible by superficial examination of reaction parameters alone rather than by examination of the mechanism by which the enzyme and substrate interact both with their environment and each other. For a more detailed knowledge the amino acid sequence of the whole substrate surface rather than the amino acids of the rennin sensitive bonds alone need to be determined. Proof of the importance of this approach has resulted from recent work[64] in which it was shown that the presence of serine at two amino acids distant from the phenylalanyl-

methionine bond of χ-casein accelerates rennin attack. When this information is known and also knowledge of the conformation of the interacting molecules it will be possible to postulate mechanisms of inhibition and activation on the atomic level rather than in the vague terms of the Michaelis-Menten theory.

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