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**A Study of Factors Contributing to Gel Formation and to
Syneresis of Gels with Particular Reference to Rennet
Casein Systems.**

A Thesis

**Presented in Partial Fulfilment of the Requirements for
the Degree of Doctor of Philosophy in Food Technology
at**

**Massey University,
Palmerston North,
New Zealand.**

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"EXHILARATION IS THAT FEELING YOU GET JUST AFTER A GREAT
IDEA HITS YOU AND JUST BEFORE YOU REALISE WHAT'S WRONG
WITH IT"

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ABSTRACT

An "artificial milk" system, prepared under controlled conditions, was developed. This was used for the study of the initial phase of rennet action on casein which results in gel formation. It was also used for a study of factors contributing to syneresis of rennet-casein gels.

"Artificial milks" containing calcium alone (60 - 125 mg% added calcium)* were shown to contain micelles very similar in gross structure and physical organisation to those in normal skim milk. It was demonstrated by electron microscopy that the presence of phosphate ion in the "artificial milks", whether as Na_2HPO_4 or as H_3PO_4 , sharply reduced the size of casein micelles which were then quite unlike those in skim milk.

Rennet action at 0°C on both sodium caseinate and calcium caseinate "milks" was found to significantly reduce the viscosity and hydration constant 'K' of the caseinate systems. An ultracentrifugal technique using haemoglobin as an indicator, showed that rennet action on casein micelles in calcium caseinate "milks" released, at or about gel point, a quantity of "immobilised" water which amounted to about 70% of the weight of the "wet" protein.

Electron Micrographs of calcium-caseinate "milks", calcium caseinate - calcium phosphate "milks" and skim milk indicated that gel formation was essentially a micelle-surface phenomenon. No indication was found for the distortion of the micelles due to rennet action, nor did rennet action appear to reduce the size of the micelles.

Whole casein was found to contain both cystine and cysteine. However, sulphhydryl - disulphide or disulphide exchange reactions did not appear to play a significant role in gel formation or in the syneresis

* added as $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$

of rennet-casein systems. The studies indicated that the presence of para-kappa casein in its entirety, probably on the micelle surface, was necessary for the development of a 3-dimensional gel network.

Modification of para-kappa casein through scission of the disulphide bond was found to give a precipitate rather than a gel network after rennet action.

Calcium was shown to be important for both gel formation and syneresis. In its absence, neither phenomena occurred in rennet-casein systems. Despite this observed fact, evidence has been presented to show that syneresis was not a continuation of the gelation phenomena. Factors which greatly affected syneresis were found not to affect gel formation. Both rennet modification of casein and the presence of calcium was found to be necessary for syneresis to occur. In the absence of either, no syneresis was observed even when the pH was lowered below the isoelectric point.

The level of calcium in the "milks" was found to greatly influence both the rate and the extent of syneresis. A sharp drop in syneresis was observed in rennet-casein systems containing between 125 mg% and 175 mg% added calcium. It was postulated that the addition of such a level of calcium neutralised by interaction, the available negative charges on the caseinate and that this caused the gel network to acquire a net positive charge, thus giving rise to electrostatic forces promoting swelling and opposing syneresis.

Dinitrophenylation studies and studies on heated "artificial milk" systems containing reducing sugars showed that β casein played a major role in the interactions causing syneresis. α_s and kappa casein did not appear to play a significant role in this phenomenon. The studies emphasised the important and possibly direct role played by the free

amino groups of β -casein, probably the epsilon amino groups of lysine, in syneresis. The attractive forces causing shrinkage of the system after gel formation has occurred initially appear to be primarily electrostatic in character.

It appears possible that free amino groups are exposed by rennet action on kappa casein and these are involved with calcium in the gelation phenomenon.

It is postulated that both gelation and subsequently syneresis result from the formation of co-ordinate covalent linkages between calcium and the separately involved free amino groups of the kappa casein for gelation and β casein for syneresis.

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INTRODUCTION

There has been a general concentration of efforts by milk chemists in recent years in trying to understand the nature and stability of the colloidal phase of milk, namely the calcium-caseinate - calcium phosphate complex. Rose⁽¹⁾ has rightly pointed out that the inability to "control gelation, coagulation and hydration of the colloid phase" has been the greatest impediment to the development of new beverage milk products. On the other hand, destruction of the stability of the colloidal complex is necessary in the production of products such as acid casein and cheese.

The literature concerned with the action of rennin on casein is extensive - there has been a particular surge in research activity on this topic over the past decade. But, as yet, no firm conclusions can be drawn as to the exact mode of action of rennin on the calcium caseinate - calcium phosphate complex or as to what type of cross-links are formed to give the gel structure. On the other hand, the literature is sparse in dealing with the phenomena of "syneresis" or curd contraction which is so important in the manufacture of most types of cheese. This lack of precise information on two of the most fundamental and important phenomena important to the cheese industry is, no doubt, due to the meagre knowledge of the chemistry and biochemistry of the calcium caseinate - calcium phosphate complex.

To throw some light on the two phenomena which could be related to each other, or be interdependent, a study was undertaken on aspects of rennin action on casein, leading to a study of factors contributing to the syneresis of rennet-casein gels. The study was initiated on the knowledge that buffaloes' milk, used in some countries both as a beverage and for conversion into milk products, was not quite as satisfactory as cows' milk for the preparation of hard varieties of cheese such as cheddar and Gouda. This was in the main due to the curd losing excessive moisture during the manufacturing process.

The work was done on cow milk casein with the hope that the same fundamentals could be applied to buffalo milk casein, perhaps with some modifications.

PART I

The Action of Rennin on Casein.

CHAPTER I

Review of Literature on the Action of Rennin on Casein

SECTION A:

GENERAL REVIEW

Rennin is an enzyme which occurs in the fourth stomach of calves. As far back as 1872, Hammarsten⁽²⁾ had postulated that the action of rennet on casein, with the liberation of peptide-like substances, was through an enzyme present in the rennet extract. This enzyme was found to be rennin⁽⁴⁾ which was first isolated and crystallised by Berridge⁽⁵⁾. Its specificity was found to be similar to that of pepsin, its optimum pH, however, being much higher, about 4.0⁽⁶⁾*. Rennin was shown to attack other proteins, for example, haemoglobin⁽⁵⁾ and insulin⁽⁷⁾. Rennin is not the only enzyme to clot milk, other proteolytic enzymes having a similar action⁽³⁸⁾⁽¹²⁾. Mickelsen and Ernstrom⁽³⁷⁾ have recently reported on factors affecting the stability of rennin. The action of rennin on the caseinate complex may be broadly visualised as occurring in three phases, namely:

- (a) primary enzymatic phase⁽⁸⁻¹⁰⁾ in which one of the casein components acting as a protective colloid is attacked and its protective action destroyed,
- (b) secondary phase in which coagulation occurs, and
- (c) tertiary phase⁽¹¹⁾ in which casein components are slowly hydrolysed under the proteolytic action of rennin. In normal coagulation the three phases probably overlap.

Despite the evidence presented⁽⁴⁾, Berridge⁽¹²⁾ was unable to accept the protective colloid theory, stating that it did not explain "the time required for the clotting phase, nor the dependence of

the clotting on temperature". Instead a denaturation hypothesis was put forward. This was based on observations that the enzymatic stage progressed with a temperature coefficient similar to known enzymatic reactions, and that the second stage had a high temperature coefficient similar to that found in the heat denaturation of proteins.

The "protective colloid" theory, however, gained impetus with the discovery of a new casein component by Waugh and von Hippel⁽¹³⁾, which they termed kappa-casein. This component appeared to be in a complex with the α_s -casein (that is, α_s + kappa-casein formed the original entity α -casein). The authors⁽¹³⁾ concluded from their observations that kappa-casein was the "most important single factor responsible for micelle stabilisation and was the protein on which rennin acted immediately". Kappa-casein was also found to be the only protein altered during rennin coagulation of casein⁽¹⁴⁾, losing 20% of its molecular weight. The para-kappa-casein (rennin altered kappa-casein) was insoluble in the absence or presence of calcium ions. In contrast, kappa-casein itself was remarkably stable to high calcium ion concentrations⁽¹⁴⁾ of the order of 0.03 M at neutral pH.

Nitschmann and his co-workers⁽¹⁵⁾ reported that 1.5 - 2.0% non-protein nitrogen (soluble in 12% T.C.A.) was rapidly split off by rennin from whole casein. After coagulation occurred there was little or no further liberation of T.C.A.-soluble nitrogen. This N.P.N. proved to be composed mainly of a glyco-macropptide of molecular weight 6000-8000⁽¹⁵⁾. A similar glyco-macropptide was found to be released from kappa-casein⁽¹⁶⁾. Since rennin was considered to be only slightly proteolytic and as the macropptide was released very quickly from casein, Nitschmann and Varin⁽⁹⁾ postulated that the nitrogenous material liberated was not bound to the caseinate by primary valencies.

Beeby and Nitschmann⁽²⁴⁾ suggested that rennin opened secondary bonds of the kappa-casein complex, releasing the glyco-macropeptide. All enzymes capable of clotting milk have been found to liberate the N.P.N.⁽³⁸⁾. Analyses of the macropeptide⁽¹⁷⁾ indicated the presence of 15.2% galactose, 4.3% glucosamine and 11.4% of neuraminic acid. Wake⁽¹⁸⁾ confirmed earlier observations⁽¹⁶⁾ on the release of the glyco-macropeptide and traced its origin to kappa-casein. He was unable to find evidence for the rupture of a peptide bond in the release of the macropeptide.

Holter and Li⁽¹⁹⁾ working on a substituted amide of phosphoric acid, and Dyanchenko⁽²⁰⁾ had proposed that rennin cleaved phosphoamide bonds in whole casein. Fish⁽⁷⁾ was not able to demonstrate phosphoamide activity for purified rennin, although with commercial rennet, the activity could be demonstrated. However, Aiyar and Wallace⁽²¹⁾ presented evidence to indicate that purified rennin had phosphoamidase activity on whole casein, and postulated the presence in casein of -N-P-N- bonds with the phosphate group joining two arginine residues on the protein molecule. Koning and Stadhouder's⁽²²⁾* work indicated that only arginine groups increased during the first 30 minutes of rennin action. The relationship of this observation with clotting was, however, not clear.

Chuan⁽²²⁾ on the basis of electron micrographs and amperometric titrations for sulphhydryl groups proposed that milk clotting was similar to blood coagulation in which a sulphhydryl-disulphide chain reaction was involved. He also postulated a rapid, irreversible unfolding of the caseinate particle in the first stage followed by a slow aggregation in the second stage. A similar -SH-SS- exchange reaction for clotting was proposed by Rege⁽⁶³⁾.

* cited in Ref. 48

An esterase activity for rennin was suggested by the work of Jolles et al⁽²⁵⁾ and Garnier et al⁽²⁶⁾ but this theory was questioned by Cheesman, Rawitscher and Sturtevant⁽²⁷⁾ who were unable to measure liberation of protons during rennin action on casein.

More recently Hill and Laing⁽³⁹⁾ observed that photo-oxidation of kappa-casein prevented the liberation of non-protein nitrogen and that photo-oxidation of rennet-treated casein interfered with the aggregation which occurred normally in the presence of calcium ions. They attributed both of these effects to modification of the histidine residues. Earlier, Zittle⁽⁴⁰⁾ had observed that photo-oxidised α_s - casein was no longer sensitive to calcium ions; and that photo-oxidised kappa-casein⁽⁴¹⁾ lost its ability to stabilise α_s - casein and did not precipitate on treatment with rennin. Higgins and Fraser⁽⁴²⁾, on the basis of titration data and spectral differences between the azo-derivatives of casein and para-casein, had suggested the participation of histidine in the gelling of para-casein. Subsequently Hill and Craker⁽⁴³⁾ observed that blocking of lysine side chains on the α_s and β caseins with 1-dimethylamino-napthalene 5-sulphonyl chloride (dansyl chloride) had no effect on their stabilisation by kappa-casein, nor on the coagulation of rennin-treated kappa- α_s , and kappa- β complexes. Lysine did not appear to be important for these processes. The lysine residues on the kappa-casein were apparently not involved in the reaction between rennin and casein since dansylated kappa-casein released N.P.N. on rennin action. However, dansylation of kappa-casein did cause inhibition of coagulation. Hill and Craker⁽⁴³⁾ interpreted this observation as being due to the participation (in the coagulation of rennin-treated whole casein) of one or more lysine side chains on the kappa-casein fraction.

Rennin action on kappa-casein does not appear to liberate new N-terminal amino acids in the para-kappa-casein produced⁽¹⁸⁾⁽⁴⁴⁾. A new N-terminal methionine residue has, however, been observed on the glyco-macropeptide⁽⁴⁵⁾. Earlier a new C-terminal phenylalanine residue had been identified on the para-kappa-casein⁽²⁵⁾, which prompted a suggestion that rennin cleaved a phenylalanine-methionine bond⁽³⁾. Hill⁽⁴⁶⁾ found that rennin was unable to hydrolyse the phe-met. bond in the dipeptide or its derivatives, but did so on the synthetic pentapeptide containing the amino acid sequence similar to the region in kappa-casein containing the phe-met bond⁽³⁾. This reaction was found to be moderately rapid at pH 3.7 and 4.7 (time for half reaction 4 hours) and slower at pH 5.9 (12 hours). Hill⁽⁴⁶⁾ suggested that serine played a catalytic role in the reaction but that the nearby hydrophobic side chains probably acted as binding sites, since specificity studies on rennin⁽⁷⁾ had indicated its tendency to hydrolyse bonds adjacent to hydrophobic side chains. The attack on the pentapeptide was, however, slower by a factor of 2 - 3. Hill⁽⁴⁶⁾ attributed this to the absence of catalytic action of the histidine residue, that is, the nearby serine and histidine side chains catalysed the enzymic attack on the phe-met bond of kappa-casein. Such a catalytic role has been postulated for the side chains in the active centre of enzymes.

Viscometric studies of renneted casein have also given rise to contradictory and confusing results. Sterling⁽²⁸⁾ suggested unfolding and, therefore, an increasing asymmetry of the spherical casein micelles during the enzymatic stage. He stated that this unfolding could not be observed with sodium caseinates, the alkaline caseinates being already in an elongated state due to preparation procedures. On the other hand Higgins and Hayes⁽²⁹⁾ observed a

decreased intrinsic viscosity following enzymatic action on sodium caseinate solutions, indicating increased particle symmetry. Scott-Blair and co-workers⁽³⁰⁾⁽³¹⁾ attributed the decrease in observed viscosity to the lower molecular weight of the sodium caseinate after rennet action and to steric effects resulting from change in shape of the coiled casein particles after the release of the glyco-macropptide. In a recent review, Conway and Dobry-Duclaux⁽³³⁾ have pointed out that the viscosity of solutions of polyelectrolytes may be increased in three ways, namely:

- (a) clouds of ions around individual particles would be deformed as a result of shearing and this deformation would take up energy,
- (b) Possible interference between the ionic atmospheres of the different particles, and
- (c) change in shape of the coiled-up particles when the electrical free energy is changed by ionisation and the presence of neutral salts. Scott-Blair and Oosthuizen⁽³⁰⁾ on the basis of observations that small amounts of sodium chloride reduced the viscosity of sodium caseinate (ruling out electroviscous effect from 'a' above) and that Newtonian flow behaviour was shown by casein solutions up to 9% protein (therefore ruling out 'b'), concluded that it was the change in shape of the coiled-up particle causing the decrease in viscosity.

On viscometric evidence, Sohngen and Wieringa⁽³⁴⁾ proposed that the first stage of rennin action involved casein dehydration. Richardson and Palmer⁽³²⁾, however, had indicated earlier that rennin was able to decrease the electrokinetic charge on the casein micelles and Smoluchowski⁽³⁵⁾ had shown that a suspension of charged particles

was more viscous than one of uncharged particles. Thus the decrease in viscosity could have been due to true dehydration or to decrease in electrokinetic charge or both factors. Hankinson and Palmer⁽³⁶⁾ found that hydration and zeta potential were both partially destroyed by rennin. At low rennin concentrations they observed a large decrease in relative viscosity with little change in electrokinetic potential. This was interpreted as being due to true dehydration. Increasing the rennin concentration decreased both hydration and electrokinetic potential. They proposed that several partially hydrated particles subsequently clump to form a particle bigger than the hydrated particle. This explained the increase in viscosity just prior to gelling. Hankinson and Palmer⁽³⁶⁾ concluded that rennin action was primarily that of dehydration and secondarily that of reducing the zeta potential. Fish⁽⁷⁾ reported on the specificity of rennin as a proteolytic enzyme, finding that it had a strong tendency to hydrolyse bonds in proteins adjacent to hydrophobic side chains. Klotz⁽⁴⁷⁾ has pointed out that hydrophobic residues on proteins may be expected to play an important part in the maintenance of tertiary structures and that they stabilised large regions of water around themselves in the form of ice-like structures. It follows from the above that rennin action on any bond adjacent to hydrophobic residues might firstly entail the disruption of these water regions. Lindquist⁽⁴⁸⁾ has stated that the primary and secondary phases of rennin action on casein could be separated by lowering the temperature (refrigerator temperatures)⁽⁴⁹⁾⁽⁵⁰⁾, but these phases probably overlap under normal conditions of casein coagulation. * He suggested that the first stages of the secondary phase involved rearrangement of serine and phosphoric acid-bound calcium and of colloidal complexes of calcium, magnesium,

phosphate, and citrate, which resulted in a decrease in the amount of water of hydration bound to these complexes. New forces then aggregated the casein micelles. Christ⁽⁷³⁾, Konow⁽⁷⁴⁾, and Cheeseman⁽⁷⁵⁾ have also postulated that rennin action removes the hydrate water shell and that only then does "real" syneresis begin, involving hydrogen bonding between the protein units.

Several electron microscopic studies⁽⁵¹⁾⁽⁵²⁾⁽⁵³⁾ have been carried out to study changes before and after coagulation. The general conclusions were⁽⁴⁸⁾ that casein micelles are joined by short, fine-threaded formations to nodular fibrils. These form an assembly of strings, partly adhering to each other, and, in a more advanced stage, orientated into a three dimensional network. Using polarised light and phase contrast microscopy, Swiatek⁽⁵⁴⁾ made a fluorescence-microscopic examination of the gel structure and showed that the threads consisted partly of ball-shaped protein particles and partly of hygroscopic, inorganic material concentrated in the corners and constrictions of the structure.

SECTION B: REVIEW OF LITERATURE ON THE FACTORS AFFECTING THE
ACTION OF RENNIN ON CASEIN.

1. Effect of Concentration of the Enzyme:-

Nitschmann and Bohren⁽⁵⁵⁾ showed that at rennin concentrations above 0.8 µg N/ml in the reaction mixture, the terminal point of the primary phase, measured by release of NPN, was independent of the amount of rennin. The rate of reaction was, however, very strongly dependent on the concentration of the enzyme. Below 0.8 µg N/ml the terminal point of the primary phase was dependent on the amount of rennin as the maximum amount of glyco-macropetide was never obtained. This was probably due to irreversible inhibition of the rennin.

2. Effect of pH:-

Foltman⁽⁵⁶⁾ and later Mickelsen and Ernstrom⁽⁵⁷⁾ showed that loss of rennin activity increased with pH above pH 6.0, and was highly temperature dependent. Foltman⁽⁵⁸⁾ found that at low pH values, below pH 6.3, milk coagulated before the primary reaction, that is, release of N.P.N., was completed. At constant temperature (25°C) a lowering of pH from 6.7 to 6.3 shifted the coagulation point towards the terminal point of the primary phase. Nitschmann and Bohren⁽⁵⁵⁾ showed that at pH 5.5 the velocity of the primary phase was three times faster than at 6.7, whereas between pH 2.0 - 3.5 the tertiary proteolytic reaction was very fast. At pH 2.0 they were unable to detect the primary action. The pH optimum for the primary reaction was said to be between 3.5 and 5.5. At pH 5.4 or lower, little or no calcium was needed to cause precipitation

of the caseinate following rennin treatment⁽⁵⁹⁾⁽¹⁷⁴⁾.

Presumably this was because the pH values were close to the isoelectric point of the para-casein.

3. Effect of Temperature:-

Foltman⁽⁵⁸⁾ showed that at constant pH (7.0) a rise of temperature shifted the coagulation point towards the terminal point of the primary phase so that they coincided at 40°C. The effect was similar to dropping the pH from 6.7 to 6.3. Nitschmann and Bohren⁽⁵⁵⁾ reported that the rate of primary reaction was eight, four, and two times faster at 30°C, 20°C, and 10°C respectively than at 1°C.

At refrigerator temperatures, milk does not coagulate although the primary phase may be completed⁽⁴⁹⁾⁽⁵⁰⁾.

Berridge⁽⁴⁹⁾ found that the temperature coefficient for the coagulation phase was 1.3 - 1.6/1°C, while Pyne⁽⁶¹⁾ reported a figure of 2 per 1°C in calcium caseinate solutions. These coefficients were analogous to those found for thermal denaturation of proteins and led to Berridge's denaturation hypotheses⁽¹²⁾. Kelley⁽⁶²⁾ found that coagulation time decreased with increasing temperature up to 40°C, above which the rate of destruction of the enzyme exceeded its increase in activity.

4. Effect of Various Inorganic Compounds:-

Most of the work on the effect of inorganic compounds has been done in relation to clotting rather than on the primary phase of rennin action. Thus, monovalent ions such as sodium, potassium and ammonium do not precipitate para-casein whereas magnesium, calcium, barium, cobalt, nickel, etc. cause rapid

precipitation of para-casein at room temperatures⁽⁶⁰⁾ and neutral pH. Calcium chloride added to milk at a concentration of 0.142% appeared to give the quickest clotting. Above and below this concentration, coagulation was slower.

5. Effect of Various Organic Compounds:-

Rege⁽⁶³⁾ found that addition of glycerol at levels between 0.2 - 6.0% v/v delayed clot formation in reconstituted skim milk. The delay was equivalent to about a 40% increase on the "control" clotting time at 6.0% v/v of glycerol addition. This was attributed to the possible solvation effect on proteins by this polyhydroxy compound of low molecular weight. Gelatin on the other hand was found to accelerate clotting either by serving as a bridge to enhance the association of casein micelles⁽⁶³⁾⁽⁶⁴⁾ or by providing cations which would induce precipitation of the micelles⁽⁶⁴⁾.

6. Effect of Modifying the Casein with Respect to its Inorganic Constituents:-

Casein is a phospho-protein requiring calcium ions for gelation. This led McFarlane⁽⁶⁶⁾ to suggest that the calcium ions form intermolecular links between bound phosphate groups. Later Hsu et al⁽⁶⁷⁾ showed that while enzymatic dephosphorylation of casein resulted in marked increases in clotting time at 0.01 M Ca^{++} concentration, the same casein did clot rapidly following rennin treatment in the presence of a calcium ion concentration of 0.02 M. This suggested that in addition to the postulated links of calcium to phosphate groups, other types of inter-molecular bonds were involved in the clotting reaction⁽³⁹⁾. Pepper and

and Thompson⁽⁶⁸⁾ also found that enzymatically dephosphorylated kappa-casein was still able to stabilise α_s casein in the presence of calcium ions. The authors concluded that α_s -kappa complex formation was not necessarily dependent on the presence of phosphate-calcium interactions.

Yamauchi et al⁽⁶⁹⁾ found that dephosphorylated whole casein and dephosphorylated α_s casein bound one-third of the calcium normally bound by the respective native proteins, while dephosphorylated kappa-casein bound nearly half of the calcium bound to native kappa-casein. They observed that dephosphorylated α_s casein was still able to coagulate in the presence of calcium ions⁽⁶⁹⁾ which suggested that binding of calcium to organic phosphate groups was not essential for the coagulation of α_s casein. Also dephosphorylated whole casein could still form micelles in the presence of calcium ions. It appeared therefore that while organic phosphate groups were important binding sites for calcium, other binding sites were also present. Carboxyl groups have been suggested⁽⁷⁰⁾ as alternative binding sites, as have amino and imidazole groups⁽⁷¹⁾. The pH of caseinate solutions was observed to drop on the addition of calcium ions⁽⁷²⁾ possibly due to displacement of protons on the amino groups on casein by the formation of coordinate covalent links between the amino N and the calcium⁽⁷¹⁾. Yamauchi et al⁽⁶⁹⁾ found that addition of 15mM CaCl_2 to a caseinate solution at pH 7.0, dropped the pH by 0.8 for native whole casein and by 0.4 for dephosphorylated whole casein. The difference was probably due to proton contribution from phosphate groups.

SECTION C: OBJECTIVES AND EXPERIMENTAL PLAN.

The review of literature had indicated considerable confusion and difference of opinion, particularly on the results obtained by viscometric studies, on the initial or primary phase of rennin action on casein. Whether the initial action is one of dehydration of the casein micelles, prior to gel formation, has been a matter of argument. The second phase of rennin action is also largely unknown. It is still not clear what role, exactly, is played by calcium, and on what sites on the casein it binds to form, if at all, the cross links to give gel structure.

The following experimental plan was therefore devised in the hope of throwing some light on the initial phase of rennin action on casein. Simple model systems using "artificial milks" were to be used in the study to avoid the possible interference of other factors such as whey proteins, and other mineral constituents. The "artificial milks" were essentially "calcium caseinates" or calcium phosphate-calcium caseinate systems. The advantage of using such systems was that it would give day to day conformity in composition, allowing comparison between experiments.

It was believed that a combination of viscometric, ultra-centrifugal and electron microscopic studies would help to reveal the mechanism of the initial phase of rennin action on casein. It was also considered that a study of the effect of added thiol blocking and disulphide splitting reagents, and of calcium ions, on gelation and syneresis of rennet-casein gels would give an insight into the mechanisms of the two phenomena. This has been discussed in Part II of this thesis (see Ch.3 c).

CHAPTER II

Viscometric, Ultracentrifugal and Electron Microscopic Studies on the Action of Rennet on Casein

SECTION A: METHODS AND MATERIALS.

1. Preparation of Acid Casein

The method used is that described under "Preparation of acid casein - (a)" in the paper entitled "Sulphydryl and disulphide groups in casein" (attached)^{(82)*}. To keep oxidative changes on the protein, if any, to a minimum, the skim milk used was purged of dissolved air by bubbling nitrogen through it for 10 minutes before acid precipitation. This nitrogen treatment was sustained throughout the preparation process.

2. Preparation of "Artificial Milks"

The preparation of a stable "artificial milk" has been described by Aiyar⁽⁸⁴⁾ (paper attached). To ensure complete solvation of the protein, all solutions were held in the refrigerator at temperatures below 5°C for at least 12 hours with constant stirring, after which they were warmed to 30°C and held at this temperature for at least 30 minutes before starting the experiment. Toluene (2 drops/100 mls) was used as a preservative in all solutions.

When orthophosphoric acid was used as a source of phosphate the following was done:

Orthophosphoric acid solution containing 11 mg 'P'/ml (4.8% H_3PO_4) was added right at the start to the casein slurry and the mixture titrated to pH 6.8 with 0.2 N NaOH. At pH 6.0

'ARTIFICIAL MILK' AS A BASIS FOR RESEARCH

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SUMMARY: A method for the preparation of a stable 'artificial milk' is described, using acid casein as the protein base and incorporating calcium and phosphate in the system. The 'milk' was primarily designed as a research tool, but the method could be used for the preparation of a beverage.

Various workers (Clark, 1927; Powell and Palmer, 1935; Grindrod, Price and Sommer, 1956) have used 'artificial milks' as basic materials for research on factors such as the action of rennin and the effect of heat on calcium caseinate-calcium phosphate sols. Use of 'artificial milks' simplifies experimental procedures for the study of phenomena such as syneresis of rennet-milk gels, of which little is known, and in which workers in this laboratory are interested. Whether or not 'artificial milks' represent conditions of true milk remains to be seen, but it is believed that much useful information can be obtained by using model, simplified systems.

In 'artificial milk' systems used by others (Clark, 1927; Powell and Palmer, 1935) the acid casein was usually solubilized in cold calcium hydroxide solution. Sodium and/or potassium hydroxide solutions were also added for quicker solubilization of the caseinate, and the pH was adjusted later by citric and/or phosphoric acid to 6.6-6.9.

Experience in this laboratory shows that solubilizing casein at high pH (in excess of 9.0)

has a detrimental effect on the caseinate, even if precautions are taken to do the mixing at 2°C. The final 'milks' prepared in this laboratory by the methods used by others (Clark, 1927; Powell and Palmer, 1935; Grindrod *et al.*, 1956) showed significant quantities of precipitates, after 24 h holding in the refrigerator, ranging from 300-500 mg in a 3% casein solution.

High pH, in excess of 9.0, is known to affect the sulphur amino acids and in particular cystine. Whole casein appears to have both cystine and cysteine (Wallace and Aiyar, 1969) and these amino acids seem to be present only in the κ -casein fraction of whole casein (Waugh, Ludwig, Gillespie, Garner, Kleiner and Noble, 1960). κ -casein is now recognized as being the stabilizing factor (Waugh and Von Hippel, 1956) for casein micelles in milk, and it would be most undesirable in the preparation of 'artificial milks' to use conditions whereby κ -casein could be irreversibly modified.

Use of low temperature during the preparation of 'artificial milks' is also undesirable. It has been shown (Waugh, 1958) that at low temperatures (4°C) β -casein does not enter into reaction with α -caseins or κ -caseins in the formation of stable micelles, but remains separate, and when the temperature is raised to 18°C and above, β -casein precipitates.

On the basis of the above information, a new approach was taken to the formulation of 'artificial milks' and resulted in the development of a product which is extremely stable.

Experimental and Results

The composition of the 'artificial milk' is as follows:

	Quantity	Percent. (w/v)
Acid casein (hydrochloric, moisture-free basis, preferably spray dried)	3 g	3
Calcium (as calcium chloride)	125 mg	0.125
Phosphate (as Na ₂ HPO ₄)	75 mg	0.075
Distilled water	up to 100 ml	

The levels of calcium and phosphate approximate the average values found in normal milk.

Hydrochloric casein, preferably spray dried, was found to be most suitable. Spray dried sulphuric acid casein was also satisfactory, provided it had been efficiently washed. Lactic casein may not be suitable when lactic starters are used, as the stability of the caseinate system could be affected by proteinases produced by the starter bacteria.

The 'milk' is prepared as follows:

Whole acid casein and a quantity of solid calcium chloride (CaCl₂·2H₂O) of required equivalent calcium concentration, are mixed and some distilled water (30-35 ml) at room temperature is added to form a slurry. 0.1 N NaOH is then added dropwise to the thoroughly agitated slurry. When the system has reached a pH of 6.0 the temperature is raised to 35°C-37°C, the addition of sodium hydroxide being continued until the pH reaches 6.7. At this point all of the casein has dissolved. Disodium hydrogen phosphate (of required equivalent phosphate concentration), dissolved in 20-30 ml of distilled water, is then added dropwise to the system with constant agitation. This causes an initial drop in the pH presumably due to the reaction of free calcium ions and the added phosphate with consequent release of hydrogen ions (Jenness and Patton, 1959).

$3\text{Ca}^{++} + 2\text{HPO}_4^{--} \rightarrow \text{Ca}_3(\text{PO}_4)_2 + 2\text{H}^+$
Sodium hydroxide is therefore added concurrently with the phosphate solution to maintain the pH above 6.4. The addition of sodium hydroxide is continued until a rise in pH indicates the completion of the above reaction. When all the disodium hydrogen phosphate solution has been added the final pH is adjusted, if necessary, to 6.7-6.9 by the addition of 0.1 N NaOH.

'Milks' prepared in the manner described showed no sedimentation when held in the refrigerator for more than a month (a few drops of toluene were used as a preservative). When

warmed to 40°C, there was no coagulation of the solution, contrary to the findings of Powell and Palmer (1935), who prepared 'artificial milks' using calcium hydroxide for solubilizing the casein. The same workers used only 77% of the average calcium and phosphate levels found in normal milk, in the preparation of their 'artificial milks' to avoid coagulation at 40°C. Similarly, Grindrod *et al.* (1956) were unable to prepare a stable solution at room temperature. 'Milk' prepared in the manner described in this paper could tolerate boiling for one hour without showing signs of sedimentation.

Reaction to rennet appeared normal and the gels so formed appeared to synerise normally when compared to skim milk gels. Such milks, with varying calcium concentrations, have been used in gel-syneretic studies with interesting results.

It is possible to blend into this base 'milk' formulation, other materials, such as milk fat and lactose for the preparation of a drinkable beverage. It is well known that 'substitute milks' are gaining importance as beverages in the United States. Sodium caseinate has been used as a protein base in these 'milks' because of its high nutritive value, but difficulties have been experienced due mainly to off-flavours which develop in the caseinate and which are imparted to the 'substitute milks'. This limits the amount of sodium caseinate which can be used in these products (about 0.8%) and consequently the nutritive value of the beverage is low. A further problem with the use of sodium caseinate is its sensitivity to calcium ion concentration and that sedimentation will occur if calcium is included in the formulation.

Acid casein has advantages over sodium caseinate as a protein base. It has better keeping qualities than neutral sodium caseinate and does not sediment in the presence of calcium if the 'milks' are formulated as described.

Reconstituted 'artificial milks' have been prepared in this laboratory as follows:

	Quantity	Percent. (w/v)
Acid casein (preferably spray dried)	3 g	3
Calcium (as CaCl ₂ ·2H ₂ O)	80 mg	80 mg%
Phosphate (as Na ₂ HPO ₄)	75 mg	75 mg%
Lactose (or other sugars in concentrations of equivalent sweetness)	4.9 g	4.9
Anhydrous butter fat	4.0 g	4.0
Glycerylmonostearate	0.5 g	0.5
Gelatine	0.3 g	0.3

The 'milk' with casein, calcium chloride, and disodium hydrogen phosphate solution is prepared as before with a final pH of 6.7-6.9. Lactose is added when the casein has gone completely into colloidal solution. The 'milk' is then warmed to 120°F, and fat and glycerylmonostearate mixed in. Gelatine, dissolved in warm water, is added and the mix is then homogenized. The resulting 'reconstituted milk' is quite stable, has a good body, and acceptable flavour. It should be possible to blend other minerals and vitamins into this system.

It will be noted that the calcium level in the reconstituted 'milks' has been reduced from 125 mg % (in the original base formula) to 80 mg %. This was done to reduce the associated chloride content which otherwise tended to give the 'milk' a rather salty taste.

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the temperature was raised to 35°C, and kept constant until the final pH was reached. The "milks" were then treated as described earlier.

"Artificial milks" containing only calcium were prepared in the same manner without any form of phosphate addition. Calcium was added in the form of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$. For higher than 125 mg % calcium "milks" (used in syneresis studies) the process was slightly modified - this is described under the appropriate section (Ch. V: D. 1.)

3. Preparation of Sodium Caseinate Solutions

Sodium caseinate solutions were prepared by making a slurry with the required quantity of acid casein in distilled water and titrating to pH 6.8 with 0.1 N NaOH. Subsequent treatments were similar to those given to "artificial milks".

4. Rennet used in these studies was a commercial preparation supplied by the New Zealand Rennet Co., Eltham, New Zealand.
5. All chemicals used in the study were of A.R. or L.R. grade. Glass distilled water was used in the preparation of all solutions.

SECTION B: VISCOMETRIC STUDIES ON THE ACTION OF RENNET ON CASEIN

1. Introduction

The dependence of viscosity on concentration has been thoroughly investigated for many spherocolloids. A theoretical treatment of this subject was first given by Einstein (1906)⁽⁷⁶⁾ who derived the equation:

$$\eta_{rel} = 1 + 2.5 \phi$$

where ϕ represented the relative volume concentration of spherical, colloidal or coarser particles. According to the equation, the viscosity of a spherocolloid should increase linearly with its concentration⁽⁷⁶⁾. This equation was modified⁽⁷⁶⁾ for more convenient use as follows:

$$K = \eta_{sp} \times \rho / c \quad \text{where}$$

ρ = density of the dispersed phase

c = grams substance in 100 mls. solution.

If the value of K equalled 0.025, the hydration of the colloid was considered negligible. A larger value meant that the particles were hydrated or solvated. It is noteworthy that both the equations do not take particle size into consideration, which meant that the viscosity of spherocolloids was independent of the particle size.

The modified Einstein formula was used in this work for calculation of the hydration constant "K".

2. Experimental

A Brookfield viscometer was used to measure all viscosities. Use was made of the special accessories* provided with the instrument for the measurement of low fluid viscosities.

* U L adapter

Viscosity measurements on the rennet-treated casein were made at 0°C to avoid complications from protein aggregations. For this purpose the special accessories of the viscometer (including the spindle head) were equilibrated at about 0°C overnight (16 hours) before viscosity measurements were undertaken.

25 ml. of the cold casein solution with an added drop of distilled water, from a 1 ml. bulb pipette, was then taken in the cold cylinder and this was quickly fitted on to the viscometer, over the spindle head. A thermos flask of suitable internal diameter and containing a mixture of cold distilled water and crushed ice (prepared earlier from distilled water) at a temperature of 0°C was then placed under the viscometer. The viscometer was lowered into this flask so that the cylinder was completely immersed in the ice cold water.

After allowing 5 minutes for equilibration of temperature, readings were taken on the viscometer with the spindle head rotating either at 30 RPM or 60 RPM. The absolute viscosity was then calculated using appropriate factors based on the speed of the spindle head (as per instructions for the viscometer),

$$\text{for example, at 60 RPM, } \eta_{\text{abs}} = (\text{Reading} - 0.4) \times 0.1$$

$$\text{at 30 RPM, } \eta_{\text{abs}} = (\text{Reading} - 0.1) \times 0.2.$$

The specific viscosity η_{sp} was then calculated using the following formula⁽⁷⁶⁾:

$$\eta_{\text{sp}} = \frac{\eta_{\text{coll}} - \eta_{\text{sol}}}{\eta_{\text{sol}}},$$

where η_{sp} is the specific viscosity which measures the increase in viscosity produced by the dissolved substance.

$$\eta_{\text{coll}} = \text{absolute viscosity of the colloidal solution,}$$

$$\eta_{\text{sol}} = \text{viscosity of the solvent or continuous phase.}$$

The value of η_{sp} was then used to calculate the hydration constant "K".

In the experiment one drop of rennet was added to 25 mls. of casein solution and the viscosity was measured as detailed above after fixed reaction time intervals. Values for the constant "K" were calculated from this data.

3. Results

(a) Preliminary work on sodium caseinate solutions

Results are given in Table I and plotted in Figures 1 and 2.

Table I. Effect of Casein Concentration on the Absolute Viscosity of Sodium Caseinate Solutions (pH 6.8) at 0°C.

Casein conc. (gms/100cc)	Viscometer reading at 30 RPM*	Absolute viscosity (η_{abs}) cps	Specific viscosity (η_{sp})	Abs. viscosity of water, 0°C (cps)
0.25	9.5	1.88	0.093	1.72
0.50	10.0	1.98	0.151	1.72
0.75	10.9	2.16	0.256	1.72
1.00	11.5	2.28	0.326	1.72
2.00	14.7	2.92	0.700	1.72

* At least 3 viscometer readings were taken per sample.

The straight line relationship between protein concentration (up to 2%) and absolute viscosity in sodium caseinate solutions (Fig.1) indicates that the latter behaves like a spherocolloid, probably because of the nature of protein-protein interactions. Also it is interesting to note that when the straight line in Fig. 1 is extrapolated to zero protein concentration, the value of η_{abs}

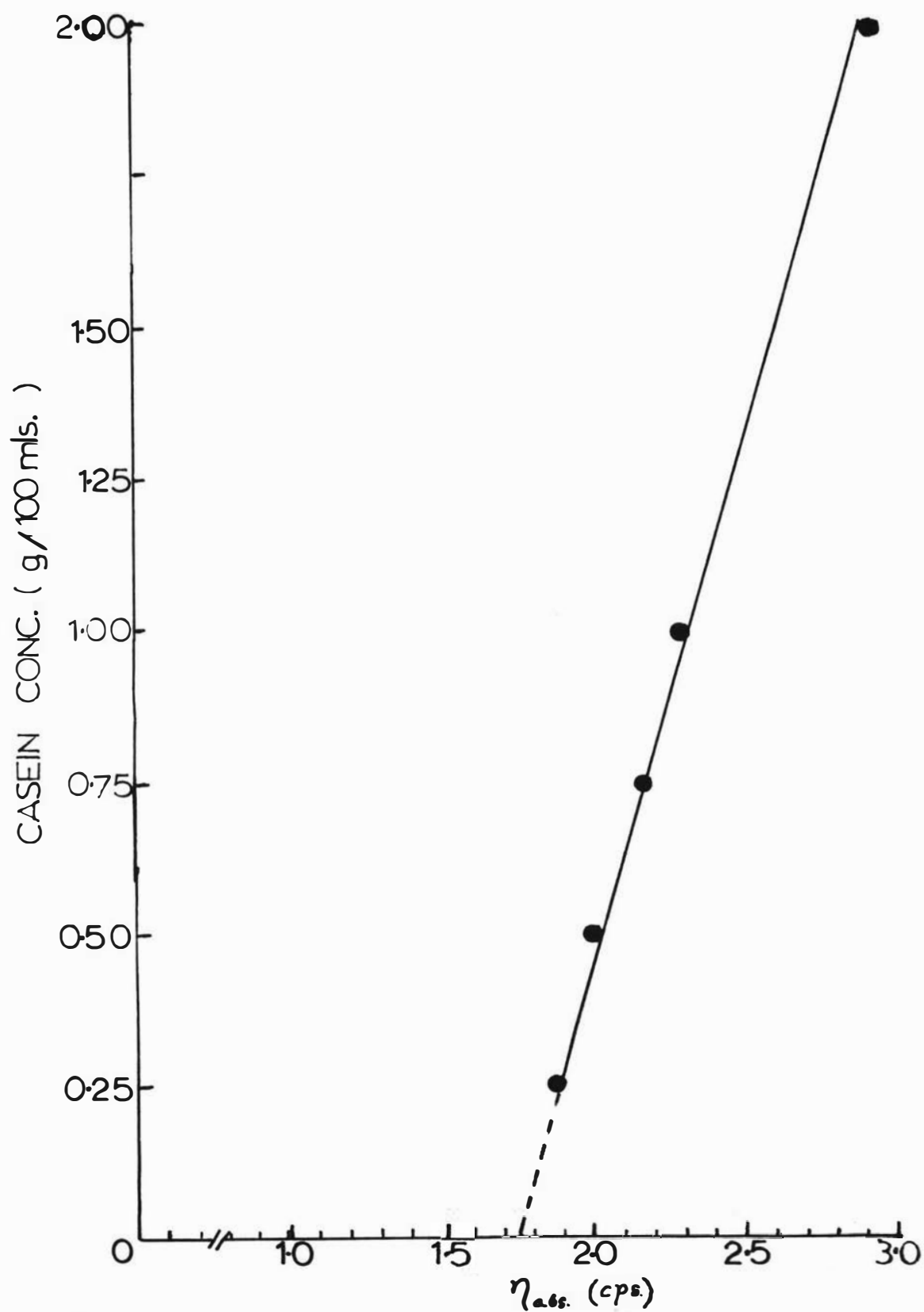


FIG.1.- EFFECT OF CASEIN CONCENTRATION ON THE ABSOLUTE VISCOSITY OF SODIUM CASEINATE SOLS. (pH 6.8, 0°C)

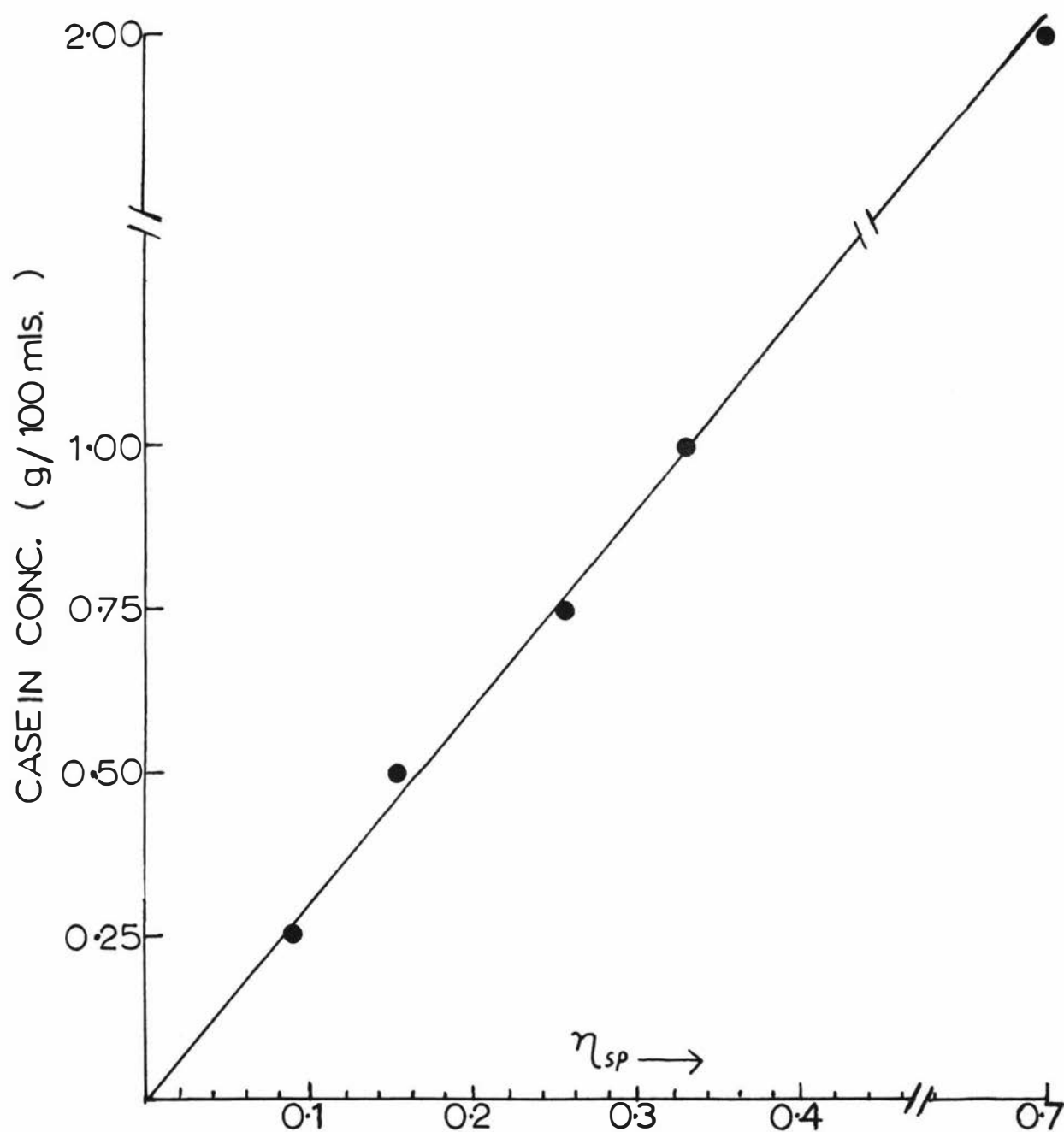


FIG. 2.- RELATION BETWEEN SPECIFIC VISCOSITY AND CASEIN CONCENTRATION IN SODIUM CASEINATE SOLS. (pH 6.8, 0°C)

becomes about 1.70. This value is similar to that obtained for distilled water at 0°C, that is, 1.72.

A linear relation was also found between the specific viscosity and casein concentration in sodium caseinate solutions, the line passing through the origin (Fig. 2). It would appear therefore, that sodium caseinate behaves like a spherocolloid⁽⁷⁶⁾ in the concentration range used in this work.

(b) Determination of the hydration constant "K" for sodium caseinate and sodium-para-caseinate solutions.

The change in viscosity of a rennet-treated 2% sodium caseinate solution (pH6.8) was determined at 0°C. The determination was done over a period of time, with the viscometer cylinder immersed in the ice water. Crushed ice was continuously added to the thermos flask to maintain the temperature at 0°C. For calculation of the constant "K", the density of the dry protein 'P' was taken as 1.37, as calculated by McMeekin et al⁽⁷⁸⁾, and absolute viscosity of the solvent as 1.72.

The results are shown in Table II.

Table II. Effect of Rennet Action on the Value of the Constant "K" for 2% Sodium Caseinate Solution at pH 6.8 and 0°C.

Time after rennet addition (minutes)	Viscometer reading at 30 RPM	η_{coll} (cps)	$\eta_{sp} = \frac{\eta_{coll} - \eta_{sol}}{\eta_{sol}}$	"K" = $\eta_{sp} \times P/c$ ($= \eta_{sp} \times 0.69$)
0	15	2.98	0.730	0.50
30	14.5	2.88	0.67	0.46
60	13.8	2.74	0.59	0.41
120	13.3	2.64	0.53	0.37
1000	12.0	2.38	0.38	0.26

Note: η_{coll} = abs. viscosity of the caseinate solution.

η_{sol} = abs. viscosity of the continuous phase (1.72).

P = density of the dry protein = 1.37.

c = concentration of the caseinate, gms/100 mls.

The results indicate that the value of the constant "K" drops appreciably for the para-caseinate solutions from the original value of 0.50 calculated for sodium caseinate. Viscosity of the non-renneted sample remained stable for at least 24 hours. However, the final value (0.26) after 1000 minutes of rennet action at 0°C is still much higher than 0.025 at which point a spherocolloid is considered to hold negligible water of hydration⁽⁷⁶⁾. This may be due to several factors.

(a) Rennet action may not fully dehydrate the caseinate particles, but may cause sufficient dehydration to destabilise the system, so that it gels in the presence of calcium ions.

(b) The interaction between the individual casein components to form a complex may be such that a loose complex structure is formed.

The complexes probably carry hydrated water not only on the surface, which is removed wholly or partially by rennet, but also internally, which may not be wholly removed by rennet action.

- (iii) The para-caseinate particles are still sufficiently charged to give an electroviscous effect.

3. (c) Determination of the constant "K" for Calcium Caseinate and Calcium Para Caseinate Solutions

The change in viscosity of a 5% calcium caseinate solution containing 90 mg % calcium at pH 6.8 was determined at 0°C, as before. The density of the dry caseinate complex was taken as 1.43 as calculated by Ford et al⁽⁷⁷⁾.

The results are shown in Table III.

Table III. Effect of Rennet Action on the Value of the Constant "K" for a 5% Calcium Caseinate Solution at pH 6.8 and 0°C.

Time after rennet addition (minutes)	Viscometer reading at 60 RPM	η_{coll}	$\eta_{sp} = \frac{\eta_{coll} - \eta_{sol}}{\eta_{sol}}$	$K = \eta_{sp} \times P/c$ ($= \eta_{sp} \times 0.28$)
0	49	4.86	1.80	0.50
20	44.4	4.40	1.55	0.43
40	43	4.26	1.45	0.40
60	42	4.16	1.40	0.38
120	40.5	4.01	1.30	0.36
1000	39.0	3.86	1.25	0.35

Note: η_{coll} = absolute viscosity of calcium caseinate solutions.

η_{sol} = absolute viscosity of the continuous phase.

P = density of the dry calcium caseinate complex.

c = concentration of the protein, gms/100 mls.

Results from Table III indicate, as in the case of the sodium caseinate solution, a significant drop in the value of "K" from 0.50 for the unrenneted sample to 0.35 after rennet has acted for 1000 minutes. Once again, this value is far above the value of 0.025 at which point the hydration of spherocolloids is considered negligible.

SECTION C: USE OF AN ULTRACENTRIFUGAL TECHNIQUE FOR THE
MEASUREMENT OF THE RELEASE OF "IMMOBILISED" WATER FROM
CASEIN MICELLES BY RENNET ACTION.

1. Introduction

The technique developed to measure release of "immobilised" water from the caseinate by rennet action depended on the use of a coloured compound whose concentration could be measured spectrophotometrically in a caseinate system before and after rennet action. This meant that within certain selected concentrations, the coloured compound would have to follow Beer's Law with respect to the relation between optical density and concentration. The principle of the method was based on subjecting the mixture of casein and the coloured compound to a high centrifugal force in the ultracentrifuge under conditions whereby the casein micelles would sediment but not the coloured compound. By comparison with suitable standard curves, it was found possible to measure the dilution of the coloured compound in the supernatant. Obviously, no coloured compound could be used which would react chemically with the caseinate. Haemoglobin was selected as the likely substance to meet the conditions required, for the following reasons:

- (a) The molecular weight of haemoglobin is 68000⁽⁸⁰⁾. In comparison the casein micellar weight runs into millions - 80% of the micelles range from 10 to 625 millions and a significant number reach 3000 million⁽⁷⁹⁾. It was hoped that this great disparity in weight would help separate the

two under suitable conditions in ultracentrifugation with the caseinate coming down as a pellet.

- (b) Haemoglobin has been reported⁽⁸⁰⁾ to be resistant to trypsin and other proteolytic enzymes. Furthermore, rennin has an optimum activity pH of 4.0 whilst for pepsin (which may be present in rennet extracts as an impurity) it is about 2.0⁽⁶⁾. Neither of these was therefore expected to cause significant changes in haemoglobin at about neutral pH.
- (c) The sulphhydryl groups on haemoglobin are reported to be unreactive at pH 6.8⁽⁸¹⁾, which is the pH at which "artificial milks" were prepared for this study. The sulphhydryl groups of haemoglobin cannot be oxidised by ferricyanide at neutral pH; on cysteine the sulphhydryl group is readily oxidisable⁽⁸¹⁾. Furthermore, the sulphhydryl groups on casein also seem to be well protected from the environment⁽⁸²⁾⁽⁸³⁾. It was unlikely therefore that haemoglobin would interact with the caseinate.
- (d) Being a coloured protein it would be possible to measure its concentration by means of spectrophotometry. Preliminary work indicated that it was possible to centrifuge a haemoglobin solution in distilled water at 326,000 g for 35 minutes without sedimenting the haemoglobin. A Beckman ultracentrifuge was used for the purpose.

2. Experimental and Results

- (a) Wavelength of maximum absorption for haemoglobin solutions
A 0.05% (w/v) solution of haemoglobin was prepared in glass distilled water and in 0.005 M CaCl_2 solution for determination of wavelength of maximum absorption. The reason

determining this in the presence of calcium was that later experiments on the ultracentrifuge were to be carried out with mixed solutions of calcium caseinate and haemoglobin and that the wavelength of maximum absorption might change in the presence of calcium.

Optical density measurements were made sequentially at different wavelengths in a Beckman Spectrophotometer (model D.U. 2400). The wavelength of maximum absorption was found to be 404 μ . (Figure 3). Results also indicated that calcium ions had no effect either on the wavelength of maximum absorption, nor on the optical density readings for each wavelength.

(b) Calibration curve for haemoglobin solutions in distilled water and in presence of calcium chloride

Solutions of haemoglobin were prepared in both distilled water and in 0.005 M CaCl_2 solution at the following concentrations:

70 mg%, 140 mg%, 210 mg%, 280 mg%, 350 mg% (w/v).

The solutions were held in the refrigerator before use.

0.5 ml. of each of the above solutions was then mixed with 3.0 mls. of water or 3.0 mls. of 0.005 M CaCl_2 . The final haemoglobin concentrations ranged from 10 mg% to 60 mg%.

Each system was then centrifuged at 326,000 g for 35 minutes, in a Beckman Ultracentrifuge. For each system two temperatures were used, 1°C. and ambient temperature (21 - 22°C). In all the tubes some slight sediment was noticed. This appeared to be coarse and fibrous in nature and essentially

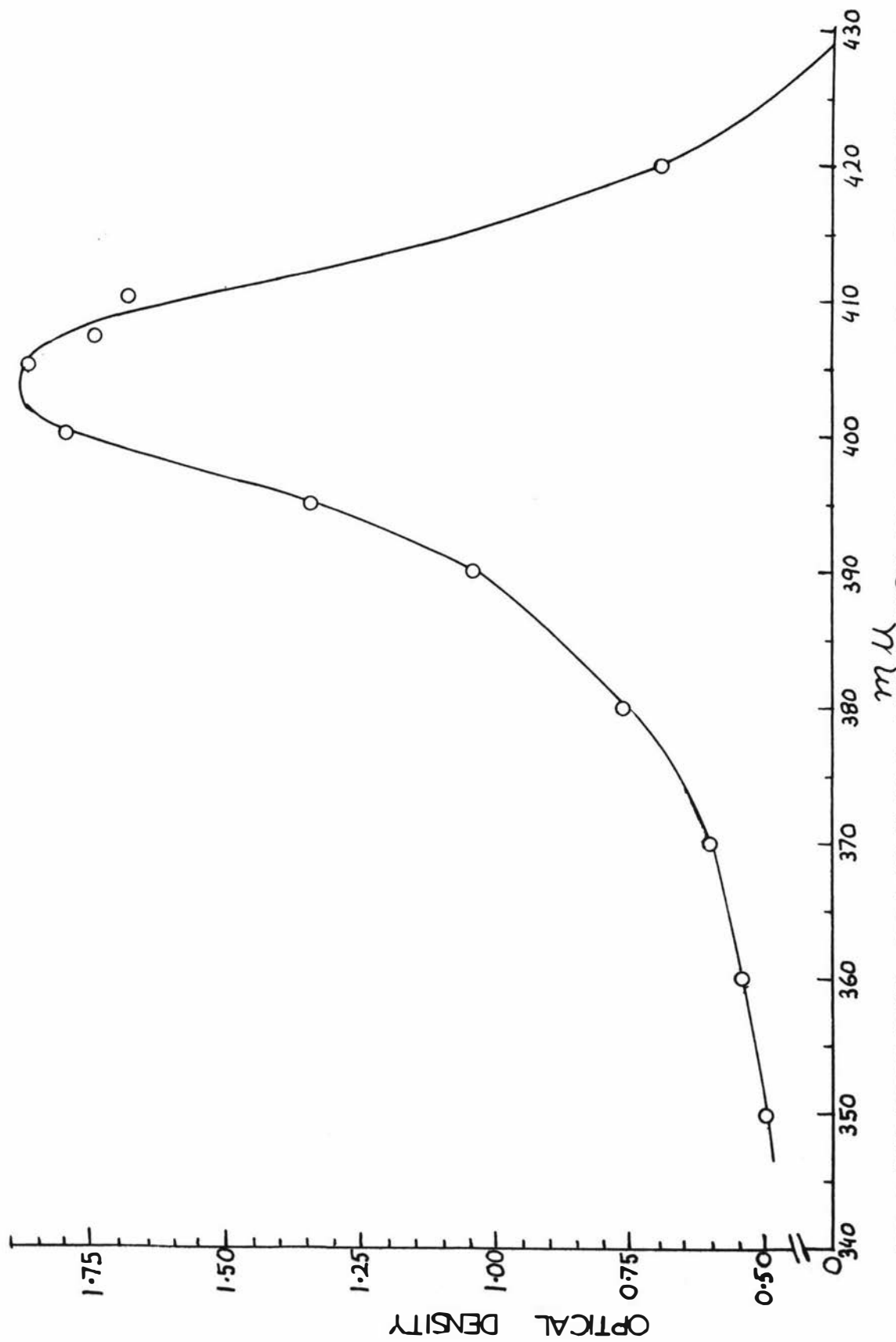


FIG. 3.-WAVELENGTH OF MAXIMUM ABSORPTION FOR HAEMOGLOBIN SOLUTIONS
 (0.05% w/v IN DISTILLED WATER AND 0.005 M $CaCl_2$)

extraneous. In a second run, 40 mls. of each of the above haemoglobin solutions (70 mg% - 350 mg%) were mixed with a drop of commercial rennet (diluted 1:1) and incubated at 20°C for one hour. 0.5 ml. aliquots of the solutions were then mixed with 3 ml. of distilled water or 0.005 M CaCl_2 solution as before and subjected to ultracentrifugation at 326,000 g for 35 minutes.

The optical densities of all ultracentrifuged samples were then measured on the Beckman Spectrophotometer at 404 mμ. The results from these measurements were used for drawing the calibration curve.

Results are given in Table IV.

Table IV. Relation between Haemoglobin Concentration and Optical Density readings at 404 mμ. (Calibration curve).

Concentration of Haemoglobin (mg % w/v) (in distilled water, or 0.005 M CaCl_2 , before and after rennet treatment)	Optical Density at 404 mμ	
	1°C	21-22°C
10	0.43	0.45
20	0.80	0.80
30	1.20	1.20
40	1.55	1.50
50	1.87	1.90
60	2.35	2.35
70	3.00	3.00

Results from Table IV (Figure 4) indicate a linear relationship between optical density readings at 404 mμ and haemoglobin concentration up to a final concentration of 60 mg%. Also

there was no difference between the distilled water and calcium chloride-containing haemoglobin solutions, nor did rennet treatment affect the optical density readings. As indicated in the Table, temperature of centrifugation was also without effect.

(c) Calibration curve for haemoglobin solution in an "artificial milk" system

It had been noticed in preliminary work that ultracentrifugation of an "artificial milk" system at 326,000 g for 35 minutes or longer would not reduce the slight residual opacity of the supernatant. To measure release of "immobilised" water in the "artificial milk" system, therefore, it was necessary to draw up a calibration curve using different concentrations of haemoglobin in an "artificial milk" system, subjecting the mixture to 326,000 g for 35 minutes, and measuring haemoglobin in the supernatant at 404 mμ in the Beckman Spectrophotometer. This was in order that the effect of the residual opacity of the supernatant from the centrifuged "artificial milk" would be allowed for in the calibration curve.

To do this the following method was used:

To 40 mls. "artificial milk" (3% calcium caseinate containing 100 mg % calcium) was added a drop of 0.03 M NaCl (in the hydration experiments a drop of commercial rennet, diluted 1:1, was added and this contained NaCl equivalent to about 0.03 M). This was then dispensed into 3 ml. lots in polyethylene ultracentrifuge tubes, and 0.5 ml. lots of haemoglobin solutions (70 mg %, 140 mg %, 210 mg %, 280 mg %

and 350 mg %) were then added to the tubes to give final haemoglobin concentrations in the mixture of 10 mg %, 20 mg %, 30 mg %, 40 mg % and 50 mg % respectively. The sixth tube was the control. These solutions were centrifuged at 1°C. as well as 21-22°C.

It was found that at low temperatures, the supernatant soon after centrifugation was clear but by the time the samples had been placed in the Beckman for optical density measurements at 404 mμ the temperature had risen to room temperature (21-22°C.) and they had become extremely turbid. No optical density readings were possible. Also, depending on the temperature of measurement, the turbidity varied. It was presumed that β-casein was causing the trouble since it is soluble and in monomeric form at low temperatures but forms colloidal aggregates as the temperature is raised. Polyacrylamide gel electrophoresis (described in Part II of this thesis) confirmed this supposition. (Plate 1 A). It was therefore decided to carry out centrifugation at room temperature (21°C.) only.

Results are indicated in Table V.

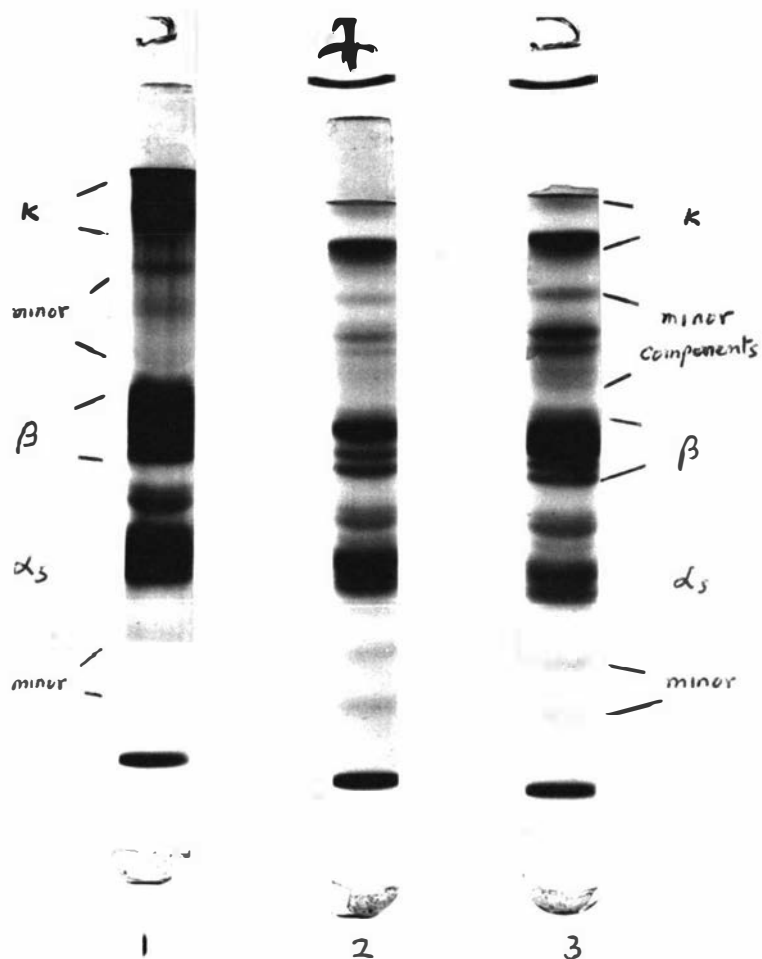


PLATE 1 A: P.A.G.E.* patterns of calcium-caseinate 'artificial milks' after ultracentrifugation at 326000 g at 1° and 21°C for 35 minutes.

1. 3% casein 'milk'.
2. Supernatant after centrifugation at 21°C.
3. Supernatant after centrifugation at 1°C.

Note. Sample 3 having a much higher concentration of β -casein.

* Polyacrylamide Gel Electrophoresis

Table V. Relation between Haemoglobin Concentration (in 3% Calcium Caseinate Systems) and Optical Density Readings at 404 mμ.

Haemoglobin Concentration in 3% Calcium Caseinate Solutions	Optical Density at 404 mμ after Ultra- centrifugation	
	distilled water as blank	calcium caseinate supernatant as blank
10	1.10	0.42
20	1.45	0.80
30	1.90	1.20
40	2.20	1.55
50	2.65	2.00

Results showed a linear relationship between haemoglobin concentration in the supernatant and optical density (Figure 4) indicating that there was no measurable interaction between the caseinate and the haemoglobin. A comparison of the results when the calcium caseinate solution was the blank with those using distilled water as blank indicates that the responses to concentration in both cases were linear (Figure 4). The two curves were also parallel but that using the distilled water blank was displaced by about 0.70 optical density units due to the residual opacity in the system. Using the calcium caseinate as the blank, the optical density values were found to be similar to those found for CaCl_2 and distilled water systems containing equivalent concentrations of haemoglobin. (Compare with Table IV).

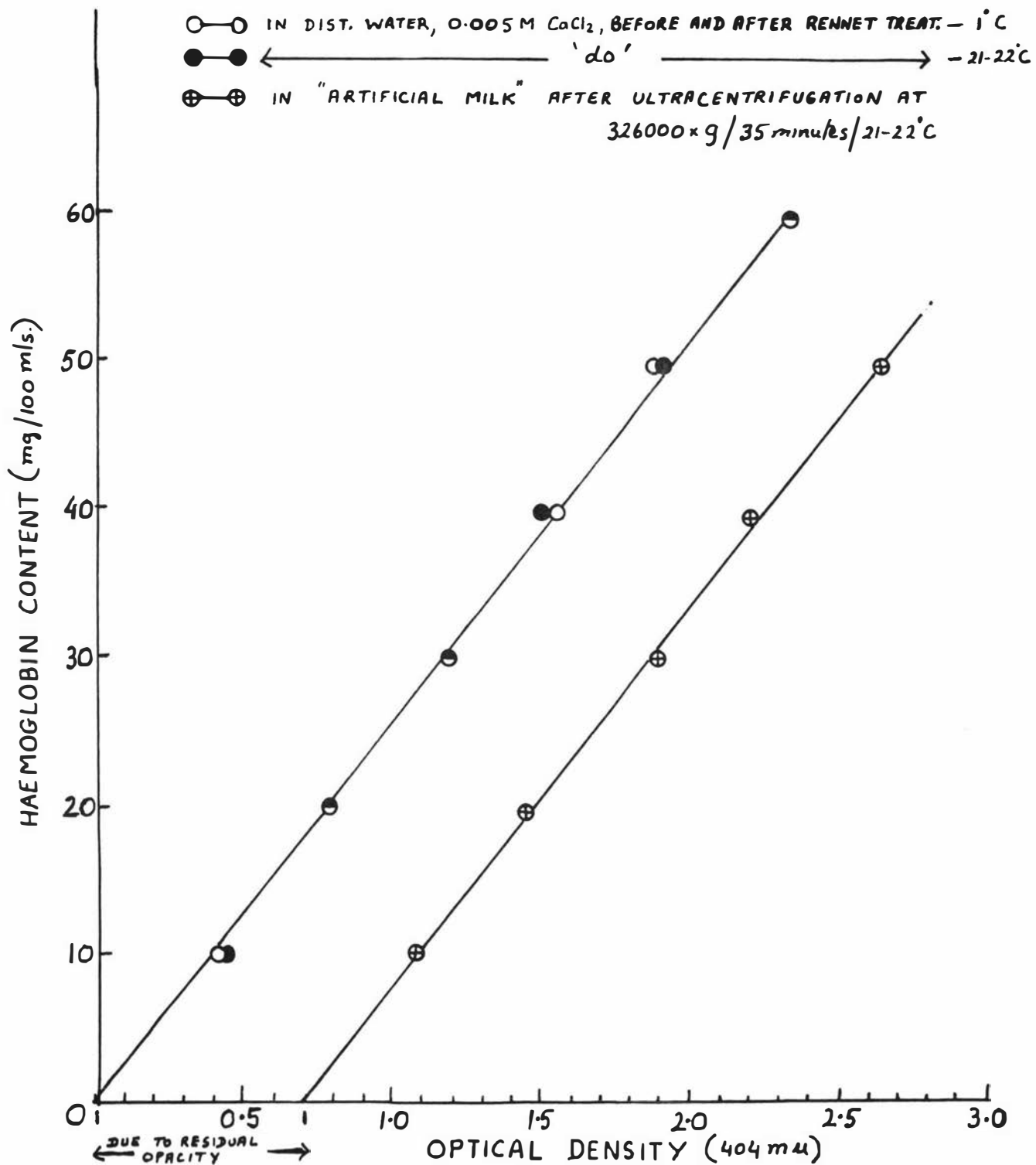


FIG. 4.- CALIBRATION CURVE FOR HAEMOGLOBIN

(d) Measurement of the Release of "Immoblised" Water from Calcium Caseinate by Rennet Action

A drop of 1:1 diluted commercial rennet was added to 40 mls. "artificial milk", containing 100 mg% calcium, at pH 6.8 and 20°C. 3 mls. of this mixture was quickly dispensed into polyethylene ultracentrifuge tubes, each containing 0.5 ml. of 210 mg% w/v haemoglobin solution in distilled water. The final haemoglobin concentration in the mixture was 30 mg%. The control tube contained 0.5 ml. distilled water mixed with 3 mls. of the rennet-treated "milk". Two of the centrifuge tubes contained the same "artificial milk" treated similarly except that instead of rennet a drop of 0.03 M NaCl was added. The contents of one tube were mixed with 0.5 ml. 210 mg% haemoglobin solution whilst to the other was added 0.5 ml. distilled water. The coagulation time for rennet-treated samples, as found independently, was 20 minutes. This would have elapsed by the time the ultracentrifuge was started. The samples were subjected to 50,000 R.P.M. (326,000 g) for 35 minutes.

The supernatant at the end of the ultracentrifugation was found to be clear - this was in contrast to supernatants from untreated "artificial milk" samples described earlier. The optical density of these supernatants was measured in the Beckman Spectrophotometer and results are given in Table VI.

**Table VI. Determination of the Release of "Immobilised" Water by
Rennet Action on Casein Micelles.**

No. Treatment	O.D. of "milk" supernatants relative to appropriate blanks at 404 mμ	Hb. content (mg % w/v)	Release of water (as % of wet casein weight)
1. Control	1.20	30	-
2. Rennetted sample	1.05	27	80

Note: Hb. = haemoglobin

Results indicated in Table VI show a significant drop in the optical density values for a 30 mg % haemoglobin solution in a para-caseinate system when compared to the standard curve (Figure 4). The optical density reading of 1.05 is equivalent to a haemoglobin concentration of 27 mg %, whereas it originally contained 30 mg %. This dilution of the haemoglobin is equivalent to a release of 0.36 mls. "immobilised" water from 0.09 g dry casein present in the ultracentrifuge tubes; that is, the water released was equal to 80% of the weight of the wet casein.

With distilled water as blank the O.D. of the rennetted sample was 0.05 O.D. units higher than when the para-caseinate solution was used as the blank. This does not affect the observation that rennetting resulted in a significant drop. Calculations in this case show that "immobilised" water equal to 74% of the weight of the wet casein would have been released.

(e) Effect of varying Casein and Haemoglobin concentrations on the Optical Density Measurements.

Results reported in the preceding paragraphs had indicated an apparent release of "immobilised" water from the calcium caseinate by rennet action, to the extent of 74-80% of the weight of the wet casein complex. The lowering of optical density in the rennet-treated samples could, however, have been due to occlusion or adsorption of haemoglobin by the calcium-para-caseinate during gel formation and/or ultra-centrifugation.

To study this possibility the following experiments were carried out:

- (i) To 40 mls. of "artificial milk" at pH 6.8 containing 2%, 3%, 3.5% casein respectively and 100 mg% w/v calcium each, was added a drop of 0.03 M NaCl. 3 ml. portions were then dispensed into polyethylene ultracentrifuge tubes containing 0.5 mls. of 210 mg% w/v haemoglobin solution. The mixtures contained final haemoglobin concentrations of 30 mg%. The control had no haemoglobin mixed with it. After ultracentrifugation at 326,000 g for 35 minutes the optical density of the supernatants were measured (Table VII). Here the casein concentration differed but haemoglobin concentration remained constant.
- (ii) In a second experiment, the casein concentration in the "milk" was kept constant at 3% (100 mg% w/v calcium, pH 6.8) but the haemoglobin concentration was varied thus:

To 40 mls. of "artificial milk" at ambient temperature was added a drop of commercial rennet diluted 1:1. 3.0 mls. of the renneted "milk" was dispensed into ultracentrifuge tubes containing 0.5 mls. each of 70 mg%, 210 mg%, and 350 mg% w/v haemoglobin solutions respectively, giving final haemoglobin concentrations in the mixture of 10 mg%, 30 mg%, and 50 mg% (w/v) respectively. The control sample had no haemoglobin mixed with it. After ultracentrifugation at 326,000 g for 35 minutes, the optical density of the supernatants was measured.

Results are given in Table VII.

Table VII. Effect of Varying Casein and Haemoglobin Concentration on the Optical Density Measurements.

No.	Supernatant from:	Haemoglobin concentration in the "artificial milks" (mg % w/v)	Optical density at 404 mμ of the supernatants	Hb concentration from standard curve (mg % w/v)	"Immobilised" water released (% of wet casein complex)
1	2% caseinate	30	1.15	30	-
2	3% caseinate	30	1.20	30	-
3	3.5% caseinate	30	1.20	30	-
4	3.0% para-caseinate	10	0.40	9.5	67
5	3.0% para-caseinate	30	1.10	28.0	74
6	3.0% para-caseinate	50	1.85	47.0	70

Note: Hb = haemoglobin

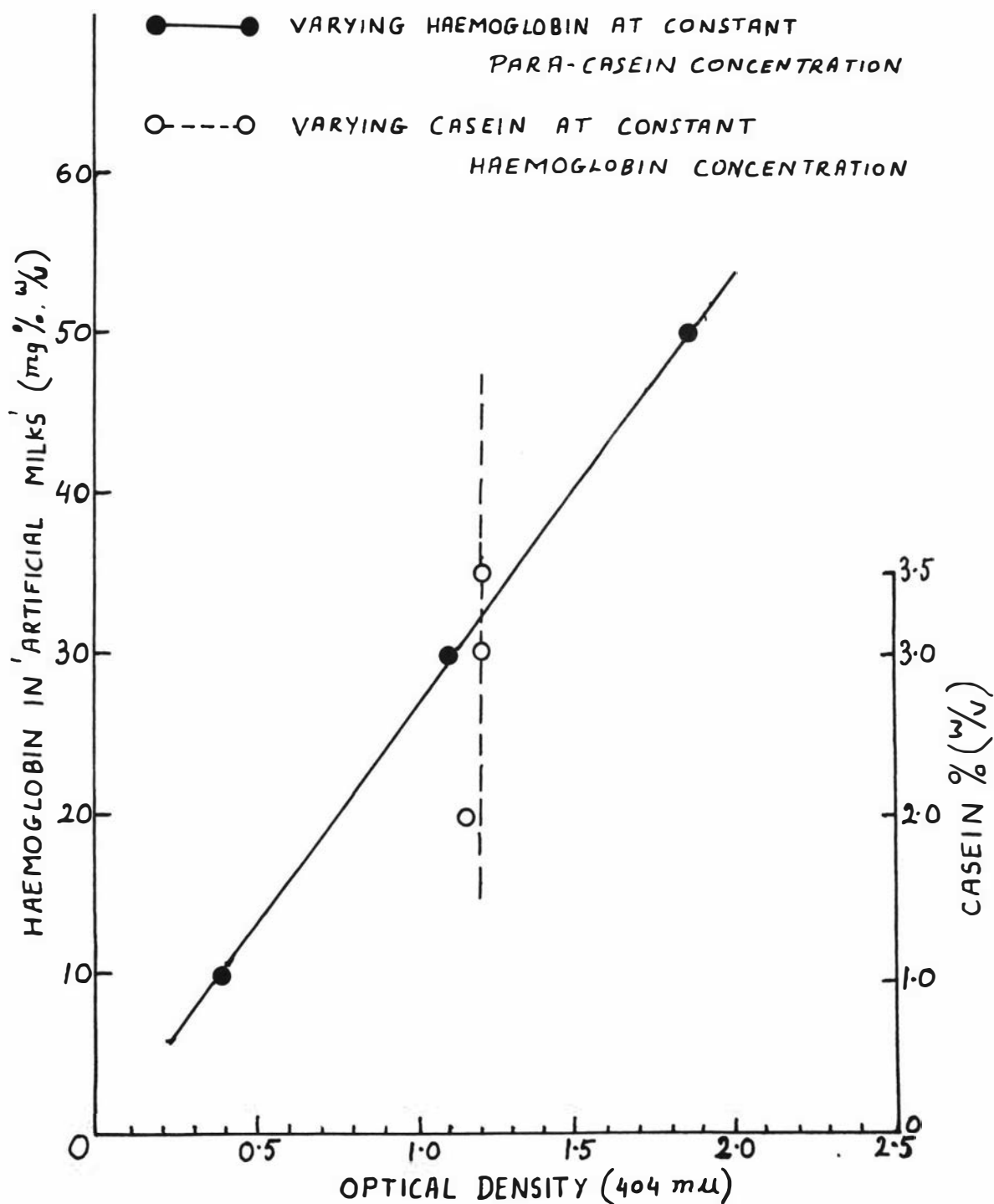


FIG. 5.- EFFECT OF VARYING CASEIN AND
HAEMOGLOBIN CONCENTRATION ON OPTICAL
DENSITY MEASUREMENTS

Results given in Table VII show that little or no adsorption or occlusion of the haemoglobin occurred in the systems studied. The data plotted in Figure 5 indicates a linear relationship, maintained between the haemoglobin concentration and optical density. This would not be expected if occlusion or adsorption had been taking place. Furthermore, the para-caseinate systems indicated that rennet action had reduced the supernatant haemoglobin concentration from 10 to 9.5, 30 to 28, and 50 to 47 mg% w/v respectively, corresponding to a release of "immobilised" water equivalent to 67, 74, and 70% respectively. These figures agree favourably with the 74% and 80% reported in the preceding section.

SECTION D. A STUDY OF THE PHYSICAL ORGANISATION OF CASEIN MICELLES
AND THE PROCESS OF GELATION BY ELECTRON MICROSCOPY,
USING "ARTIFICIAL MILK" SYSTEMS AND SKIM MILK.

1. General

Electron microscopy of the "artificial milk" systems was undertaken to determine the gross structure of the casein micelles in "artificial milks" and to compare it with those in skim milk. As far as the writer is aware, electron microscopic studies of "artificial milk" systems have not hitherto been published.

2. Experimental

(a) Preparation of "artificial milks"

This has been described earlier in Chapter II.

(b) Technique for Electron Microscopy

(i) Preparation of support film for samples

A collodion support film was prepared by dipping a clean glass slide into a 0.8% solution of collodion in amyl acetate and allowing it to dry at room temperature. The film was removed over a water surface. Copper grids with minute honeycomb openings were placed on this film and the whole lifted off the water surface with the help of a filter paper. Drying was carried out in the oven at 100°C. The collodion film surface (one not having the grids) was then heavily carboned in a vacuum chamber to a light grey colour compared to the filter paper. The grids were then carefully removed from the filter paper and placed in front of an atomiser. The atomiser was

connected to a nitrogen cylinder, for the 'application' of the sample, and negative stain.

(11) Fixing, application and "negative" staining of skim milk and "artificial milk" samples.

1 ml. of the "milk" sample was taken in a test tube containing 5 mls. of 3% gluteraldehyde in 0.1 M phosphate buffer at pH 7.2. The tubes were held at 40°C for 3 hours for fixation of the micelles. Preliminary work had indicated that "fixing" in gluteraldehyde was as good, and sometimes better, than osmium tetroxide for the preservation of the micelle structure. While this work was being completed, a paper by Carroll et al⁽⁸⁵⁾ came to the author's notice, in which it was stated that gluteraldehyde was superior in preserving casein micelle structure. Results from the present work support this view.

After fixation, the protein was spun down in a B.T.L. bench centrifuge at 5000 R.P.M. for 3 minutes. The sediment was resuspended in 5 mls. of distilled water. Using a syringe, 10 drops of the sample was mixed with 10 drops of neutralised 2% phosphotungstic acid, and 1 drop of 0.5% bovine serum albumin in distilled water. The serum albumin contributed towards better spreading of droplets when sprayed on to the collodion film. Two drops of this mixture were then applied to the atomiser for spraying on to the grid covered by the collodion and carbon films.

An alternative method was to spray 2 drops of the "fixed" protein suspension in distilled water, on to the grid, followed by the spraying of 2 drops of a mixture of

neutralised phosphotungstate and bovine serum albumin.

The grids were then examined in the Electron Microscope.*

This method was more commonly used.

(iii) Fixing, application and negative staining of rennet-treated skim milk and "artificial milk" systems.

To 40 mls. of the sample was added 1 drop of diluted commercial rennet, diluted 1:10 for calcium caseinate samples and 1:1 for calcium caseinate-calcium phosphate and skim milk samples for roughly equivalent gelling times.

Differences in gelling time appear to be primarily associated with the differences in the level of "available" calcium.

A preliminary experiment had indicated the gel point for each of the samples. 1 ml. portion of the mixture was withdrawn at various intervals up to the gel point and pipetted into test tubes containing 5 mls. of 3% gluteraldehyde in 0.1 M phosphate buffer at pH 7.2. The treatment subsequent to this was similar to that reported in (ii) above.

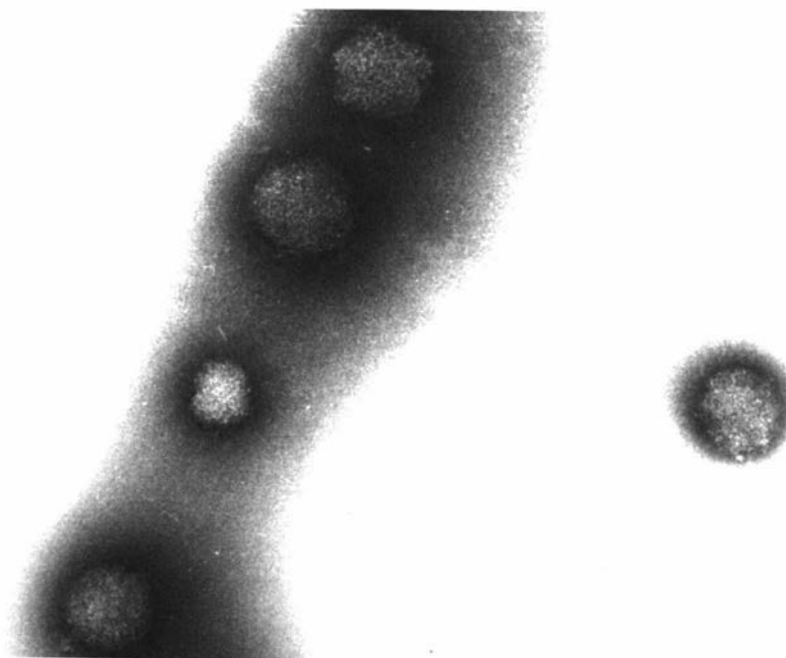
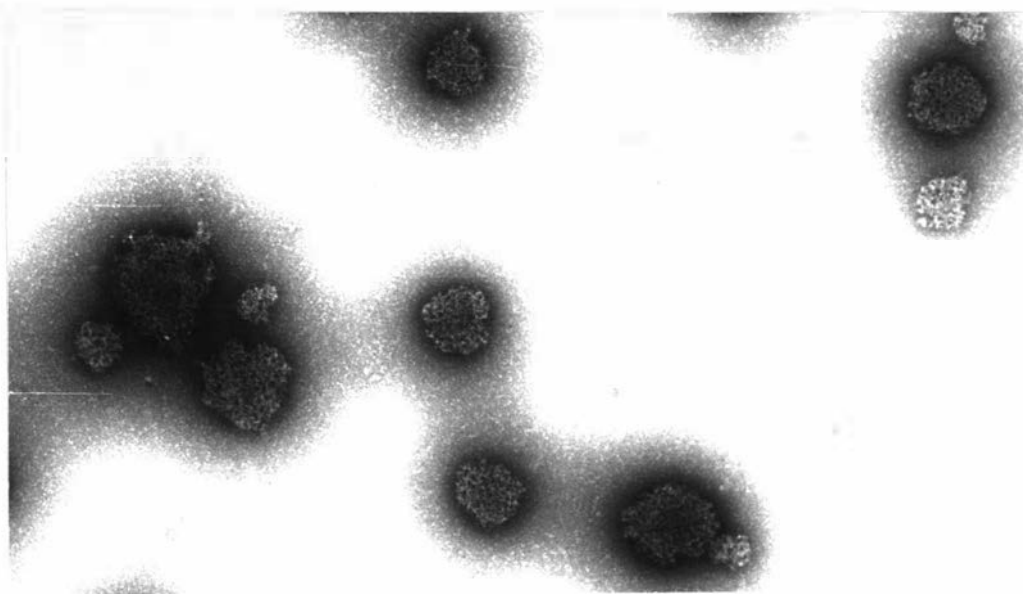
A skim-milk and an "artificial milk" sample containing 62.5%^{mg} calcium were also treated in the cold, below 5°C, with 2^A drops of neat rennet for a long enough time to ensure that the primary phase of rennet action would have been over. This was checked by quickly warming a portion of the milk to 30°C in a test tube to check for coagulation. If coagulation occurred immediately it was assumed that the primary stage of rennet action was over. 1 ml. portions from these samples were then fixed and stained in the usual way for examination in the electron microscope.

* PHILLIPS EM 200, Made in HOLLAND

3. Results

(a) Comparison of "artificial milk" systems with skim milk.

A comparison of the electron micrographs, Plates 1 and 2, indicates clearly that at the 62.5 mg% calcium addition, the "artificial milks" show casein micelles essentially similar in all respects to those in skim milk in their physical organisation and appearance. The size ranges also appear to be similar to micelles in skim milk, ranging from less than 25 μ u to over 150 μ u in diameter, with nearly half of them in the 25 μ u to 100 μ u range. The average size of casein micelles in the "artificial milks" appear to be slightly larger than those in skim milk; about 120 μ u as against about 100 μ u. An increase in the calcium content of "artificial milks" to 125 mg% gave rise to much larger micelles of average diameter about 150 μ u as a comparison between Plates 3 and 3a indicates. (Note: the magnification in Plate 3 is 154,000 against 85,000 for Plate 3a). As the "artificial milks" were devoid of phosphate ion, it seems clear that ionic calcium alone is sufficient for the formation of casein micelles. This is also supported by the fact that raising the calcium in the "artificial milks" increases the size of the micelles. When phosphate is added to the system either as disodium hydrogen phosphate or as orthophosphoric acid, the micelles are greatly reduced in size, as indicated in Plates 3b and 3c. The vast majority of the micelles are below 50 μ u in diameter and their appearance is quite unlike the micelles in skim milk, (Plate 1). after treating with phosphate.

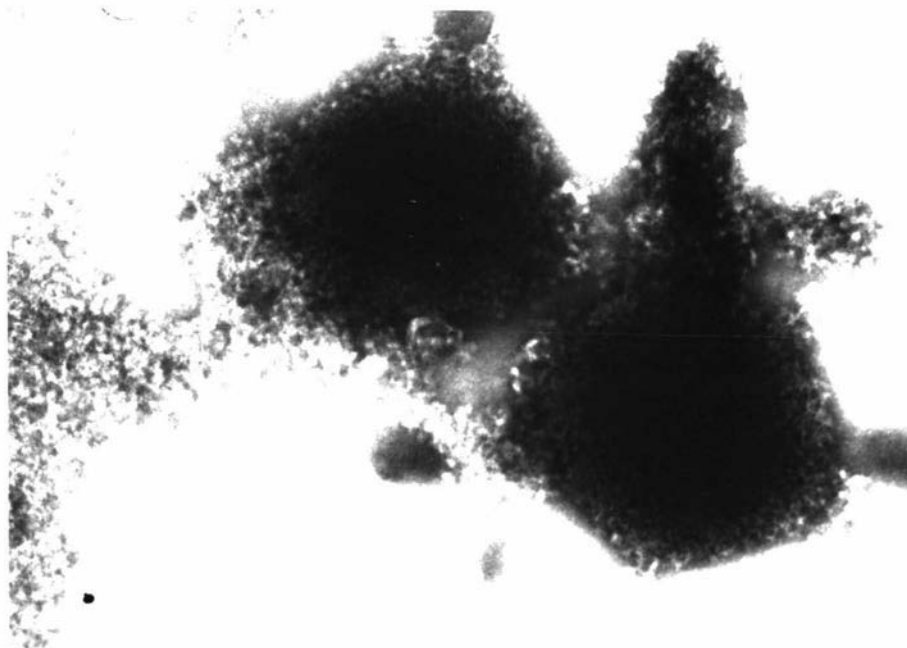
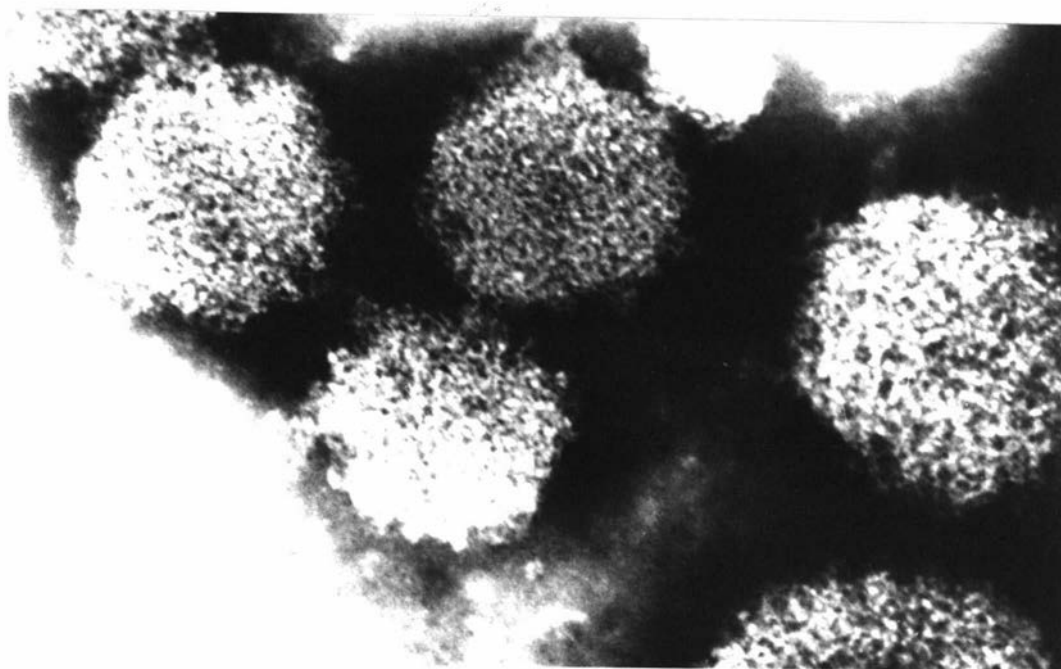


Electron Micrograph of Casein Micelles

PLATE 1: Skim milk ---- x 47600.

PLATE 2: 'Artificial milk' (62.5 mg % calcium) ----- x 47600.

Note: Size distribution and physical organisation is similar in the two systems.

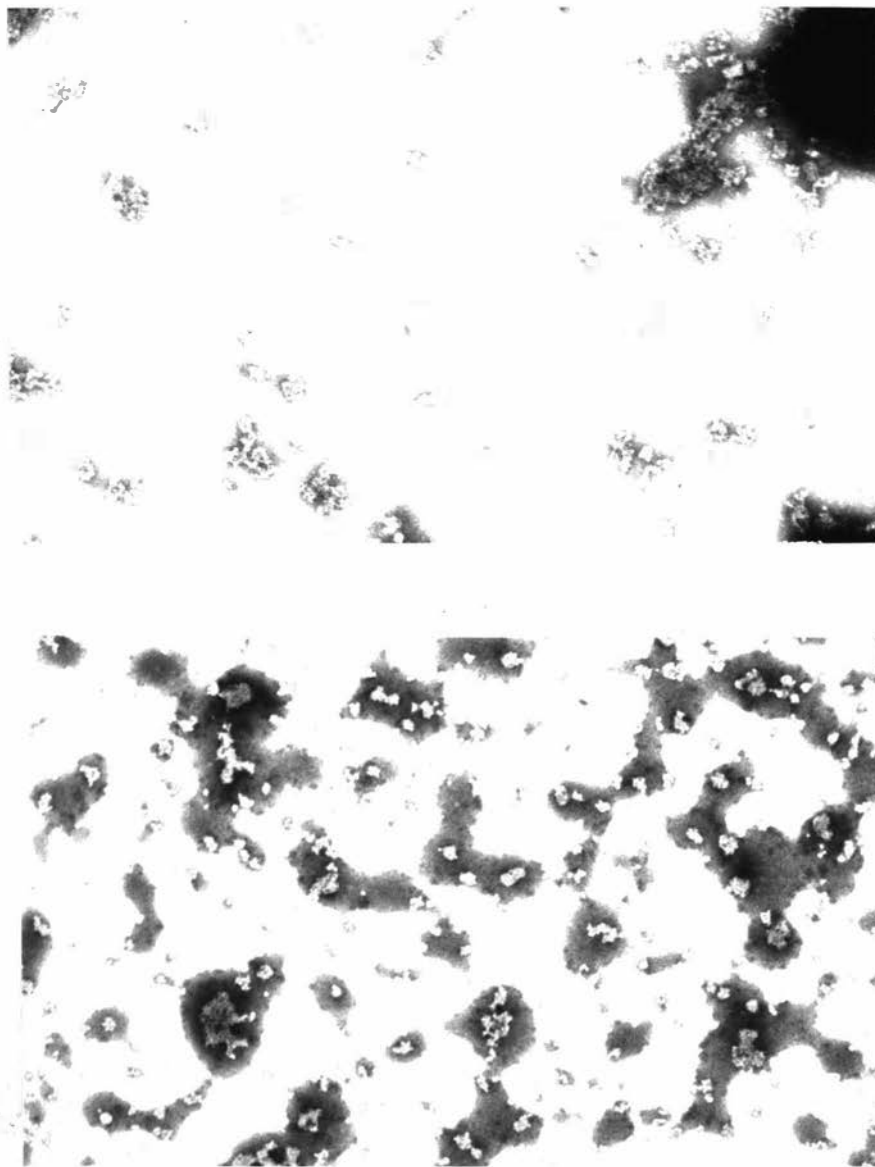


Electron Micrographs of Casein Micelles in 'artificial milk' systems

PLATE 3: 'Artificial milk' (62.5 mg % calcium) ---- x 154000.

PLATE 3a: 'Artificial milk' (125 mg % calcium) --- x 85000.

Note: Micelles much bigger at higher calcium level.



Electron Micrographs of Casein Micelles in 'artificial milk'
containing both Calcium and Phosphate

PLATE 3b: 'Artificial milk' (125 mg % calcium, 55 mg % 'P' as Na_2HPO_4)
---- x 47600.

PLATE 3c: 'Artificial milk' (125 mg % calcium, 55 mg % 'P' as H_3PO_4)
—— x 35000.

Note: Micelle size greatly reduced in the presence of phosphate.

It seems clear that the addition of phosphate causes dispersion of the micellar components with the formation of units greatly reduced in size. The reduction in size is presumably due to the sequestering action of the added phosphate ions on calcium thus reducing the effective calcium ion concentration in the "artificial milk" systems. This emphasises the view expressed earlier that calcium ions play an important positive role in the formation of the micelle. Furthermore, it would appear that calcium phosphate has a limited cementing role to play in the maintenance of the integrity of the micelle.

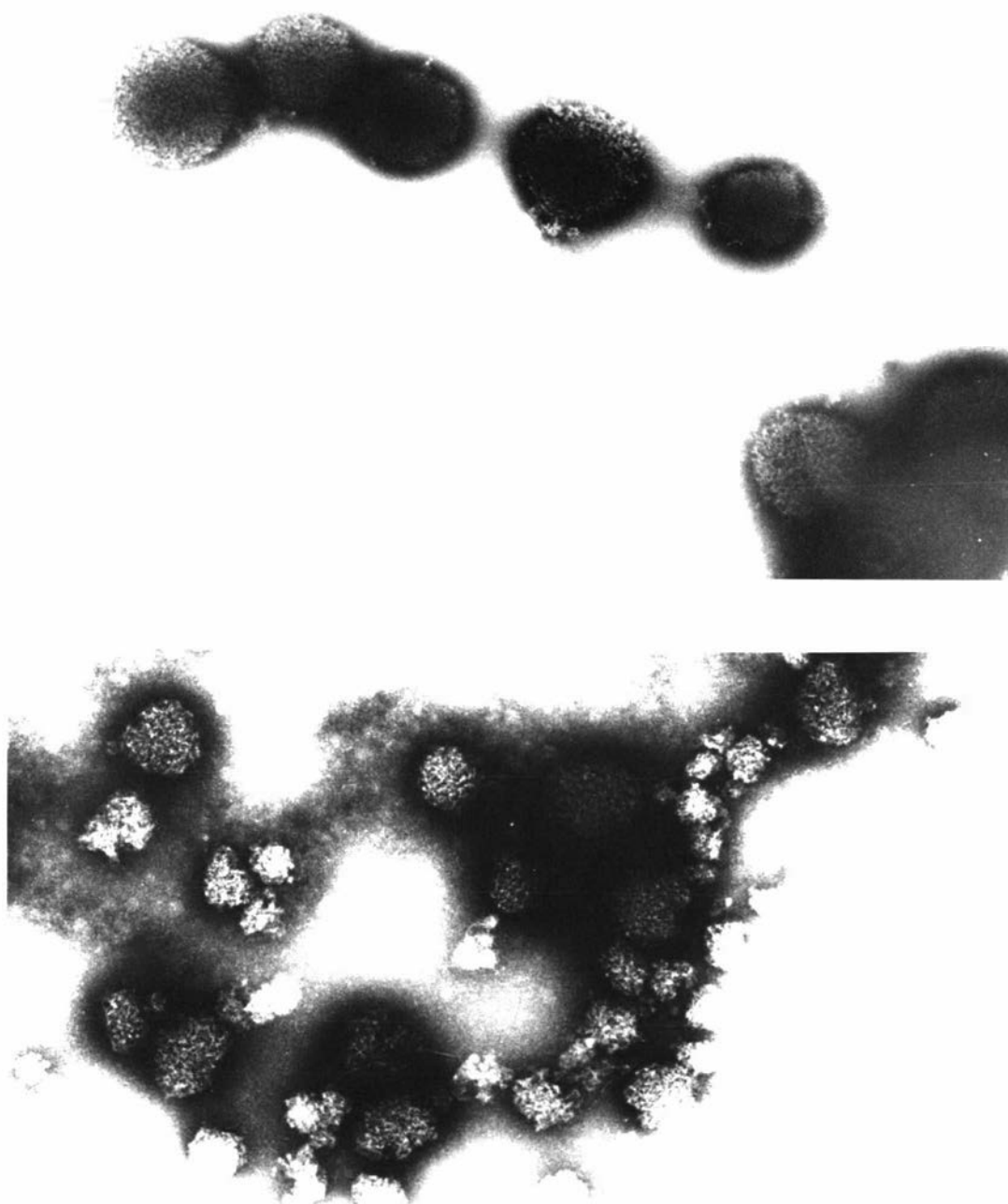
The physical organisation and appearance of casein micelles in skim milk and calcium-containing "artificial milks", as shown by Plates 1 and 2, is similar to the electron micrographs of casein micelles from skim milk produced by Shimmin and Hill⁽⁸⁷⁾ despite the different methods used in the preparation of the sample. Shimmin and Hill⁽⁸⁷⁾ used osmium tetroxide for fixation and uranyl acetate for negative staining as against gluteraldehyde and neutralised phosphotungstic acid used in this study. The modes of fixation by osmium tetroxide and gluteraldehyde are vastly different⁽⁸⁸⁾. The similarity is therefore significant and reduces the possibility that artifacts have been produced by the preparative methods used.

Rose and Colvin⁽⁹⁰⁾ had postulated that the electron-dense particles observed in electron micrographs were probably granules of colloidal calcium phosphate and not the protein aggregates suggested by Shimmin and Hill⁽⁸⁷⁾. However, electron micrographs of "artificial milks" containing calcium but not phosphate, as shown in Plates 2 and 3a, do not support this contention. The

same authors in a separate publication⁽⁸⁹⁾ indicated that they were unable to find a significant relation between ionic calcium and average micelle diameter in the small number of milk samples from individual cows that were studied. They suggested that other soluble forms of calcium such as the complex ions (Ca citrate) and $(Ca_2 \text{ citrate})^+$ as well as mono and dicalcic salts of phosphoric acid must be at least as effective as calcium ion in influencing micelle size. However, these ions would be absent in the calcium containing "artificial milks" used in this study. It would thus appear that calcium ion alone plays a more important role in micelle formation than has been attributed to it by Rose and Colvin⁽⁸⁹⁾. The similarity of micelles in the "artificial milks" containing calcium and skim milk, indicated by Plates 1 and 2, show that results obtained using the former could be related to normal milks.

(b) Electron Microscopy of rennet-treated samples.

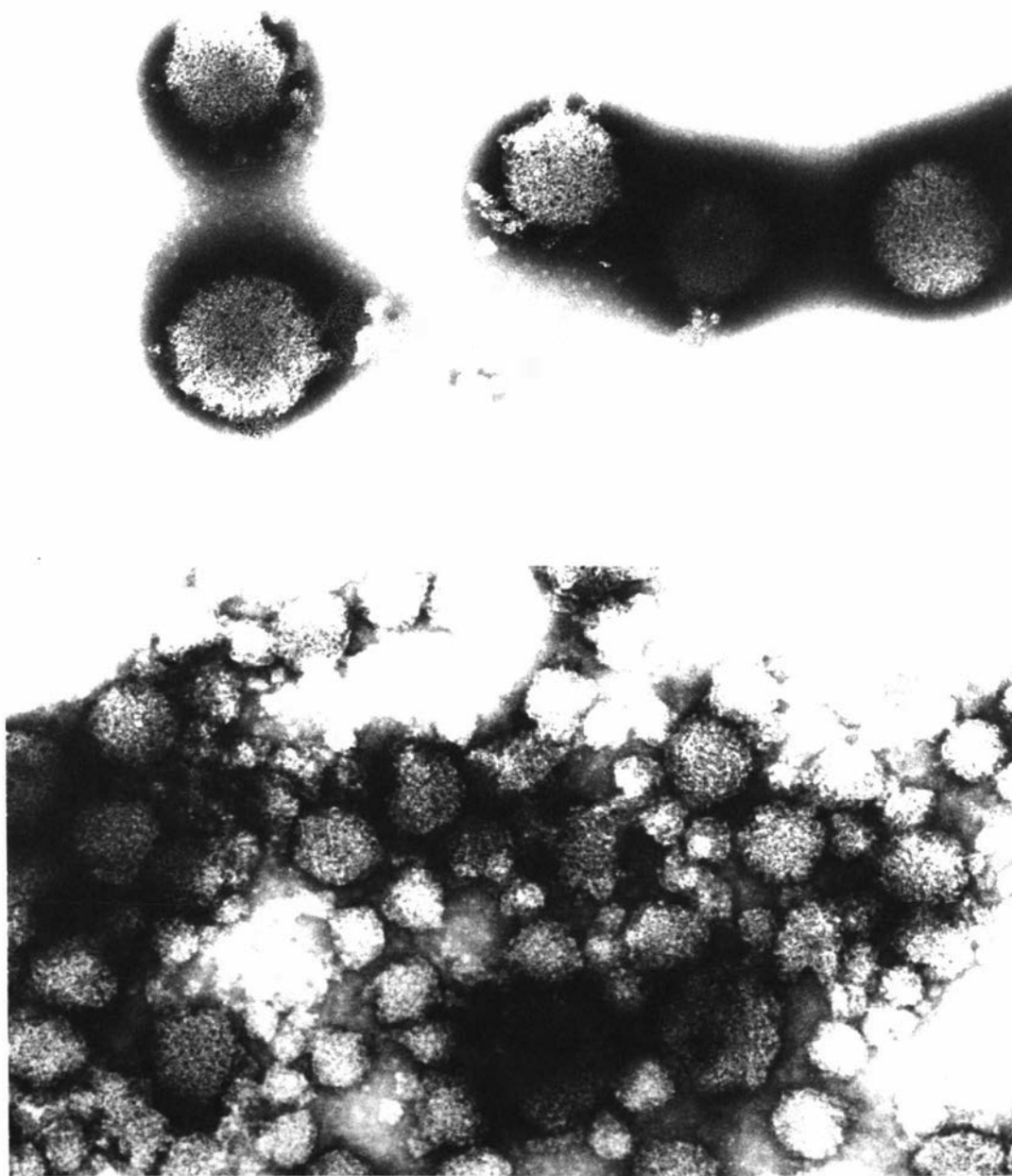
Electron micrographs of rennet-treated "artificial milks" containing 62.5 mg% calcium (Plates 4, 6, 8 and 10) and those of rennet-treated skim milk (Plates 5, 7, 9 and 11) show the various stages in the primary phase of rennet action on casein micelles up to gel point. The gelling process clearly appears to proceed by formation firstly of "beaded" micellar threads wherein only the micelle surfaces appear to be involved. The micellar strands then further interact with each other to form a 3-dimensional network, enclosing in the process the whole volume of liquid. The process of "beaded" thread formation is clearly demonstrated in Plates 8 and 9. Both the "artificial milks" and the skim milk samples appear to behave in a similar manner. It is also evident from the micrographs that right through the process, up to the gel point,



Electron micrographs of casein micelles after rennet treatment

PLATE 4: 'Artificial milk' (62.5 mg % calcium, 2½ minutes) --- x 47600.

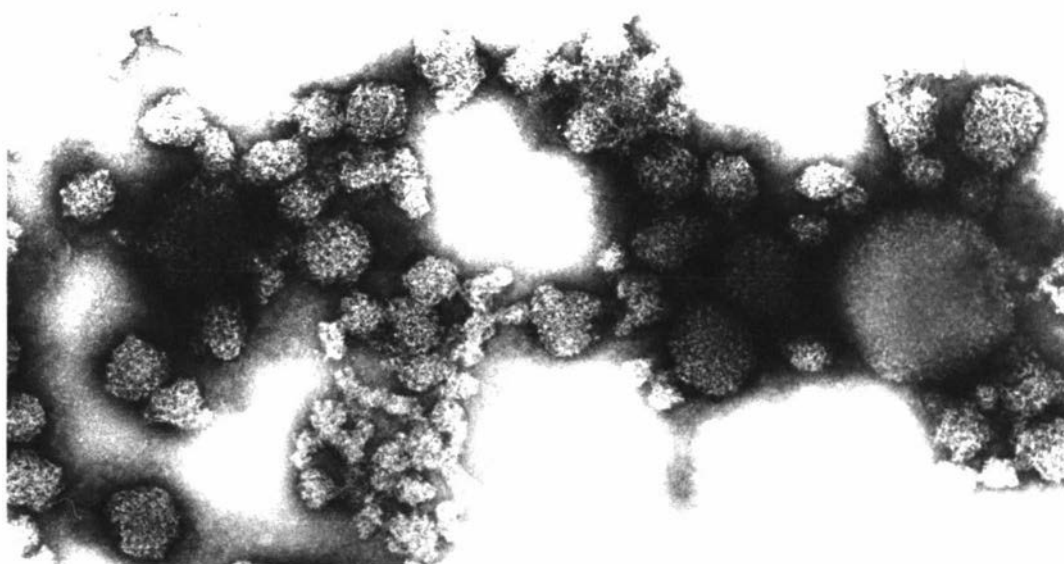
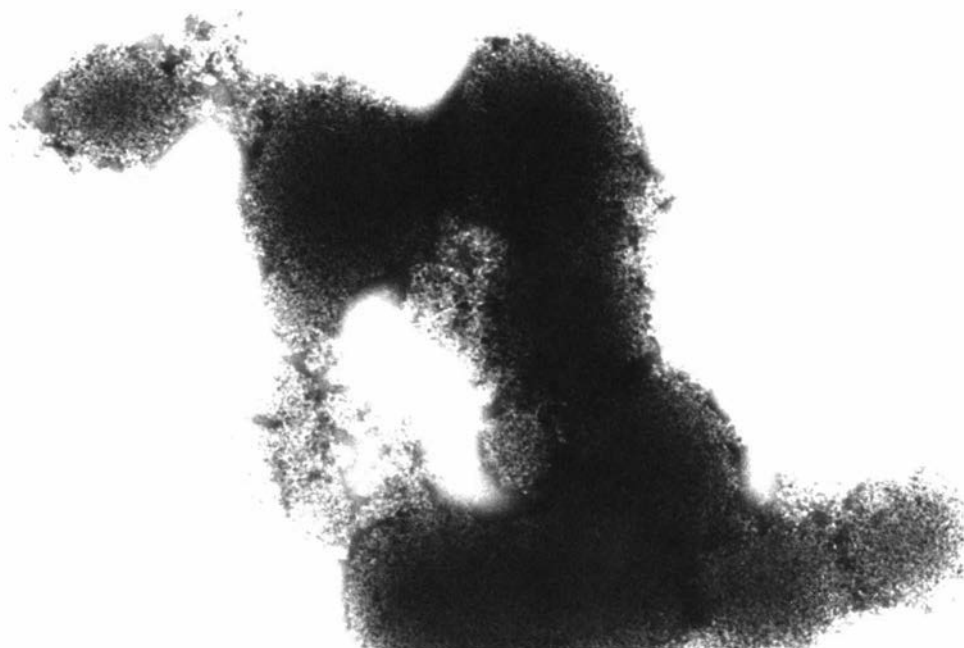
PLATE 5: Skim milk (4 minutes) — x 85000.



Electron micrographs of casein micelles after rennet treatment

PLATE 6: 'Artificial milk' (62.5 mg % calcium, 5 minutes) ----- x 47600.

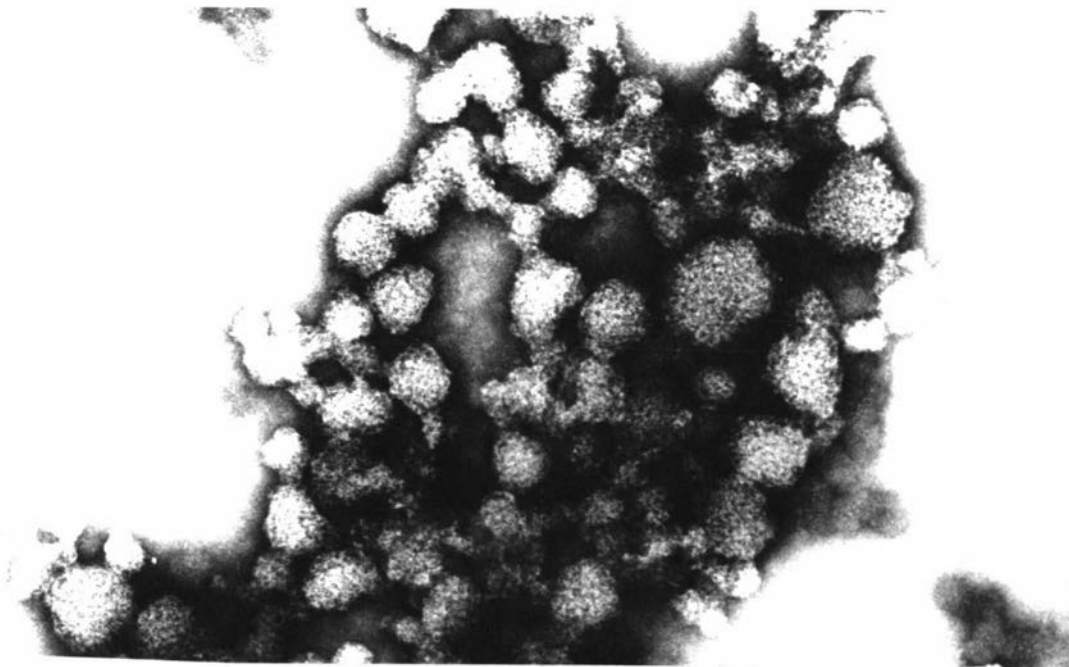
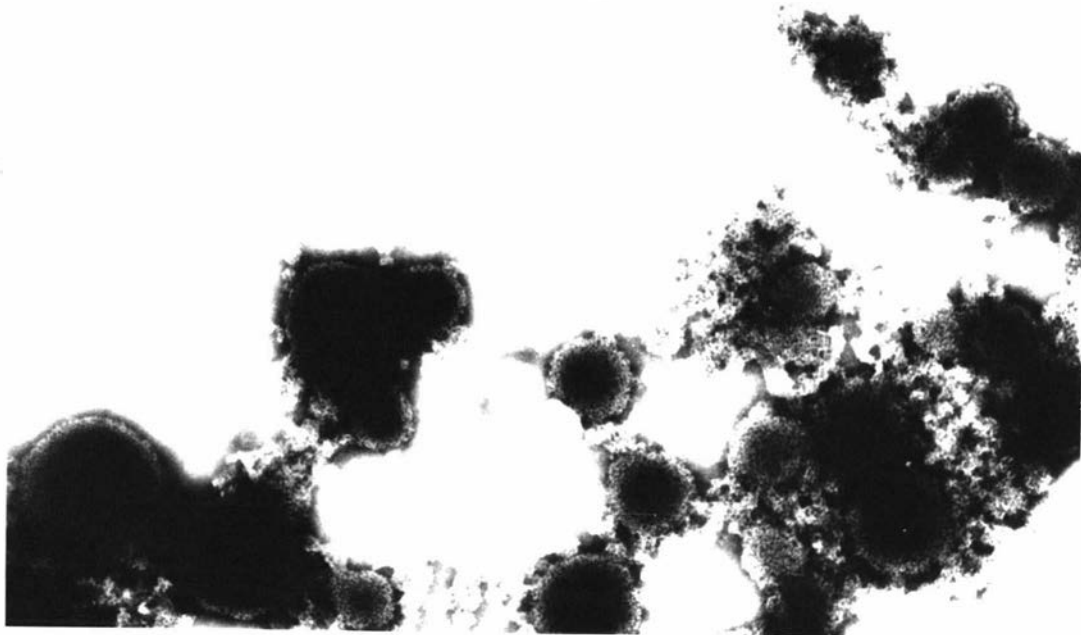
PLATE 7: Skim milk (8 minutes) ---- x 85000.



Electron micrographs of casein micelles after rennet treatment

PLATE 8: 'Artificial milk' (62.5 mg % calcium, 7½ minutes) — x 47600.

PLATE 9: Skim milk (12 minutes) — 85000.



Electron micrographs of casein micelles after rennet treatment

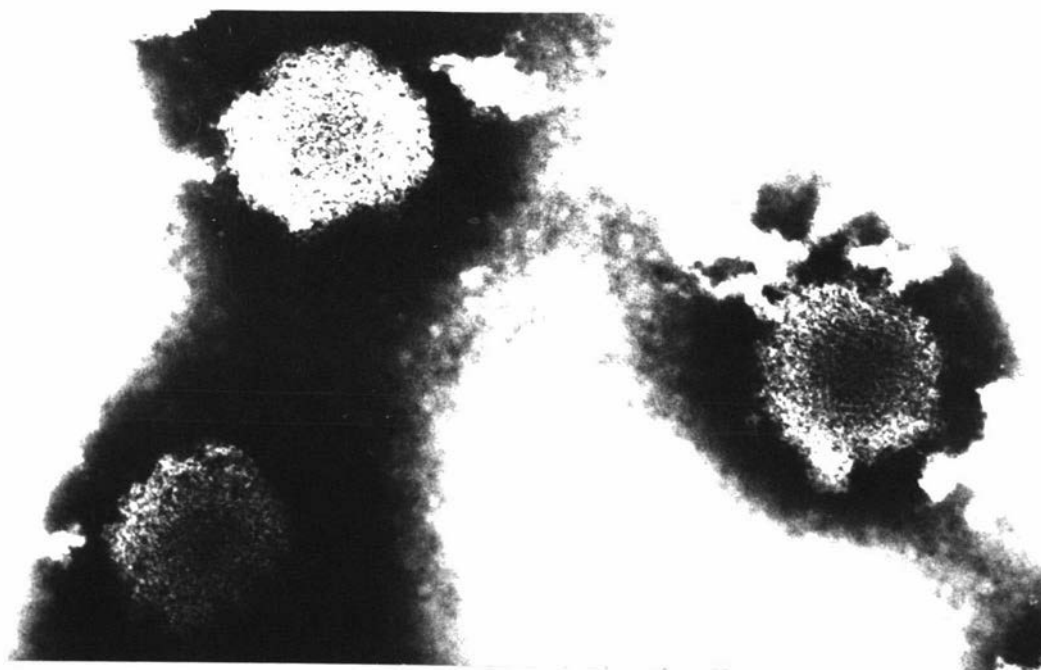
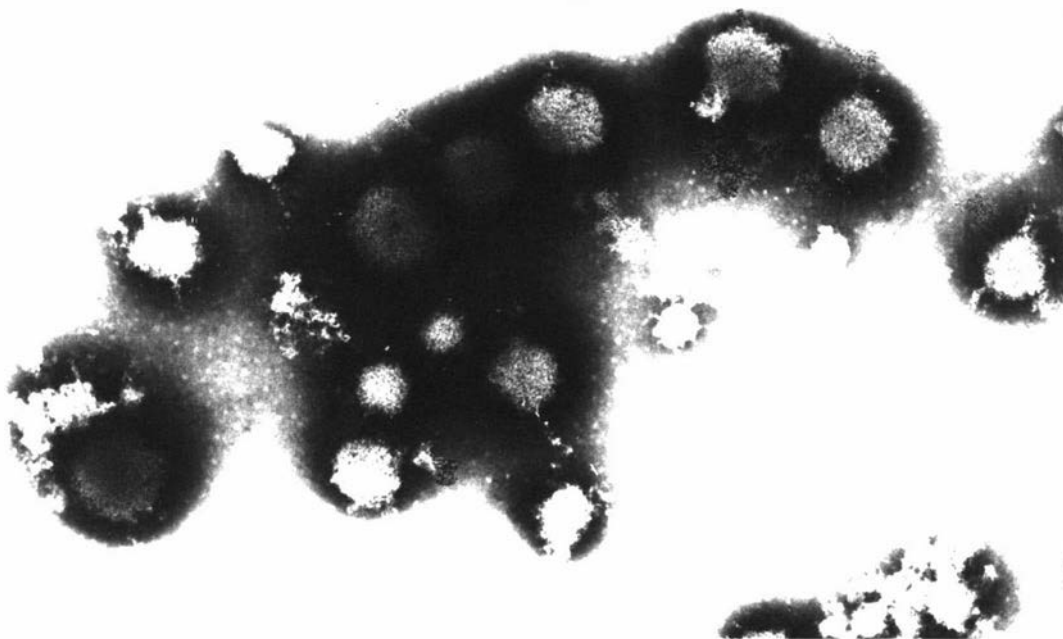
PLATE 10: 'Artificial milk' (62.5 % calcium, 10 minutes) ----- x 35000.

PLATE 11: Skim milk (16 minutes) —— x 85000.

the spherical casein micelles in both systems maintain their gross physical organisation. There does not seem to be any obvious dispersion of the inner components of the micelle, gelation appearing to be a surface phenomenon only. There is also no evidence of any great distortion of the micelles due to extensive denaturation. The denaturing action of rennet on the caseinate thus appears to be insignificant.

"Artificial milk" and skim milk samples which had been treated in the cold with rennet until the primary phase of the reaction was over, also showed complete micelles as indicated in Plates 12, 13 and 14. The size of the micelles was also apparently not changed. This indicated that at gel point no essential change occurred within the micelle and that gelation was a surface phenomenon. It had been shown earlier that rennet action significantly reduced viscosity of both sodium and calcium caseinate systems. The electron micrographs of rennet-treated samples do not show any major change in the size of the casein micelles. The fall in viscosity through rennet action on the caseinate must therefore have been largely due to release of "immobilised" water. The viscosity of spherocolloids is considered to be independent of particle size⁽⁷⁶⁾. This also lends support to the above contention.

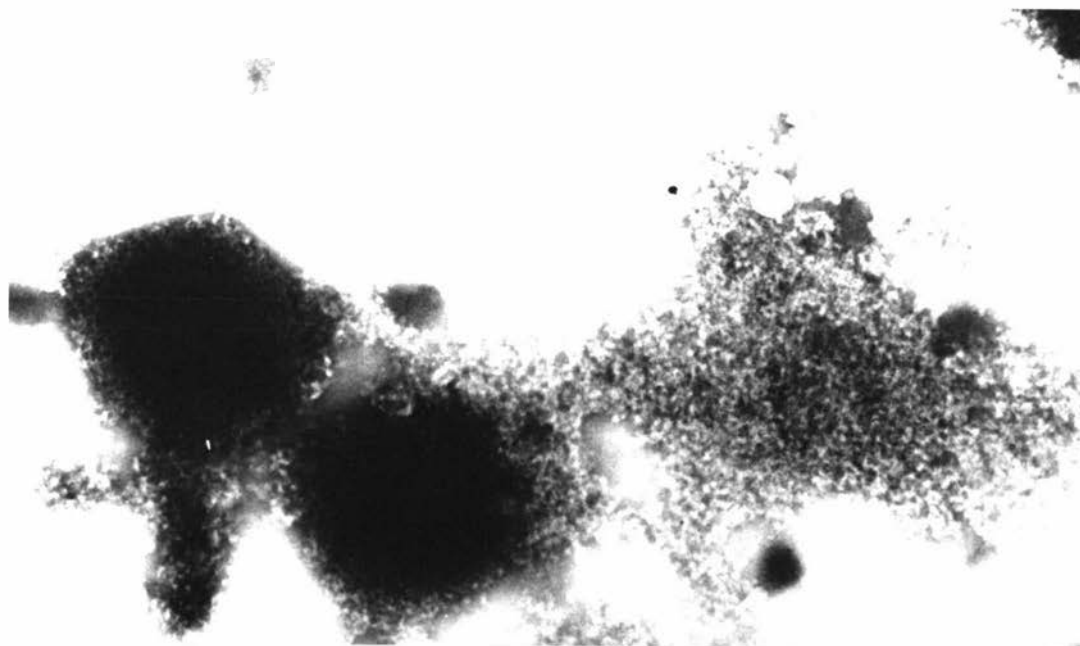
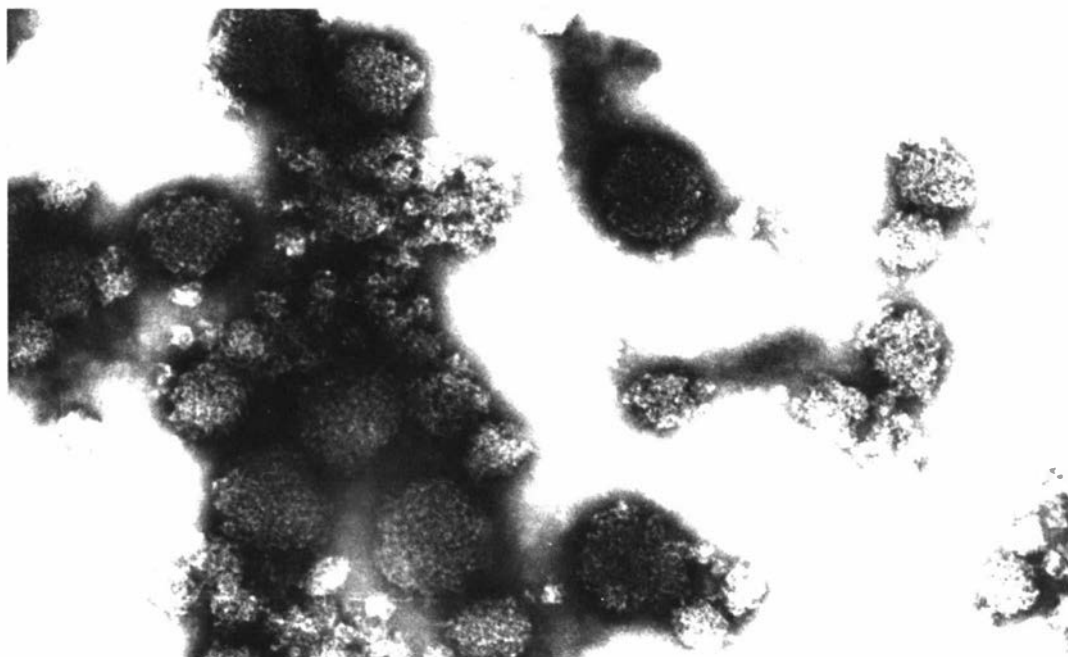
The current ideas on the physical organisation of the micelles⁽⁹¹⁻⁹³⁾ require that a considerable portion of kappa-casein, if not the whole molecule, with its reactive glycomacropeptide and sulphur amino acids, be on the micelle surface. Since, as indicated by the electron micrographs, gel formation appears to be essentially a surface phenomenon, it is possible that para-kappa-casein molecules on different micelles may be involved in



Electron micrographs of casein micelles after rennet treatment
at 4°C for 1 hour - 45 minutes

PLATE 12: 'Artificial milk' (62.5 mg % calcium) ---- x 35000.

PLATE 13: 'Artificial milk' (62.5 mg % calcium) — x 85000.



Electron micrographs of casein micelles

PLATE 14: Skim milk, after rennet treatment at 4°C for 2 hours -
30 minutes ---- x 85000.

PLATE 15: 'Artificial milk' (125 mg % calcium) ---- x 85000.

the cross bonding. Under these conditions, it appears less likely that the inner micellar components, α_s and β casein, play a part in the initial gel formation, although molecules very near the micelle surface may interact.

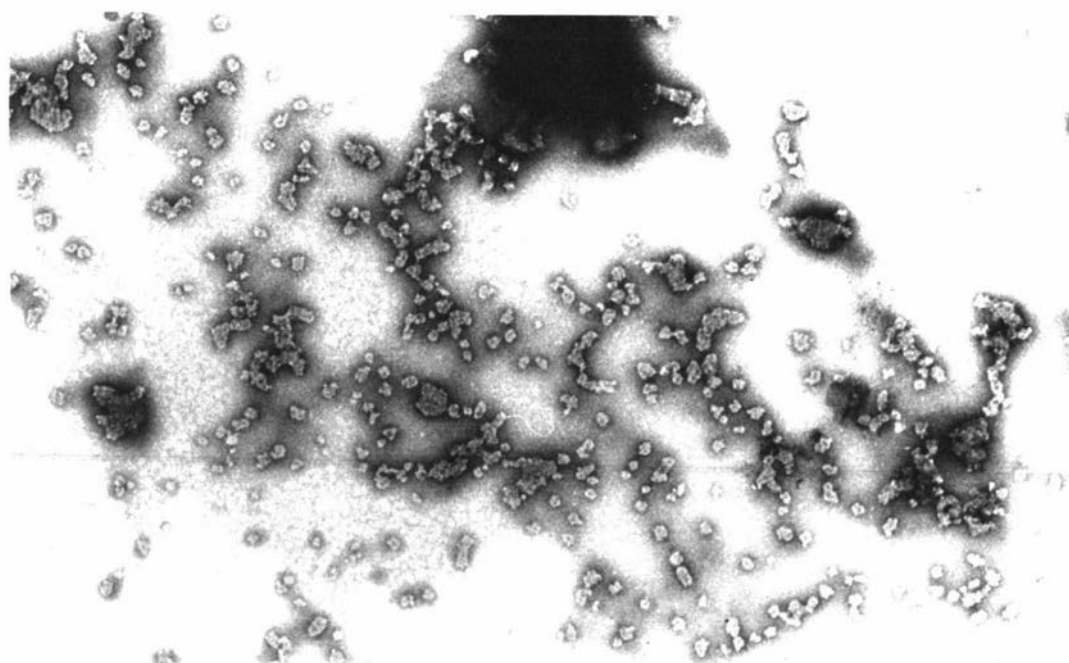
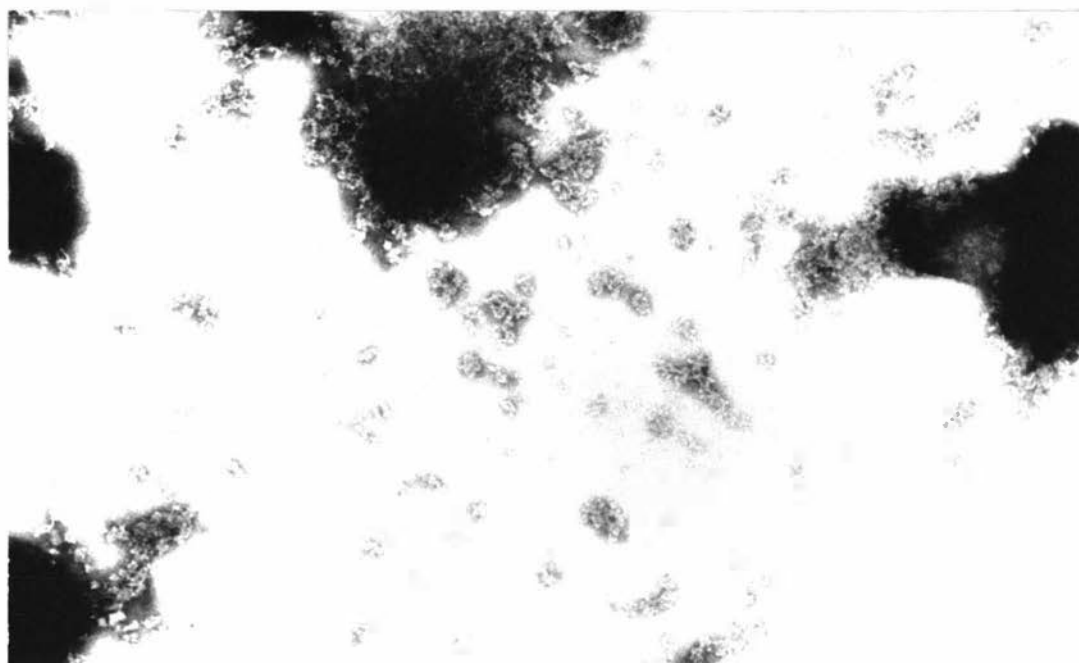
(c) Electron Microscopy of Rennet-treated "Artificial Milk" samples containing Phosphate.

The "artificial milk" samples used in one series contained 125 mg% calcium and 55 mg% phosphorus added in the form of Na_2HPO_4 . The size of the micelles was reduced considerably as indicated in Plate 16, presumably due to sequestering of calcium ions by the added disodium hydrogen phosphate.

The primary phase of rennet action in this system was followed up to the gel point and is shown in Plates 17-21. The electron micrographs clearly show the formation of typical "beaded" threads consisting of casein micelles joining together as rennet action proceeded. The micellar "threads" then joined each other to form the 3-dimensional network, (Plate 21). Here again, it is clear that there is no break up of the physical organisation of the casein micelles nor a dispersion of the inner components of the micelle prior to coagulation. The reaction again appears to be one between micellar surfaces.

The series of photomicrographs illustrates the effect of rennet action even more clearly than do the series with skim milk or with the "calcium only stabilised" "artificial milks". The difference in size of the micelles does not appear to affect the general pattern.

Differences in micelle size does however raise the question as to how much kappa-casein is involved with the surface of these smaller micelles.

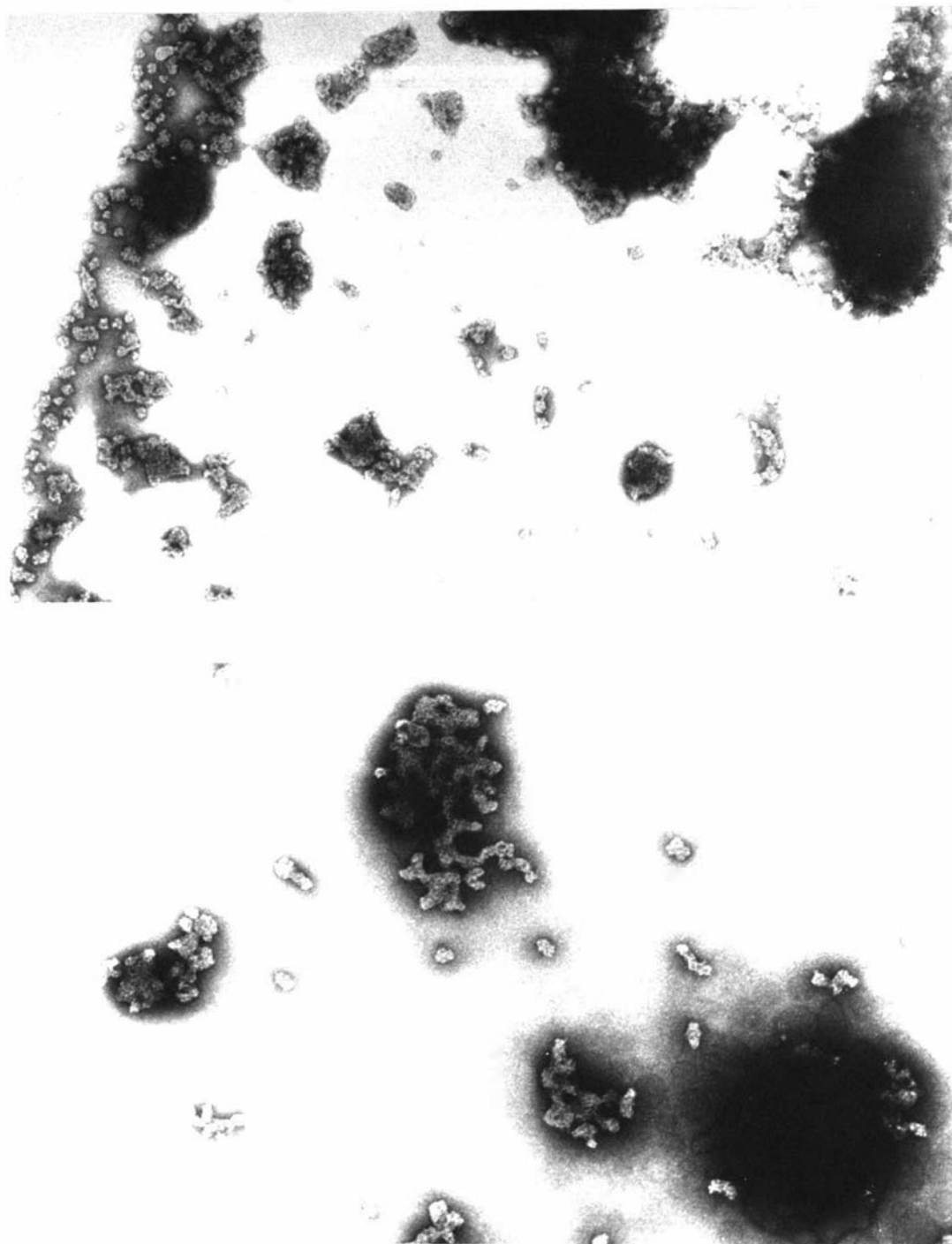


Electron micrographs of casein micelles in 'artificial milks'
containing both calcium and phosphate

PLATE 16: 'Artificial milk' (125 mg % calcium, 55 mg % 'P')----- x 47600.

Note: Micelle size greatly reduced due to Na_2HPO_4 addition to a neutral calcium caseinate solution. Compare with Plates 15 and 2.

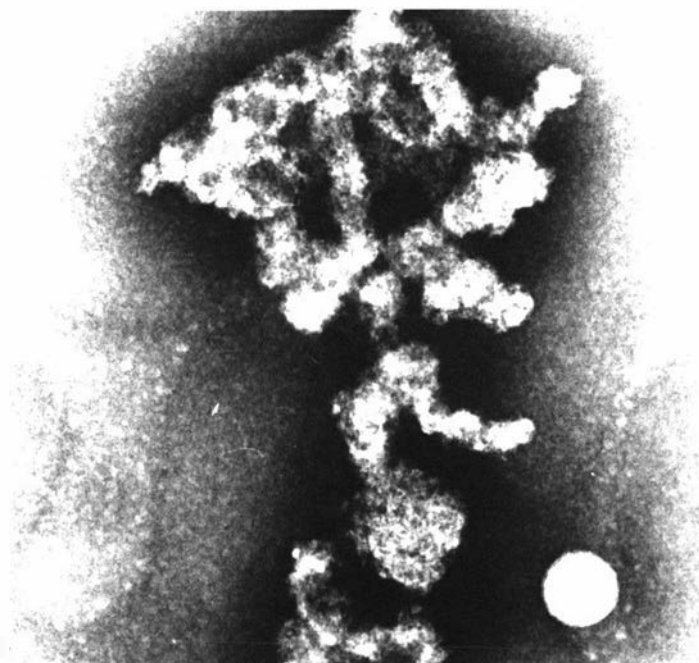
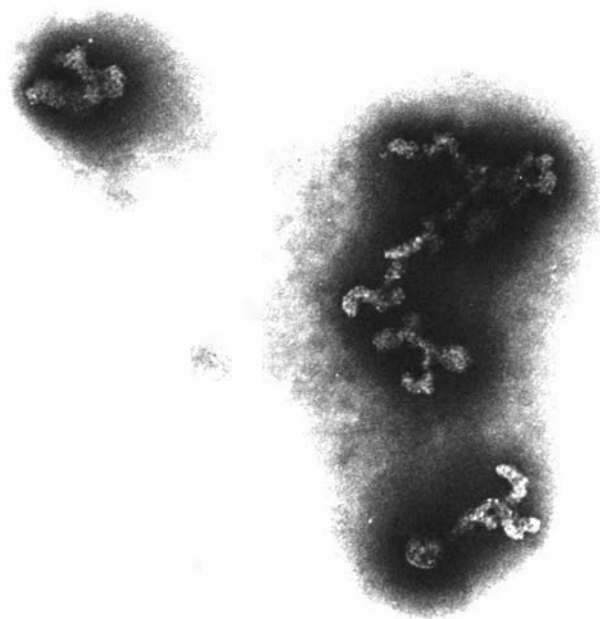
PLATE 17: 'Artificial milk' (125 mg % calcium, 55 mg % 'P') after rennet treatment ($2\frac{1}{2}$ minutes) ----- x 47600.



Electron micrographs of casein micelles in 'milks' containing
both calcium and phosphate after rennet treatment

PLATE 18: 'Artificial milk' (125 mg % 'Ca', 55 mg % 'P', 5 minutes)
—— x 47600.

PLATE 19: 'Artificial milk' (125 mg % 'Ca', 55 mg % 'P', 7½ minutes)
---- x 47600.



Electron micrographs of casein micelles in 'milks' containing
both calcium and phosphate after rennet treatment

PLATE 20: 'Artificial milk' (125 mg % 'Ca', 55 mg % 'P', 10 minutes)
---- x 47600.

PLATE 21: 'Artificial milk' (125 mg % 'Ca', 55 mg % 'P', 10 minutes)
---- x 154000.

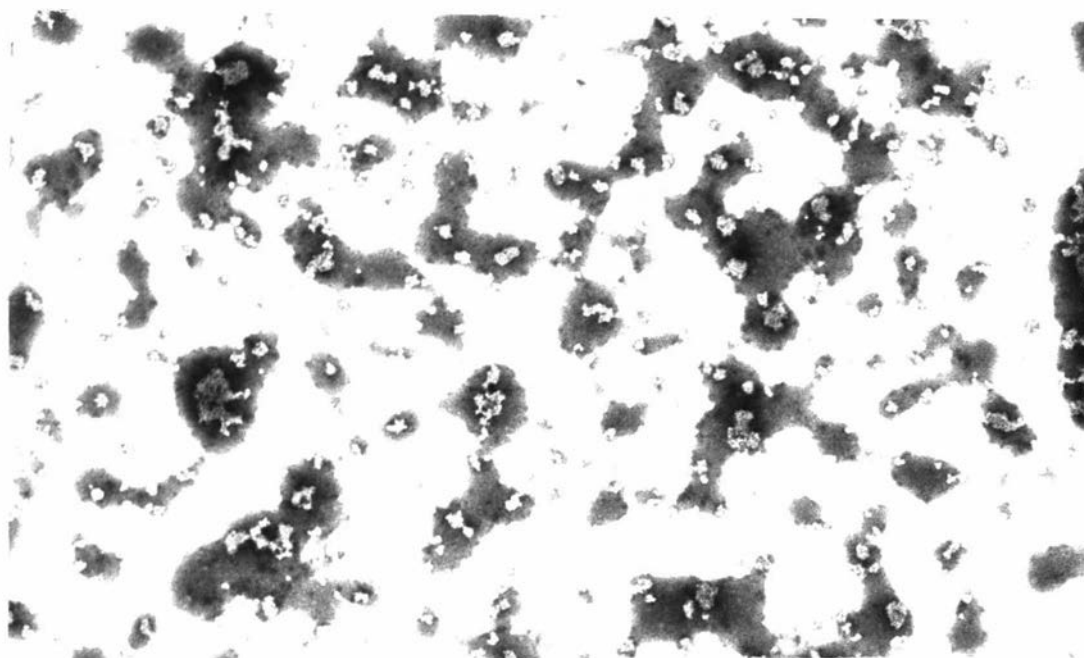


PLATE 22: Electron micrograph of casein micelles in 'artificial milk' systems containing both calcium (125 mg %) and phosphate (55 mg % added as orthophosphoric acid)
---- x 35000.

Note: Micelles only slightly bigger than those in 'milks' containing Na_2HPO_4 (Plate 16), and still much smaller than those in calcium caseinate milks and skim milk (Plates 2 and 3).

It is believed⁽¹⁵⁶⁾ that in normal milk the growth of a micelle to a larger size is restricted by the accumulation of more kappa-casein on the micelle surface. Small micelles also appear to contain more kappa-casein than large micelles⁽¹⁵⁶⁾. The relationship of these observations with the observed small micelles in "artificial milks" containing phosphate is, however, obscure and requires further investigation.

SECTION E: DISCUSSION ON THE PRIMARY PHASE OF RENNET ACTION
ON CASEIN.

Results from viscometric and ultracentrifugal studies suggest that, during the primary phase of rennet action on casein leading to gel point, substantial "dehydration" of the casein micelles occurs. It is, however, difficult to envisage the type of interaction existing between the caseinate and the solvent (water) prior to rennet action which allows the "bound water" or "immobilised water" to stabilise the caseinate suspension against aggregation. ("Immobilised water" is a better term since the studies do not indicate the nature of the "water" associated with the micelles.)

The primary factors giving stability to the casein micelles in milk are probably their degree of hydration and the net surface charge of the particles. The fact that casein particles can be flocculated at their isoelectric point indicates that charge is important for stabilisation. However, Payens⁽⁹¹⁾ has stated that the electrical surface potential is rather low. He stated that the zeta potential for casein particles of about 8 mv indicated a low electrical repulsion between micelles and concluded that stabilisation by charge appeared not to be a dominant factor at neutral pH. Results from viscosity studies done at low temperatures and reported here indicate that during the primary phase of rennet action on casein there was a significant drop in viscosity. Since the electron micrographs showed no apparent change in micelle form or size as a result of rennet action the change noted in viscosity as a result of this action must be due to an increase in the available free water. The use of the

constant "K" as indicator of the degree of hydration of spherocolloid particles has already been discussed. The value of "K" does fall rapidly from 0.50 to about 0.30 during the first two hours of rennet action (prior to onset of gelling) but never at any time approaches the value of 0.025 which is considered to indicate complete dehydration of the colloid. The drop in the value of "K" was similar for both sodium caseinate and calcium caseinate "artificial milks". These results suggest that there was an appreciable release of "immobilised" water during this primary phase of rennet action but that there is still a large amount of water associated with the residual micelle structure.

Interpretation of viscosity data is complicated by the fact that electroviscosity contributes to viscosity measurements. It is difficult to distinguish between viscosity due to electrical effects and that due to hydration and shape of a colloid. A system containing charged units would, due to electrostatic attractions, behave more like a solid than an uncharged system. Discharging the particles by addition of electrolytes would significantly reduce the viscosity⁽⁷⁶⁾. The possible effect of such addition of electrolytes on hydration has, however, not been investigated. Scott Blair and Oothuizen⁽³⁰⁾⁽³¹⁾ suggested that the release of the glycomacropeptide from kappa-casein reduced electroviscous effects and diminished viscosity. A change in shape of coiled-up particles due to "discharging" was suggested. However, electron micrographs shown in this study do not indicate any extensive structural changes in the casein micelle and do not support their contention⁽³⁰⁾. The physical organisation is essentially similar in both rennet-treated and untreated samples at 4°C and at 30°C. Booth⁽⁹⁵⁾ on theoretical

considerations predicted much smaller effects on viscosity due to the charge on a colloid, contrary to what was earlier believed. He considered that when the thickness of the double layer was small compared to the radius of the particle, the electroviscous effect was negligible. Tanford and Buzzell⁽⁹⁶⁾ confirmed this when they observed that over a considerable range of pH between 4.3 and 10.5 and ionic strength, the intrinsic viscosity (reduced viscosity at infinite dilution) of bovine serum albumin was constant at 0.037. Only a small increase of this value could be observed as the molecular charge was strongly increased at high or low pH values. Hankinson and Briggs⁽⁹⁴⁾ had earlier stated that electroviscosity could account for 59% and 38% of the specific viscosity of 1% calcium caseinate and sodium caseinate solutions respectively but later Hankinson and Palmer⁽³⁶⁾ modified this statement and suggested that at low rennin concentrations, the enzyme action was primarily that of dehydration as little change in the electrokinetic potential was observed on the caseinate after rennet action.

Thus it would seem that the observed drop in viscosity during the primary phase of rennet action on casein is largely due to loss of "immobilised" water from the micelles, with possibly electroviscosity playing only a minor role.

Ultracentrifuge studies on the rennet-treated "artificial milks", using haemoglobin as an indicator, showed that the primary phase of rennet action on the caseinate released "immobilised" water to the extent of about 70% by weight of the wet caseinate complex. Only part of the "immobilised" water is probably truly bound to the proteins in the caseinate complex. A large proportion of the

"immobilised" water released by rennet could be in the form of ice-like regions, associated with hydrophobic residues on the casein components. The formation of stable ice-like regions of water around protein hydrophobic side chains has been fully discussed by Klotz⁽⁴⁷⁾. Payens⁽⁹¹⁾ has emphasised that hydrophobic interactions must play a major role in the interactions between the caseins to form the micelle, particularly since casein has an abnormally high content of apolar amino acids⁽⁹⁸⁾. It is also well known that all types of casein associations are promoted by an increase in temperature⁽⁹¹⁾. Theoretical investigations of Nemethy and Scheraga⁽⁹⁷⁾ have shown that the spontaneous formation of hydrophobic bonds in protein is an endothermic process. It is interesting to note that rennin shows a particular preference to hydrolyse bonds in proteins adjacent to hydrophobic side chains⁽⁷⁾. To do this it must upset the stable ice-like regions with consequent release of "immobilised" water. It is possible to argue that rennin, while releasing the "immobilised" water from the micelle surface, might also penetrate the micelle and in the process weaken the ice-like regions normally stabilising the micelle structure. Such a penetration of rennin into the micelle was put forward as a possibility by Shimwin and Hill⁽⁸⁷⁾ who, from a study of their electron micrographs, stated that the distance between the sub-units in the micelle was about 60Å. Assuming a diameter of 35-40Å for the rennin molecule from its molecular weight of 31,000, they suggested that rennin could penetrate the micelle and that sorption-desorption processes may possibly be speeded up because of the closeness of the substrate molecules within the micelle. Electron micrographs in this study, however, do not show any significant differences between rennin-treated and untreated casein micelles.

An "immobilised" water content of 70% in the wet caseinate complex is by itself not excessive and impossible. Hankinson and Briggs⁽⁹⁴⁾ calculated from viscosity studies a bound water content of 2.4 g water per gram casein when the latter was dispersed in calcium hydroxide at pH 6.6. This amounted to 70% of the weight of the wet casein complex. Sodium caseinate was found to bind much more water - 7.2 g/1 gm casein ($\approx$ 88% by weight of the wet caseinate). Tumerman and Webb⁽⁹⁹⁾ considered the micelles as swollen, microscopic polyelectrolyte gels "containing in excess of 66% water of which one fourth is chemically bound". Thus these figures are comparable to the figure reported in this study. Kirchmeier⁽¹⁰⁰⁾ had considered the water shell surrounding the casein micelles and the micelle aggregates to be the greatest inhibitor of syneresis (cf Hankinson and Palmer⁽³⁶⁾ and Cheesman⁽⁷⁵⁾). Lindquist⁽⁴⁸⁾ suggested that the first stages of the secondary phase involved rearrangement of protein bound calcium in a manner which led to decreased amount of water "bound" to the complexes. This supposedly gave rise to new forces aggregating the casein micelles. Results from this work support this view.

PART II

A STUDY OF THE FACTORS CONTRIBUTING TO THE SYNERESIS
OF RENNET-CASEIN GELS

CHAPTER III

Review of Literature on the Syneresis of Gels

SECTION A: GENERAL REVIEW.

1. Definition of syneresis:-

Syneresis has been defined⁽⁷⁶⁾ as the spontaneous liberation of the liquid from a gel. It is not to be confused with dehydration occurring upon drying as it can occur in the presence of moist air and of free water and at low temperatures. It may be regarded⁽⁷⁶⁾ as an internal dehydration or desolvation connected with shrinking, or as a reversal of swelling. Kuhn⁽¹⁰²⁾ depicts syneresis as a continuation of the disintegration process, following setting, by structural changes and desolvation. Each gel type behaves differently⁽¹⁰¹⁾; thus rubber, silicic acid and viscose give increasing amounts of syneresis with increasing concentration of gel while the opposite is true for starch, agar and cellulose acetate. Mechanical influences affect very strongly the spontaneous exudation of liquid from gels. If a gel showing no noticeable syneresis is subjected to the slightest pressure it loses liquid, as for example, soaps. Similarly, it has been observed⁽¹⁰¹⁾ that a 6% starch gel exuded no liquid on standing for 8 days. But when a rod, held upright in the gel was moved slightly, the gel structure was destroyed and fluid was released immediately. The fluid produced by such mechanical disintegration cannot be attributed to the phenomena of syneresis. On the other hand, when a rennet-casein gel is carefully cut it does not collapse and the individual cubes produced continue to show syneretic behaviour.

2. Gel formation and structure, mechanism of syneresis and water holding capacity of gels.

Syneresis of a gel may be influenced by the conditions under which the gel was formed. Ferry⁽¹⁰³⁾ describes a gel as consisting mostly of fluid, behaving as a rigid solid and yet retaining many properties characteristic of the fluid component, such as, compressibility, vapour pressure and electrical conductivity. The essential structure in many protein gels such as gelatin and fibrin, appears to be a 3-dimensional network held together by primary bonds and/or secondary forces localised at certain points on the solute molecule, and by non-localised secondary attractive forces. Gelation differs from coagulation or flocculation in that the complete system becomes solidified, there is no visible separation of the solid and the liquid phase⁽¹⁰⁴⁾. Ferry⁽¹⁰³⁾ has discussed the three main theories of gel structure.

Gels may have either a honeycomb structure or a brush heap structure of minute ultramicroscopic fibrils with the dispersion medium being held by capillary forces. The latter structure is the most probable one⁽¹⁰⁴⁾ for holding large amounts of liquid in bound form or by mechanical immobilisation. The more asymmetric the molecules the lower the concentration needed for gel formation⁽⁷⁶⁾. Gels have been classified as fine⁽¹⁰³⁾⁽¹⁰⁵⁾, described as being transparent, gelatinous, very friable, very elastic and slow to synerise, and coarse, described as being opaque, non friable, plastic and easily compacted under pressure with expulsion of solvent.

Meat and fruits contain 75-85% water, the bulk of it being "free water" in the physico-chemical sense, that is, having the same freezing point, vapour pressure and dissolving power as normal water⁽¹⁰⁶⁾.

Proteins normally do not bind water much more than 20% of their dry weight as true "hydration water". Therefore, large scale immobilisation of water may be due to the presence of a flexible network of filaments and membranes formed by primary cross linkages and electrostatic forces. It may also be due to the formation of ice-like water structures around non-polar regions of the solute⁽¹⁰⁷⁾. Cross linking may occur through hydrogen bonding as in thermo-reversible secondary valence gels or by salt bridges or multivalent cations as in thermo-irreversible principal valence gels⁽¹⁰⁶⁾. Increasing electrostatic repulsion or weakening of the H-bonds gives rise to swelling and if repulsion is sufficiently high then the gel converts to a colloidal solution. On the other hand, if electrostatic attraction increases or inter linking occurs through divalent cations, then enough space would not be available for the retention of "immobilised" water. Part of this water then flows out in syneresis. For maximum imbibing of "immobilised" water, the cohesive forces should not be considerably greater than is just necessary to maintain the network structure. Jirgenson and Straumanis⁽⁷⁶⁾ regard syneresis as the ageing of unstable gels. The movable structural elements in the fresh gel is said to come into contact with each other and with the rigid elements to form new bonds. This would continue to increase the coarseness of the gel; thus they implied that syneresis was a continuation of the gelation phenomena.

Katchalaky⁽¹¹¹⁾ regards high polymer molecules as polyelectrolytes, carrying repeating ionisable groups. He considers polyelectrolyte gels as rheologically homogeneous and comprising at least two components, one of which is a network of ionisable macromolecules. Protein gels were classified as a polyampholyte type of polyelectrolyte gel. If the

protein gels or biogels were formed primarily by covalent cross-linkages such as the stable -S-S- bonds, the resulting network structure was expected to be robust and almost independent of the nature of the imbibing medium and of inner stresses. The network was not torn apart by the absorbed solvent nor did the swelling of the gel lead to its dissolution.

Electrostatic forces may play a predominant part in polyelectrolyte gels⁽¹¹¹⁾ as was shown by proteins at their isoelectric point. The attractive forces operate in the gel formation in the presence of equal numbers of positive and negative charges. Gel formation would occur in fairly concentrated protein solutions when intermolecular, as against intramolecular attractions, would develop. The electrostatic forces are able to operate in the cross-linking, despite the dissociative action of water due to its high dielectric constant. Apart from the number and valency of the charged groups, their spatial arrangement in the molecular chain units and network was considered to be important⁽¹¹¹⁾ for stabilisation of the network. Equality of positive and negative charges by itself was not sufficient for effective cross-bonding⁽¹¹¹⁾. In randomly kinked gelatin which is made up of fibrillar molecules, overall electrostatic attraction at the isoelectric point was negligible compared to other forces. However, in highly organised collagen the close proximity of the oppositely charged groups gives a 3-dimensional network at the isoelectric point.

3. Factors influencing syneresis

Munro and Monteith⁽¹⁰¹⁾ reporting on the syneresis of silica gels, state that at pH 6.5, 20°C and 8.4% silica concentration, neutral salts increased the rate and final volume of syneresis. Potassium salts

appeared to be more potent than sodium salts and sulphates appeared to be better than chloride. Neutral silica gels were generally weakly synergetic while basic gels set rapidly and synerised quickly⁽¹⁰²⁾. Acid silica gels synerised more slowly⁽¹⁰⁸⁾ than basic gels but final quantity was greater. Increasing temperature increased syneresis - a factor of 1.8 per 10° rise in temperature⁽¹⁰⁸⁾. This increase was attributed to increased Brownian movement resulting in increased internal contacts between the structural elements⁽⁷⁶⁾. Electrolytes in general tended to increase syneresis probably by discharging the mobile structural elements since similar charges repel and would not allow formation of new bonds⁽⁷⁶⁾.

With albumin gels⁽¹⁰⁹⁾, gelation and syneresis were promoted to a greater extent by sulphate ions than by chloride or nitrate. The opacity and ease of syneresis of albumin gels formed by the addition of sodium chloride passed through a minimum at a concentration of about 0.3 M NaCl⁽¹⁰⁹⁾. This was probably due to a decrease in attractive forces⁽¹⁰³⁾ to a minimum with increasing salt concentration.

Rapid gelation and high concentrations of structural materials result in the formation of fine gel structures⁽¹⁰³⁾ which tend to synerise slower.

HARR⁽¹⁰⁶⁾ states that of the total water held by meat, only 4-5% is bound as true hydration water. This amount was hardly influenced by the structure of, or by charge on, the muscle proteins. However, the "free water" immobilised within the tissue is strongly influenced by the spatial molecular structure of the muscle tissue. A loosening of the microstructure increases the amount of immobilised water, as for example when raising the protein net charge by acid or base addition.

Sodium chloride increases water holding capacity of meat 3-fold at pH 5.5, the effect probably being due to the chloride ion as sodium acetate at a similar ionic strength did not give the increase⁽¹⁰⁶⁾. Chloride ions probably react with protein positive charges, expanding the network. Alkaline earth metals such as calcium and magnesium decrease water holding capacity in meat by decreasing the swelling. Ion exchange with Na^+ gave increased swelling. Citrates and phosphates used in the processing of meat reduce loss of water probably by sequestering calcium⁽¹⁰⁶⁾.

Studies on water binding in sausages show that addition of glycine helps enormously to increase the water retention during processing⁽¹⁷⁵⁾. The suggestion was that the dielectric constant was increased by the added glycine. This tended to give rise to swelling of the meat through weakening of the attractive forces in the network. However, other amino acids also give equal, if not greater, increment to the dielectric constant of a medium, depending on the number of carbon atoms separating the charged free $-\text{NH}_2$ and $-\text{COOH}$ groups⁽¹¹⁰⁾⁽⁸⁰⁾. Polyglycine peptides produce a much greater dielectric increment in the medium than glycine alone and it remains to be seen whether they are able to give more water retention than glycine alone.

Haurowitz⁽⁸⁰⁾ has pointed out that the solubility of proteins was increased by addition of glycine and other dipolar molecules, probably by raising the dielectric constant of water. The solubility was, however, reduced by organic solvents such as ethanol which lowered the dielectric constant of water. While use of these observations has been made in the fractionation of plasma proteins⁽⁸⁰⁾, the exact role played by the additives such as glycine in the greater water retention of gels such as in sausages is largely unknown.

Reversible gels may be formed by adding an equal volume of methanol to a 40% solution of serum albumin at pH 7.5 or by ethanol addition to aqueous solutions of casein at pH 3-4 and pH 9-12 when casein concentration required for gel formation was reduced by two-thirds. This effect of organic solvents was attributed to an increase in electrostatic potential and long range attractive forces of the proteins through depressing the dielectric constant of the medium⁽¹¹¹⁾.

SECTION B: REVIEW OF LITERATURE ON THE SYNERESIS OF RENNET-MILK
GELS.

1. Factors influencing gel formation and structure of rennet-casein gels.

This has been fully discussed under Chapter I of this thesis. Electron micrographs shown in this work had indicated that, in essence, gelation involved joining of the spherical casein micelles to form "beaded" threads. These threads then linked with each other to form a three dimensional network, enclosing the whole liquid. Prior to, and during the process of "beaded" thread formation and cross-linking, "immobilised" water equivalent to about 70% of the weight of the wet casein micelles was apparently released into the system.

2. Mechanisms of syneresis of rennet-milk gels.

Literature on the subject of syneresis of rennet-milk gels has been very scant. In particular, the knowledge on the mechanism of syneresis of rennet-casein gels is very obscure. Yet, this phenomenon is fundamental and of great economic importance in the manufacture of all varieties of cheese. Elucidation of the mechanism would help greatly in the scientific control of cheesemaking operations.

The limited work done indicates that syneresis of rennet-casein gels proceeds stepwise, similar to the coagulation phenomena. Hankinson and Palmer⁽³⁶⁾ considered the shell of water surrounding the casein micelles and the micelle aggregates to be the greatest inhibitor of syneresis and this conclusion was supported by Kirchmeier⁽¹¹²⁾. Cheeseman⁽⁷⁵⁾ studied the effect of various additives on syneresis of milk and caseinate systems and concluded that casein dehydration,

probably initiated by rennin action, was an important factor affecting the subsequent rate and extent of syneresis through protein-protein interaction. Cheeseman⁽⁷⁵⁾ also noted that certain additives such as calcium, up to 50 mM, decreased clotting times but also decreased syneresis rate and maximum syneresis. Other additives such as urea, up to 10%, increased clotting times but also increased the rate and extent of syneresis. This indicated that syneresis was not a continuation of the gelation phenomena.

After the initial dehydration, syneresis was stated to proceed by formation of ionic or hydrogen bonds⁽⁷³⁻⁷⁵⁾⁽¹¹²⁾. During the final stages of syneresis, sulphhydryl groups were also stated to be involved⁽⁷³⁾. Milk treated with 3.75% N-ethyl maleimide, a reagent specific for SH-groups, was found to give reduced syneresis. Schober et al⁽¹¹³⁾ reported similar findings. Tuszynski, Burnett and Scott Blair⁽¹¹⁴⁾, however, were unable to detect any effect of N-ethyl maleimide added to milk, at any stage during coagulation or of softening of the curd. This ruled out involvement of -SH groups in the normal clotting of milk. Stoll⁽¹⁰⁴⁾ also ruled out the possibility of -SH groups taking part in syneresis of rennet-milk gels. He commented that Christ's⁽⁷³⁾ observation of reduced syneresis in the final stages may have been due to bacterial growth, since the experiments were carried out at temperatures conducive to such growth, over a number of hours. Stoll⁽¹⁰⁴⁾ in an extensive study of the syneresis of rennet-milk gels states that syneresis is not a continuation of the clotting reaction (cf Cheeseman)⁽⁷⁵⁾. He suggests also that osmotic forces and ionic strength have an effect on syneresis. The interior of the curd, where acid is produced and pH is lower than in the whey in the early stages of syneresis⁽¹²⁹⁾, would be the site of greatest osmotic activity in a curd-whey system. The

curd, therefore, would be expected to swell. Salts added to whey should increase syneresis. The force within the curd, however, which causes syneresis, may be strong enough to produce a phenomenon similar to "reverse osmosis"⁽¹¹⁵⁾, a phenomenon which, in fact, has been used for the concentration of whey⁽¹¹⁶⁾. Reverse osmosis is produced by applying pressure in excess of the osmotic pressure, so that the direction of the normal osmotic flow can be reversed⁽¹¹⁶⁾. In rennet-milk gels, two conflicting forces may operate. One may cause the curd to shrink and the other cause the curd to swell. Stoll⁽¹⁰⁴⁾ considers the latter to be the weaker force and the balance between the forces determines the rate and extent of syneresis. Structural hindrance to the shrinkage of the gel was also considered as a factor. The view that syneresis of rennet-milk gels is caused by contraction of a gel structure is supported by Stoll's⁽¹⁰⁴⁾ observation that treatments which cause dispersion of the curd resulted in decreased syneresis. Stoll reporting on the effect of pH on syneresis indicated that decrease in charge was important as also was the removal of calcium from the structure. These effects allowed structural elements to draw closer together.

3. Factors influencing syneresis of rennet-milk gels.

(a) Effect of previous treatment of the milk

Cheeseman and Mabbitt⁽¹¹⁷⁾⁽¹¹⁸⁾ showed that pre-concentration of the milk to one-third the original volume, at around 34°C for about 20 minutes in a laboratory climbing film evaporator, and redilution to original volume gave, after rennet treatment, a curd which had high fat retention properties. This was attributed to a reaction between casein micelles and the fat globule membrane.

This reaction was promoted if the milk had been cooled and stirred before processing. They found no effect on the fat retention property when milk was preheated before concentration for 10 minutes in the temperature range $45^{\circ}\text{C} - 80^{\circ}\text{C}$. Beyond this, denaturation of the whey proteins was presumed to give poor curds⁽¹¹⁸⁾. Concentration at a temperature of 54°C instead of 39°C also produced no change in the fat retention properties of the curd. Work with pure proteins⁽¹¹⁸⁾ showed that both β -casein and kappa-casein were equally effective in fat retention. The concentrated-rediluted milks also gave a lower rate and extent of syneresis at pH 6.0 when examined by the method of Cheeseman⁽⁷⁵⁾. At pH 5.5 and below there appeared to be no significant difference in whey retention when compared to controls. The high whey retention property was attributed to casein micelle - fat globule interaction. The presence of a greater number of micelle-globule complexes possibly interfered with casein-casein interaction in syneresis.

Stoll⁽¹⁰⁴⁾ found that pasteurisation to 145°F for 30 minutes did not affect syneresis of curd in Cheddar cheese manufacture but heating to $165^{\circ}\text{F}/30$ minutes gave a slower rate and extent of syneresis at all pH levels up to 6.0. He attributed the lower syneresis at 165°F to serum protein denaturation and not due to decrease in soluble calcium phosphate in milk.

Generally, pre-heat treatment of milk, in particular high pasteurisation temperatures, has been found to reduce syneresis of the resulting curd⁽¹¹⁹⁾⁽¹²⁰⁾, falling sharply beyond 85°C . The reason for the reduced syneresis has not been made clear in the literature. Heating to high temperatures gives a slower rate of rennet coagulation⁽⁶⁰⁾ but the addition of CaCl_2

partially restores the rate⁽¹²¹⁾, probably by increasing the soluble calcium⁽¹²²⁾.

(b) Effect of pH or acidity on syneresis

Whitehead and Harkness⁽¹²³⁾ felt that the most potent factor in moisture expulsion from rennet curd was the gradual development of lactic acid by the starter. Cheeseman⁽⁷⁵⁾ adjusted the milk pH using, separately, hydrochloric acid, lactic acid and a starter. He observed the rate and extent of syneresis to be similar in all cases. This indicated that the nature of the acid was of no importance. Between pH 6.0 and 5.54, the rate and extent of syneresis was greater with the lower pH values. However, between pH 5.3 and 4.9, the rennet-curd appeared to retain more moisture at the end of 15 minutes at 50°C. The increase in the rate of syneresis from pH 6.0 through to pH 5.3 was ascribed by Cheeseman⁽⁷⁵⁾ as being due to a reduction in the capacity to maintain the hydration lattice and/or to increasing ionisation of groups which take part in protein-protein interactions. Stoll⁽¹⁰⁴⁾ also observed that lower pH values of skim milk encouraged syneresis of the curd. Lower pH values also overcame the depressing effects of heat treatment on syneresis at pH 6.7. However, in those skim milks heated to 165°F for 30 minutes, the hysteresis effect was only partially overcome. Stoll⁽¹⁰⁴⁾ also observed that less calcium was bound by the curd at each successively lower pH, yet magnesium binding was constant. The curd at pH 5.5 did not appear to have sufficient rigidity to sustain its shape, indicating that calcium may have a stabilising effect on curd structure. Stoll⁽¹⁰⁴⁾ was also able to get an increase in syneresis by adding 10% lactic acid to skim milk gels

after cutting. This addition reduced the pH of the whey to

6.0. Acid addition increased calcium in the whey only slightly.

(c) Effect of cooking temperature on syneresis

Whitehead and Markness⁽¹²³⁾ found that variations in cooking temperature of 0.5°F and 1°F between 96°F to 100°F gave no significant change in the rate of syneresis but that a variation of more than 2°F was required. They also pointed out that temperatures above 100°F would hamper growth of starter bacteria and thereby acid production. This would tend to counterbalance any increase in syneresis by raising of temperature. Lawrence⁽¹²⁴⁾ found a 6% increase in syneresis for each 5°F rise in temperature between 91°F - 112°F, the rate of shrinkage being 50% faster at 98°F and 100% faster at 105°F than at 91°F. Between 91°F and 112°F, the extent of syneresis at the end of 3 hours was greater as the temperature was raised. Stoll⁽¹⁰⁴⁾ found that the ability of curd to synerese at an increased rate when placed at 45°C was not lost, although setting times were increased. This indicated that syneresis was not a continuation of the clotting phenomenon. Syneresis appeared to be a linear function of temperature between 21°C - 45°C. Stoll⁽¹⁰⁴⁾ states that observed effects of physical and chemical variables were more pronounced at 32°C, although syneresis was more rapid at 40°C. Sammis et al⁽¹²⁶⁾ found that high acidities and high temperatures caused rapid syneresis immediately after cutting. Syneresis of curds set at 86, 92, 98 and 104°F and heated to 104°F, was more rapid at first in the curds set at the higher temperature. After 1 hour the rate had decreased so that all curds had the same moisture contents. Rate of heat application during cooking was also important - too rapid a

heating probably causing a skin to form on the curd surface which hindered the flow of whey from the curd⁽¹²¹⁾.

(d) Effect of cutting time and rennet concentration

Skim milk set at 32°C and cut 15, 30, 45 and 60 minutes after rennet addition showed very little difference in extent of syneresis at the end of 120 minutes, being slightly less for the longer setting times⁽¹⁰⁴⁾. The rate of syneresis was slower for the shorter setting times as against the longer setting times. This again indicated that syneresis was not a continuation of the clotting phenomena.

Increase of rennet concentration 6-fold gave very little difference, 120 minutes after cutting, in the extent of syneresis at 32°C, nor was there a difference at 40°C⁽¹⁰⁴⁾. There appeared to be a marginally greater rate of syneresis with the greater concentration of rennet at 32°. Stirring overcame the small effects due to variation in cutting time and rennet concentration.

(e) Effect of size of cubes and curd blocks, stirring and volume of whey on syneresis

Whitehead and Harkness⁽¹²³⁾ using $\frac{1}{4}$ ", $\frac{3}{8}$ " and $\frac{1}{2}$ " spaced knives found significantly lower water-in-the-non-fat-substance values for curd cut into smaller cubes. Acid development was similar in all cases. The same authors⁽¹²³⁾ found that the speed of rotation of the agitator used for stirring the curd during cooking, was of no significance in syneresis. Lawrence⁽¹²⁵⁾ found that the rate and extent of syneresis was considerably increased by stirring of the curd in the whey and that stirring minimised the differences in syneresis due to the use of different cooking temperatures. Stirring of the curd outside the whey, that is dry stirring, also significantly increased syneresis against no

stirring⁽¹²³⁾⁽¹²⁵⁾. Lawrence⁽¹²⁵⁾ also found that the greater the volume of whey surrounding the curd particles, the slower the rate of syneresis. Results obtained with water were similar. Whitehead and Harkness⁽¹²³⁾ showed that curd blocks of normal size, that is 2 ft 3 in x 6 in x 6 in, gave significantly lower W.N.F.S. values in cheeses than those from larger blocks, for example 2 ft 3 in x 12 in x 12 in.

(f) Effect of total solids and composition of milk on syneresis

Comparison of syneresis between 8-14% total solids in reconstituted skim milk⁽¹⁰⁴⁾, showed that while the initial rate of syneresis, within 30 minutes, was higher with the high solids, the extent of syneresis was lower in terms of total whey exuded. However, in terms of grams solids in the original skim milk expressed per millilitre of whey exuded, there was no difference in syneresis. The grams solids/ml. whey was directly proportional to per cent solids. Stoll⁽¹⁰⁴⁾ infers that the main effect of solids-not-fat levels is to start the curd synerising at a different point on the syneresis curve. He stated that curd with higher solids does not have to lose as much whey as curd with lower solids to have the same moisture content. In this connection it is interesting to note that Peters⁽¹²⁷⁾ prepared cheese from 62% solids concentrated milk in which no syneresis was involved. Dimov and Minerva⁽¹²⁰⁾ found that curd from sheep's milk synerised to a greater extent than that of cows or buffaloes' milk. Several reports⁽¹¹⁹⁾⁽¹²⁰⁾ indicate that a high fat content impairs syneresis. Beeby⁽¹²⁸⁾ reported a greater rate and extent of syneresis with skim milk gels than with corresponding whole milk gels. Homogenisation of milk causes the cheese made from it to

contain relatively more moisture⁽⁶⁰⁾ - the effect of homogenising cream separately and then adding to skim milk minimised the effects. Cheeseman⁽⁷⁵⁾ found that additions of 1%, 2.5% and 5% milk powder to raw skim milk at pH 5.7 gave a decrease in both rate and extent of syneresis while increasing the clotting time.

(g) Effect of the concentration of various ionic species on syneresis

(1) Cations

Effect of calcium, magnesium, aluminium and sodium

Calcium

Calcium in the form of calcium chloride has often been added to milk for cheesemaking as an aid to curd formation, particularly to heat treated milk⁽¹²¹⁾. Cheeseman⁽⁷⁵⁾ showed that between 10 mM and 50 mM CaCl_2 addition, clotting time was decreased with unheated milks but so was syneresis. At 100 mM CaCl_2 addition, syneresis was markedly reduced and at pH 5.3 there was no syneresis observed under his experimental conditions. Clotting time was, on the other hand, increased at this level of CaCl_2 addition. In general, increase in ionic concentration of salts tended to restrict drainage and at high levels to affect gel formation⁽⁷⁵⁾. Contrary to results obtained by Cheeseman⁽⁷⁵⁾, Stoll⁽¹⁰⁴⁾ found that between 0.02% and 0.10% added CaCl_2 (lower concentrations than used by Cheeseman⁽⁷⁵⁾) both rate and extent of syneresis was increased at 32°C and at 40°C, as measured by Cheeseman's⁽⁷⁵⁾ technique. However, prolonged

incubation gave equivalent syneresis indicating that milks containing added CaCl_2 reached equilibrium quicker.

Magnesium

Addition of MgCl_2 was found to give similar results to CaCl_2 - both calcium and magnesium ions were equally effective in promoting syneresis⁽¹⁰⁴⁾.

Aluminium

Stoll⁽¹⁰⁴⁾ found that addition of 0.15 to 0.30% AlCl_3 retarded syneresis although the pH of the skim milks was decreased by the addition. When added after cutting, however, both rate and extent of syneresis was increased. Aluminium ion appeared to give greater stability to the para-caseinate gel.

Sodium

Cheeseman⁽⁷⁵⁾ showed that sodium chloride at a concentration of 17 mM reduced the rate of syneresis considerably but at pH levels of 5.7 and 5.3 gave a greater extent of syneresis. These milks did not gel at room temperature but did so at 50°C. At 85.5 mM and beyond, clotting did not occur. Stoll⁽¹⁰⁴⁾ found an increase in rate and extent of syneresis at 32° and 40°C up to 1% addition of NaCl. When added after cutting the curd, there was no effect on the extent of syneresis, until the concentration of sodium chloride added was raised to 2%. A 4% addition depressed the extent of syneresis. Calcium and magnesium appeared to be displaced from the curd into the whey by sodium.

(11) AnionsEffect of lactates, citrates and phosphatesLactates

The only study on the effect of added anions has been carried out by Stoll⁽¹⁰⁴⁾ who used the sodium salts. Using 1.3 and 2.6% sodium lactate, he found, using Cheeseman's⁽⁷⁵⁾ tube method, that at 32°C the lower concentration of sodium lactate effected a greater rate and extent of syneresis at 120 minutes. However, at 40°C and 2.6% sodium lactate concentration, the reverse was true. Curds were increasingly fragile with increasing amounts of sodium lactate and this correlated with increased calcium levels in the whey. This indicated the displacement of calcium from the curd structure. Magnesium levels were relatively constant in the whey. From this Stoll⁽¹⁰⁴⁾ deduced that there must be an optimum content of calcium necessary in the curd for optimum syneresis. Too much displacement inhibited syneresis. The curd structure appeared to be the limiting factor in extent of syneresis.

Citrates

Skim milks to which over 0.25% sodium citrate had been added⁽¹⁰⁴⁾, did not clot at 32°C. At 40°C a weak clot formed only in the 0.25% added sample. Addition of 0.02 - 0.04% sodium citrate reduced rate and extent of syneresis at 32° and 40°C as examined by Cheeseman's⁽⁷⁵⁾ tube method. The wheys contained more calcium indicating displacement of calcium from the curd.

Phosphates

Skin milks to which 1% disodium hydrogen phosphate had been added⁽¹⁰⁴⁾ gave curds which synerised at a slower rate, but the extent of syneresis was the same as for 0.25% and 0.5% additions. Generally clotting and syneresis was slowed by the salt but use of higher temperatures overcame some of the effects on syneresis. Cheeseman⁽⁷⁵⁾ using raw skim milk at pH 5.7 found that addition of 10 mM dipotassium hydrogen orthophosphate increased the extent of syneresis while decreasing the clotting time. Greater additions gave no difference in syneresis but increased clotting time.

(iii) Effect of chelating agent

Stoll⁽¹⁰⁴⁾ added 2.5, 5.0 and 7.5 mls. of 0.56 N E.D.T.A. (100 g EDTA, 4 g NaOH, made to 1 litre) per 500 mls. skim milk. This was equivalent to 3.94, 7.88 and 11.82% of the calcium in skim milk. At 32°C and 40°C, only the 7.5 ml. addition gave rise to lower rate and extent of syneresis.

(iv) Effect of sulphydryl blocking agent on syneresis

Christ⁽⁷³⁾ and Schober et al⁽¹¹³⁾ reported that n-ethylmaleimide inhibited syneresis. This, however, was not substantiated by Tuszyński et al⁽¹¹⁴⁾. Stoll⁽¹⁰⁴⁾ also was unable to detect any difference in syneresis either at 32°C or 40°C because of the addition of n-ethylmaleimide. He concluded that sulphydryl groups were not involved in syneresis, at least in its early stages.

(v) Effect of the addition of organic compounds and surface active reagents on syneresis

Urea

Cheeseman⁽⁷⁵⁾ found that at 2.5% - 10% addition of urea, both the rate and extent of syneresis was increased, but clotting times were also greatly increased at pH 6.0, 5.7 and 5.3. Stoll⁽¹⁰⁴⁾ found that addition of greater than 12% urea affected clot formation and curd syneresed at a slower rate. Adding 24% urea ($\approx$ 4 molal) caused considerable displacement of calcium from the curd, as well as protein. Hipp et al⁽¹³⁰⁾ had fractionated α , β and γ caseins on the basis of solubility in urea and it was possible that β and γ caseins were affected at the 24% urea level. Stoll⁽¹⁰⁴⁾ hypothesised that β casein could constitute a structural part of the clot which gave the system its rigidity.

Dioxane

Cheeseman⁽⁷⁵⁾ found that addition of dioxane between 10 to 30% to raw skim milk at pH 5.7 reduced the rate of syneresis but the extent of syneresis was not much affected. Clotting time was increased somewhat.

Sodium dodecyl sulphate

Similar effects to dioxane were found for this wetting agent by Cheeseman⁽⁷⁵⁾ between concentrations of 0.01 - 0.05%. Clotting time was, however, greatly increased and at a level of 0.1% no clot formation occurred.

(vi) Effect of added carbohydrate on syneresis

Cheeseman⁽⁷⁵⁾ found that at 2.5% - 5.0% lactose addition, both rate and extent of syneresis was greater, particularly at pH values of 5.7 and 5.3. Clotting time was little affected. At the 10% level, clotting time was reduced while the rate and extent of syneresis was higher than controls. He attributed this increase in syneresis to the affinity of lactose for water possibly causing disruption of the hydration lattice, an effect opposite to that caused by addition of salts. Stoll⁽¹⁰⁴⁾, however, found no measurable effect on syneresis from lactose addition up to 2%.

(vii) Summary of factors influencing syneresis of rennet-milk gels

In summary, it would appear that the mechanism of syneresis is still far from being clear. It is believed by some⁽³⁶⁾⁽⁷⁵⁾⁽¹¹²⁾ to start with casein dehydration, probably initiated by rennin action. There is a conflict of opinion on the role played by sulphhydryl groups in syneresis. The "reverse osmosis" theory⁽¹⁰⁴⁾ appears interesting but the nature of the cross bonding in the gel which gives rise to forces greater than the osmotic pressure of curd-whey systems has not been elucidated. High pre-heat treatments of milks generally lead to decreased syneresis. The reason for this has not been made clear in the literature. Lowering the pH, raising the cooking temperature and cutting of the gel into smaller cubes seemed to give increased syneresis, as did stirring of the cut curd. In general, added salts in low concentrations, tended to increase syneresis but at higher levels tended to restrict drainage and sometimes affected clot

formation itself. Calcium appears to play a definite role in syneresis as indicated by the effect of added anions and E.D.T.A. It appears that an optimum level of calcium is needed in the curd for optimum syneresis. Urea above 12% and organic wetting agents below 30% reduced the rate of syneresis. The evidence indicated that syneresis is not a continuation of the gelation phenomena. However, it may be dependent on the type of gel structure formed.

4. Methods of measurement of syneresis

Different methods have been used by different authors for the measurement of syneresis and these have been reviewed by Stoll⁽¹⁰⁴⁾. Whitehead and Harkness⁽¹²³⁾ used 80 gallon vats and determined the ^{*}R.F.F.S. in the final cheeses to note the difference, if any. Lawrence⁽¹²⁴⁾⁽¹²⁵⁾ used 400 ml. lots in aluminium trays and measured the whey after tipping the curd into a sieve and draining for a standard time. Only one observation per sample was possible by this method, and tipping might have affected the gel itself through disruption. Christ⁽⁷³⁾ measured the terminal volume of curd by toluene displacement after syneresis for 12 hours at 37°C. Stall⁽¹⁰⁴⁾ has stated that bacterial changes would most certainly have occurred affecting the syneresis measurements made by Christ⁽⁷³⁾. Thome and Lillegren⁽¹³¹⁾ measured the change in weight of a cylinder of curd, cut with a cork borer and held in a beaker of whey. At various intervals the curd cylinder was removed from the whey and weighed. Schulz and Kley⁽¹¹⁹⁾ estimated moisture content of the shrunken curd after 24 hours at 37°C, while Sammis et al⁽¹²⁶⁾ weighed the

* water in the fat-free substance

curd particles at intervals during cheesemaking. Removal of free surface whey from the curd particles would in this case be difficult. Beeby⁽¹²⁸⁾ developed a tracer technique to measure syneresis - he reacted skim milk with formaldehyde and used the dilution of this tracer by curd whey to calculate the whey expelled. The tracer was measured spectrophotometrically. Cheeseman⁽⁷⁵⁾ used a different technique for quicker assessment of the syneretic properties of the curd - he set 5 ml. of milk at 22-30°C and then placed the tubes immediately in a bath at 50-60°C for a given time, assuming that raising the temperature affected, in the main, only the rate of syneresis. Stoll⁽¹⁰⁴⁾ used three methods in his studies, involving amount of whey expelled from 500-1000 ml. curd volumes, change in specific gravity of the curd during syneresis and Cheeseman's⁽⁷⁵⁾ tube method, used at 32°C and 40°C respectively. The increase in specific gravity was measured in mixtures of monochlorobenzene and kerosine forming a specific gravity gradient.

SECTION C:

OBJECTIVES AND EXPERIMENTAL PLAN

The review of literature given in the preceding section brought out a number of salient points which needed clarification. In particular, it was noticeable that the most fundamental point in the mechanism of syneresis, that is, the nature of the crosslinks is still obscure. It is also not clear as to which of the proteins in whole casein are involved in the syneresis or at least play a major role in it.

The following experimental plan was devised:

The effect of various sulphydryl blocking and disulphide splitting reagents on syneresis was examined in somewhat more detail than those reported in the literature. Because of the confusion prevailing as to whether whole casein contained only sulphydryl or disulphide groups, this aspect was given attention. In an attempt to get some insight into the role played by calcium in gelation and syneresis, isoelectric casein gels, isoelectric para-casein gels and neutral -para-caseinate systems were studied in the presence of and absence of calcium. The effect of various levels of calcium on gelation and syneresis was also studied.

The effect of addition of some amino acids on gelation and syneresis was studied to find out whether modification of the dielectric constant of the medium affected the extent of syneresis of the gel. Work on other proteins by other workers indicated the possibility of amino groups being involved in a co-ordinated link with calcium. It was, therefore, thought that a line of research involving modification of amino groups on the caseinate through reaction with 1-fluoro-2:4 dinitrobenzene could be fruitful in giving

some insight into the crosslinking important in syneresis. It was further planned to heat casein systems in the presence of reducing sugars to determine the effect on syneresis.

In all cases "artificial milks" were used. These were essentially calcium caseinate solutions, though calcium caseinate-calcium phosphate solutions were also used in a limited number of cases. It was hoped to get a clearer picture by the use of model systems whereby other factors such as whey proteins, variability of composition, mineral salts, etc. would not interfere with results and a day to day similarity in the composition of the "artificial milks" could be maintained.

CHAPTER IV

A Study of the Effect of Disulphide Splitting and Sulphydryl Blocking Agents on Gelation and Syneresis of Calcium - Caseinate - Calcium - Phosphate Systems

SECTION A:

METHODS AND MATERIALS

1. Preparation of acid casein

This has been fully described in Section B in the attached
publication entitled "Sulphydryl and Disulphide Groups in Casein"
under "Preparation of acid casein - (a)".

2. Preparation of "Artificial Milks"

The preparation of a stable "artificial milk" has been described
earlier in Chapter II:A:2.

All "milks" were equilibrated at the temperature of the test for
at least 30 minutes, before rennet was added.

3. Measurement of syneresis

Methods of measurement of syneresis employed by other investigators
was reviewed in Chapter III:B:4. The method used in this study
was the simplest and the most direct - that of measuring the
volume of whey expelled from a certain volume of the curd under
various treatment conditions. The method was as follows:

40 mls. "artificial milk" was set in well-cleaned 100 ml.
beakers at 30°C or 38°C by the addition of one drop of neat
commercial rennet (in the case of "milks" containing both calcium

and phosphate) or one drop of 1:10 or 1:1 diluted commercial rennet. Usually the calcium "milks" required about 1/10 the rennet concentration needed for the calcium-phosphate "milks" for an equivalent coagulation time. At regular time intervals after the addition of rennet, the whey was removed from the top by means of a rubber suction device or pipette filler, fixed to a 10 ml. or 20 ml. pipette. The whey was drained into a 50 ml. measuring cylinder. By this means, damage to, or movement of, the gel was kept to a minimum. Smaller sized pipettes were also used whenever necessary.

The commercial rennet employed was the same as used in New Zealand cheese factories, with an activity of 62 rennin units/ml. as measured by the method of Ernstrom⁽¹³³⁾, (N.H. Clark⁽¹³⁴⁾ - personal communication). The commercial rennet preparations contained no detectable pepsin as a contaminant⁽¹³⁴⁾. Use of a purified rennin powder supplied by the N.Z. Rennet Co., Eltham, New Zealand, indicated no differences in the syneretic behaviour of "milk" gels when compared with the commercial rennet preparations and when used at equivalent strengths. The syneresis experiments were therefore carried out using the commercial rennet.

4. Polyacrylamide Gel Electrophoresis

The method followed was essentially that of Davis⁽¹³²⁾ with some minor modifications. The stock solutions were prepared as described by Davis⁽¹³²⁾. The fixative stain solution was prepared as a 0.2% solution in 7% acetic acid. The electrode compartments contained Tris-borate buffer at pH 8.7, prepared by dissolving 2g Tris^{*} in distilled water, adjusting to pH 8.7 with saturated boric

* 2-amino-2-(hydroxyl-methyl)-propane-1:3 diol

acid solution, the volume being made to 1 litre. To the buffer in the cathode compartment was added 1 drop of the marker dye solution of brom phenol blue (0.001%).

The apparatus used is shown in Plate 23. The electrode compartments were made from polyethylene boxes cut to the following dimensions: diameter 100 mm, height 80 mm, capacity approximately 500 ml. Six holes of 10 mm diameter were carefully bored, equidistant from the centre and from each other, into one of the boxes. This was eventually to be used as the cathode compartment. The holes were made so as to fit electrical grommets of outside diameter 12.5 mm and having an opening of 5 mm diameter. Rubber corks of suitable dimensions and with holes bored in them were glued on to the centre of the two electrode compartments. These carried graphite electrodes removed from torch battery cells. Small copper rings cut from copper tubing of suitable diameter were fixed to the top of the electrodes. Cathode and anode leads were soldered on to these rings. The graphite electrodes were changed after every 3 - 4 electrophoretic runs.

The electrophoresis tubes used for setting the small pore and large pore gels were made from ordinary glass tubing of outside diameter 7.5 mm and internal diameter 5 mm. The tubes were cut 120 mm long. The small pore gel solution was poured to a height of 95 mm and the large pore solution, after the small pore gel solution had set, to a height of 105 mm. Electrophoresis of the "artificial milks" or treated samples was normally carried out after diluting them 1:6 by adding 2 millilitres tris-borate buffer at pH 8.7 and 3 millilitres 9M urea or by diluting 1:12 and applying 0.05 ml. or 0.10 ml. to the large pore gel surface.

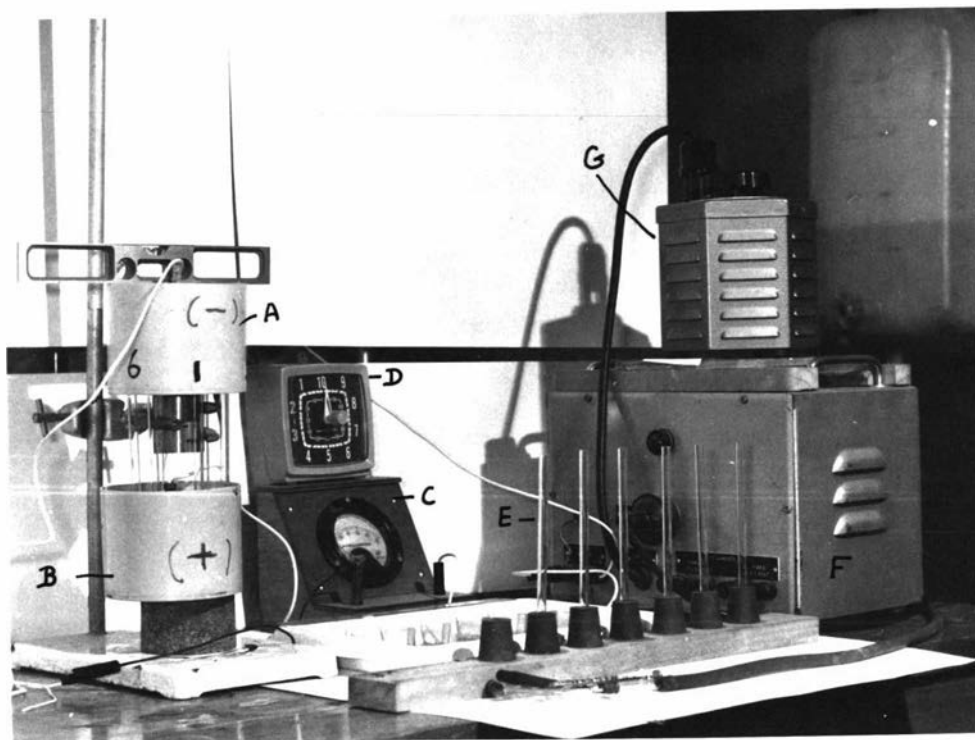


PLATE 23: Equipment used in Polyacrylamide Gel Electrophoresis

- A. Cathode compartment containing Tris-borate buffer, pH 8.7
- B. Anode compartment containing Tris-borate buffer, pH 8.7
- C. Ammeter (milliamps)
- D. Time clock
- E. Glass tubes for setting gel solutions
- F. D.C. power stabilizer
- G. Voltage control (VARIAC)

For an initial period of 20 minutes, a current of 2 ma/tube was applied (total 12 ma) after which it was increased to 3 ma/tube (total 18 ma) for the rest of the electrophoresis period, lasting usually about 2 hours or until the brom phenol blue front had moved a distance of 80 mm from the small pore gel surface. The gels were removed and stained for about 45 minutes after which destaining was carried out by several changes of 7% acetic acid solution using a gently oscillating washing tank. At the end of the washing period of about 48 hours, the gels were transferred to test tubes containing 3% acetic acid. Under these conditions they can be stored for months without change.

SECTION B: DETERMINATION OF THE SULPHYDRYL AND DISULPHIDE GROUPS
 IN CASEIN.

Experimental, results and discussion.

This has been fully dealt with in the attached publication. Because of the controversy surrounding the nature of the sulphur containing amino acids cystine and cysteine, it was thought advisable to investigate the matter prior to investigating the effects of disulphide splitting and sulphydryl blocking agents on syneresis. As indicated in the publication, acid precipitated whole casein was found to contain both cysteine (1.36 moles/100,000 g) and cystine (0.71 moles/100,000g). On the assumption that these amino acids were concentrated in the kappa-casein fraction, it would appear that 1 mole kappa-casein (molecular weight 20,000) would contain 2 moles of cysteine and 1 mole of cystine. The evidence available suggests that the sulphydryl residues of the cysteine moiety are buried within the molecule and that they are not readily available for reaction. On the other hand, the cystine appears to be on or near the surface and is readily available for exchange reactions.

Sulphydryl and disulphide groups in casein

BY G. M. WALLACE AND K. R. AIYAR

Food Technology Department, Massey University, Palmerston North, New Zealand

(Received 30 August 1968)

SUMMARY. The method of Gaitonde (1967) was adapted for the measurement of cysteine and cystine in acid hydrolysates. Analysis of 3 different casein preparations indicated the presence of both cysteine and cystine in whole casein in the proportions of 1:1 by weight and in the molecular ratio of 2:1. If all the cysteine and cystine is associated with κ -casein then it appears that one mole of the latter contains 2 moles of cysteine and 1 mole of cystine. The sulphydryl group in whole casein appears not to be highly labile.

The question of whether cysteine or cystine, or both occur in casein has been a subject of investigation for many years and is still a subject of controversy (McKenzie, 1967). Early work suggested the presence of cystine only, e.g. Kassel & Brand (1938); Gordon, Semelt, Cable & Morris (1949); Zweig & Block (1953); Hipp, Basch & Gordon (1961); Yoshino, Wilson & Herreid (1962); Waugh (1961). Christ (1956) was unable to detect any sulphydryls in casein but postulated that -SH groups were involved in the syneresis of rennet-casein gels. However, Beeby (1964) showed the presence of sulphydryl groups in freshly prepared κ -casein, the estimation being done at a high pH value (9.0) in strongly disaggregating conditions and in the absence of calcium ions. It appeared from his work that calcium and/or hydrophobic bonding masked the sulphydryl groups from -SH reagents. Hill (1964), working on whole casein and using more drastic methods for its disaggregation (e.g. proteolysis with Pronase), observed the presence of cysteine only. Subsequently, MacKinlay & Wake (1964) submitted evidence that contradicted the findings of Beeby (1964) and Hill (1964) and, later, Nakai, Wilson & Herreid, (1965) were also unable to substantiate the findings. The present paper describes a study of the cysteine/cystine balance in whole, acid precipitated casein. The work was undertaken as part of an investigation into the factors contributing to the syneresis of rennet-milk gels.

EXPERIMENTAL

All chemicals used were of A.R. grade and only glass-distilled water was used.

Preparation of acid casein

Three different casein preparations were made: (a), from freshly collected raw milk; (b) from industrially separated skim-milk; (c) from industrial acid casein. The methods of preparation were as follows:

(a) Bulk raw milk of good bacteriological quality was obtained from the Massey University dairy herd. It was cooled to 10 °C and the fat was removed by centrifugation in polyethylene containers. The skim-milk was bulked in a polyethylene bucket, the temperature having risen to 14 °C. Acid precipitation of the casein at pH 4.5 was carried out with a Radiometer Automatic Titration apparatus using *N*-HCl, care being taken to ensure adequate mixing. The fine precipitate obtained on centrifuging the slurry was washed twice with distilled water, using centrifugation to recover the precipitate. The washed precipitate was then dissolved in distilled water by addition of *N*-NaOH to pH 8.0 and reprecipitated by addition of *N*-HCl to pH 4.6. The Automatic Titrator was used for all pH adjustments. After thorough washing with distilled water, the final slurry was frozen at -15 °C and freeze-dried in an accelerated freeze-drying apparatus. The freeze-dried casein was stored at -15 °C. During the additions of acid and alkali the skim-milk was agitated by means of an electrically driven stirrer and to avoid aeration and minimize possible oxidation of sulphydryl groups in the casein, oxygen-free nitrogen was passed through 2 diffuser heads placed in the bottom of the bucket. The rate of nitrogen diffusion was adjusted to give a gentle bubbling over the entire liquid surface.

(b) Skim-milk was obtained from the Palmerston North Milk Treatment Station. The milk had been heated to about 48–50 °C for the separation in a conventional separator. Casein precipitation was done at 25 °C using the Automatic Titrator, following the same procedure as for casein (a) except that nitrogen was not bubbled through during the precipitation and solubilization processes. There was, therefore, ample opportunity for oxidation of the sulphydryls to occur, both at the Milk Treatment Station and during the isolation of the casein in the laboratory. This casein (casein (b)) was freeze-dried and stored at room temperature.

(c) Wet lactic casein was obtained from a commercial casein factory. Although the detailed history of its preparation is not known a standard commercial process was followed. The pressed curd was freeze-dried and stored at room temperature.

The 3 casein preparations (a), (b), (c) had, therefore, widely differing pretreatments, the most drastic treatment being suffered by casein (c).

Hydrolysis of casein and estimation of sulphydryls and disulphides in the hydrolysate

Hydrolysis. 2.5 g casein was hydrolysed under reflux with a 1:1 (v/v) mixture of 98% formic acid and concentrated HCl for 7 h at 105 °C. During the digestion oxygen-free nitrogen was bubbled through the digest. The mixture was approximately 6 *N* in HCl. It is important to have this high concentration of HCl in the mixture to reduce the hydrolysis time. Other investigators (e.g. Block & Bolling, 1940) have recommended a 1:1 (v/v) mixture of 6 *N*-HCl and 90% formic acid giving a final HCl normality of 3 *N* in the mixture. However, the digestion time has to be prolonged (16 h) and some destruction of cystine and cysteine takes place. Thus, when the mixture was 3 *N* in HCl acid, total SH + SS content was between 260 and 300 mg/100 g casein whereas with 6 *N*-HCl the digestion time was reduced to 7 h and total SH + SS content was significantly higher (see Results). A preliminary experiment using 6 *N*-HCl in the HCOOH-HCl mixture showed (Fig. 1) that about 80% of each of these amino acids was liberated in 2 h. (cf. Leach, 1966).

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Before heating, oxygen-free nitrogen was passed through the digestion mixture for 10 min for deaeration. The hydrolysate was transferred to a 200-ml volumetric flask and cooled immediately with running water. Twenty ml of 5 N-NaOH was then added, drop by drop, to the flask which was swirled in a bath containing water at room temperature. This procedure was essential for the removal of heat generated by the addition of the alkali and to avoid local concentrations of the alkali, which otherwise tended to destroy the sulphur amino acids. The mixture was then made up to 200 ml. The addition of the alkali raised the pH to 3.7–4.0, which allowed the precipitation of humin material and its removal by filtration using Whatman no. 540 filter paper. The clear filtrate was used for measuring cysteine and cystine levels.

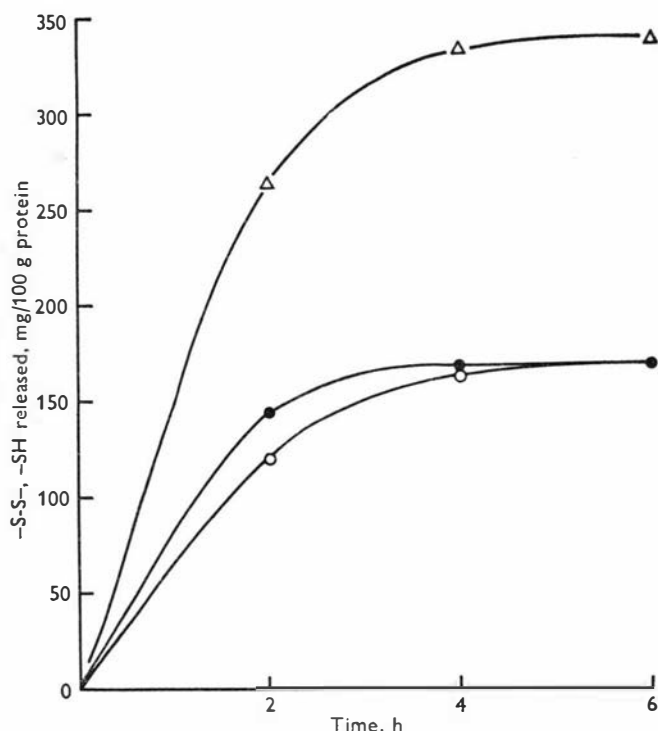


Fig. 1. Rate of release of cystine and cysteine during acid hydrolysis of whole casein, $\bigcirc$ — $\bigcirc$, cystine; $\bullet$ — $\bullet$, cysteine; $\triangle$ — $\triangle$, cystine + cysteine.

Determination of cysteine. The spectrophotometric method of Gaitonde (1967), with some minor modifications, was used to determine the cysteine quantitatively. To 1 ml of the digest filtrate in a test tube were added in turn 1 ml glacial acetic acid and 1 ml ninhydrin reagent (250 mg ninhydrin dissolved in 6 ml glacial acetic acid and 4 ml concentrated HCl). The mixture was heated for 8 min in a boiling water bath and cooled immediately and 10 ml ethanol was added. The pink colour was measured at 575 nm, the absorption maximum, in a Beckman DU Spectrophotometer using as a blank a similarly treated sample to which no ninhydrin had been added.

Determination of cystine. To 20 ml of the digest filtrate were added, drop by drop, 5 N-NaOH (4-6 ml) to adjust the pH value to 5.0. During the addition the mixture was agitated and cooled in a water bath to avoid local concentration of the alkali and to dissipate the heat quickly. Excess sodium sulphite (about 500 mg) was added as a solid and the mixture made to 40 ml with distilled water. The final pH value of the solution lay between 6.5 and 7.0.

The mixture was left at room temperature until reduction was complete (1 h) and then 1 ml was measured as described above for cysteine.

Calibration curve

To 39.4 mg chromatographically pure cysteine hydrochloride (B.D.H. Ltd) were added 50 ml of a 1:1 (v/v) mixture of 98% formic acid and 6 N-HCl. The resulting solution was diluted to 250 ml in a volumetric flask. The cysteine content of this solution was taken as 1 μ mole/ml. Further dilutions of this cysteine solution were made using 1:5 dilution of the 1:1 HCl:HCOOH as diluent. Solutions prepared in this way contained: 1.0, 0.75, 0.5, 0.25 and 0.125 μ mole/ml of cysteine. The plot of optical density against concentration was linear and passed through the origin.

Because of the protective effect of formic acid against metal catalysed oxidative destruction of cysteine (Haurowitz, 1963), the solutions of pure cysteine used for calibration, were made up in HCl-formic acid mixtures. These solutions were stable for at least 48 h.

Table 1. *Cysteine and cystine content of 3 different casein preparations*

(All values in mg/100 g dry protein.)

Sample		Cysteine	Cysteine after reduction	Cystine by difference
Casein (a)	1	165	338	173
	2	165	335	170
	3	155	328	173
	4	165	338	173
	Mean	163	335	172
Casein (b)	1	165	347	182
	2	174	337	163
	3	165	328	163
	4	165	335	170
	Mean	167	337	170
Casein (c)	1	165	338	173
	2	165	328	163
	Mean	165	333	168

RESULTS

Cysteine and cystine contents of 3 different casein preparations

The cysteine and cystine content of the 3 casein preparations described earlier are given in Table 1.

Table 1 demonstrates remarkably similar cysteine values obtained both before and

after reduction for the 3 casein preparations, despite the different methods of preparation. Accepting the claim of Gaitonde (1967) that the method in the form used is specific for cysteine, then the increased value after the reduction process could presumably only arise as a result of the reduction of cystine to cysteine. It is thus possible, indirectly, to measure the cystine content of the system.

Effects of variables on the recovery and estimation of cysteine and cystine

A check was made to see whether the pure amino acid cystine was being converted to cysteine under the conditions of hydrolysis used for casein.

About 25 mg cystine (chromatographically homogeneous, B.D.H. Ltd) was dissolved in a 1:1 mixture of 98% formic acid and concentrated HCl; the volume was made to 25 ml in a volumetric flask. Ten ml of this solution, containing about 10 mg cystine, was digested with 25 ml of the 1:1 (v/v) HCOOH:Conc. HCl mixture and refluxed for 7 h at 105 °C under a stream of oxygen-free nitrogen, as for casein. Cysteine, and cysteine-after-reduction, were then determined as described above, the digest having been diluted to 100 ml with distilled water. A second portion of 10 ml of the cystine solution was mixed with 25 ml of the 1:1 acid mixture and diluted to 100 ml with distilled water. It was tested immediately for cysteine and cysteine-after-reduction, omitting the heating at 105 °C. Results are given in Table 2. The results show that the conditions of hydrolysis did not convert cystine to cysteine. The approximately 9% cysteine equivalent was probably cysteine contamination present in the original sample as digestion for 7 h with HCOOH:HCl acid mixture at 105 °C caused no further conversion of cystine to cysteine.

Table 2. *Recovery and estimation of cystine*

(All values in mg/10 ml of original solution.)

Treatment	Cysteine	Cysteine after reduction	Cysteine in sample, %	Cystine, %
Unheated	0.99	9.60	9.3	90.7
Heated	0.97	9.84	9.0	91.0

A further check was made to establish whether cystine was reduced to cysteine in the presence of casein under the hydrolysis conditions employed and in the presence of humins. To 1.5 g casein were added 25 μ moles of cystine, and the mixture was hydrolysed under the standard conditions. The values for cysteine and cysteine-after-reduction were determined on the hydrolysate as described above. The results are shown in Table 3. These results indicate that there was (100+)% recovery of the added cystine. An increase of 4 μ moles of cysteine probably represents the cysteine impurity in the cystine mentioned earlier (Table 2), the level being of the same order.

The reduction of cystine to cysteine under the conditions used, therefore, does not appear to be a significant source of error. It is apparent also that oxidation of cysteine to cystine did not occur during the digestion as the level of cysteine contamination was similar in both heated and unheated cystine solutions (Tables 2 and 3).

To establish whether the cysteine and cystine levels found in the caseins were invariant and hence possibly the consequence of some equilibrium state, casein was

digested in the presence of thioglycollic acid. To 2.5 g of casein (*a*) were added 100 μ moles thioglycollic acid and the mixture was hydrolysed under the standard conditions. The values for cysteine and cysteine-after-reduction were determined on the hydrolysate as before and are given in Table 4. In the presence of 100 μ moles thioglycollic acid the mean value for cysteine-after-reduction was the same as that reported in Table 1, but the value for cysteine at 204 mg/100 g dry protein showed a 25 % increase. The thioglycollic acid thus appears to have caused some reduction of the cystine during the digestion.

Table 3. *Recovery of added cystine after digestion in the presence of casein*

(All values in μ M/1.5 g casein.)

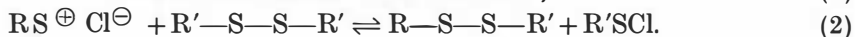
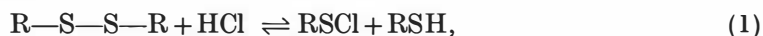
No.	Sample	Cysteine	Cysteine after reduction	Cysteine in cystine, %
1	1.5 g casein after digestion	20	40	—
2	1.5 g. casein plus 25 μ M cystine			
	Before digestion	4	40	10
3	After digestion	24	92	—
4	Difference (3-1) representing the recovery of 25 μ M added cystine	4	52	7.7

Table 4. *Casein hydrolysis in presence of 100 μ M thioglycollic acid*

(All values in mg/100 g dry protein.)

Sample		Cysteine	Cysteine after reduction	Cystine by difference
Casein (<i>a</i>)	1	204	329	125
	2	204	329	125
	3	204	348	144
	Mean	204	335	131

It has been reported (Benesch & Benesch, 1958) that disulphide exchange can take place in strong HCl (7 N-12 N) through an electrophilic attack of a sulphenium cation (RS^+) on the disulphide bond, the sulphenium ion being produced by the acid acting on the disulphide, thus:



Thus, there seemed a possibility that cystine values could have been reduced by the hydrolysis system used. This acid catalysed exchange reaction should be inhibited by the addition of mercaptans as reaction (1) is reversed and the concentration of sulphenyl chloride reduced. Theoretically, therefore, if the hydrolyses were to be carried out in the presence of thioglycollic acid or any other mercaptan, the concentration of cystine should either remain the same or increase if in fact cystine was being converted to its sulphenium chloride in previous experiments.

The (100 +) % recovery of added cystine recorded in Table 2 and the drop in cystine due to the reducing action of the added thioglycollic acid reported in Table 4, clearly eliminate any possible interference by the sulphenium chloride reaction in the cysteine-cystine balance in casein during digestion.

From all the foregoing it is reasonable to infer that during casein hydrolysis no artifact or equilibrium shift of significance was produced from the cysteine or cystine or through cysteine-cystine interconversion.

DISCUSSION

Acid hydrolysis of proteins is generally believed to cause a certain amount of destruction of the sulphur amino acids cysteine and cystine (Haurowitz, 1963). The destruction is particularly severe in the digestion of glycoproteins, and is apparently caused by the carbohydrate breakdown products (Block & Bolling, 1945). The destruction can be reduced by the use of formic acid and by exclusion of oxygen during the digestion (Haurowitz, 1963). Furthermore, acid conditions below pH 2.0 inhibit air oxidation of cysteine as well as cystine reduction through the presence of thiols or mercaptans. (Leach, 1966).

As indicated on p. 120, the conditions used in the present study did not appear to affect the total recovery of added cysteine and cystine or the balance of the two.

In this study, cystine was determined as cysteine-after-reduction in solution or in the hydrolysate with sodium sulphite. One mole cystine was found to be equivalent to 2 moles cysteine when measured by the method used. It would appear that the colour reaction with ninhydrin below pH 1.0 does not involve the sulphydryl group, because other sulphydryl-containing compounds do not respond similarly, e.g. thio-glycollic acid. Gaitonde (1967) tested a whole range of compounds, including —SH compounds and amino acids, and showed that the colour reaction was specific for cysteine under the conditions of the test and that the presence or absence of other amino acids had no effect on the specificity of the test. The increase in cysteine found after the reduction with sodium sulphite in near neutral solution could thus only have arisen through reduction of cystine.

The cysteine plus cystine content of 335 mg/100 g protein found for the 3 caseins is of the same order as figures reported for either cysteine alone (300 + mg, Hill, 1964) or cystine alone (340 mg, Gordon & Whittier, 1965). Our results indicate the presence of both cysteine and cystine in a 1 : 1 ratio by weight and so differ from results reported by Nakai *et al.* (1965), Beeby (1964), and Hill (1964).

Hill (1964) used Pronase to open up the structure of whole casein but he gave no indication whether Pronase affected the disulphide bond in any way at all. However, Davidson & Hird (1967) report the possibility of SH—SS exchange reactions during limited proteolysis. Hill found that when the casein was digested with Pronase alone he obtained a much lower sulphydryl content than when the Pronase digest had been reduced with sulphite or NaBH₄ or treated with cadmium hydroxide. He ascribed this to the sulphydryl being so strongly protected that even Pronase digestion does not fully expose the sulphydryls. However, study of table 2 of Hill (1964) shows the following: 'The average of the moles cysteine/100 000 g protein figures in column 2 (Pronase digest, pH 8.0) works out to 1.27. The average of the values in columns 3, 4 and 5 (sulphite, NaBH₄ and Cd(OH)₂ treatment) works out to 2.51 moles cysteine/100 000 g. We would argue that this difference (2.51 - 1.27 = 1.24 moles) is due to conversion of the disulphide to the sulphydryl as a consequence of the reductive treatments given and not due to simple disaggregation and exposure of more —SH groups.'

We have shown that sulphite reduces disulphide quantitatively to -SH. This difference of 1.24 mole of cysteine could thus be equivalent to 0.62 mole of cystine giving 1.27 moles cysteine and 0.62 mole of cystine/100 000 g casein. From the figures reported in this investigation (165 mg cysteine and 170 mg cystine/100 g casein) the moles/100 000 g casein work out to 1.36 moles cysteine and 0.71 mole cystine, respectively.

This re-calculation of Hill's data provides valuable confirmation of our findings.

If it is assumed that the cysteine and cystine are mostly, if not all, in the κ -casein fraction of whole casein (Waugh *et al.* 1960; Hill, 1964) then 0.71 mole cystine/100 000 g whole casein may be written as 0.71 mole/15 000 g κ -casein, assuming that κ -casein comprises 15% of whole casein (Waugh & von Hippel, 1956). This is roughly equivalent to 1 mole cystine/21 000 g κ -casein. Swaisgood, Brunner & Lillevik (1964) report the presence of $1-1\frac{1}{2}$ mole of cystine/28 000 g κ -casein and Nakai *et al.* (1965) report 1 mole of cystine/20 000 g κ -casein, which is in reasonable agreement with cystine figures reported in this work. Neither reported the presence of cysteine in casein. From a similar calculation, it appears that the concentration of cysteine is equivalent to 2 moles/22 000 g κ -casein. As the molecular weight of κ -casein is about 20 000 (see Jolles, 1966) it appears that one mole of κ -casein contains 2 moles of cysteine and 1 mole of cystine. Hill (1964) was not able to detect sulphydryls of whole casein, even under strongly dissociative conditions and did so only after partially digesting with Pronase. Thus, it appears that the sulphydryl groups are well protected in whole casein. Evidence for the structural protection of sulphydryls in other proteins is also available (Cecil, 1963). Several sulphydryl dependent aggregations, as distinct from SH-SS- interchange reactions, have been reported in the literature (Hughes, 1947; Madsen & Cori, 1956; Deutsch & Morton, 1958; Jensen, 1959) and a similar type of sulphydryl dependent aggregation in whole casein cannot be ruled out.

The fact that 3 different casein preparations used in this study gave similar results indicates that ordinary processing does not affect the sulphydryl-disulphide balance of whole casein. The sulphydryl group may not be as labile as is commonly believed.

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SECTION C: A STUDY OF THE EFFECT OF SODIUM THIOLYCOLLATE ON
GELATION AND SYNERESIS OF CALCIUM CASEINATE - CALCIUM
PHOSPHATE SYSTEMS.

1. Effect of adding Sodium Thioglycollate, after Rennet Action
on Casein at 4°C, on Gelation and Syneresis at pH 6.8 and 38°C.

(a) Experimental

"Artificial milk" containing 100 mg% calcium and 55 mg%
'P' as H_3PO_4 was prepared as described under Chapter II:A:2.
The "milk" was prepared in bulk and dispensed into beakers
in 40 ml. quantities. The "milk" samples were chilled to 4°C
and then reacted with 2 drops of neat commercial rennet for
60 minutes. The samples, except for the control, were then
reacted with 10 mg, 30 mg, and 100 mg of sodium thioglycollate
at 4°C for 30 minutes after which the beakers were transferred
to a water bath maintained at 38°C. The whey expelled at
various time intervals was collected and its volume
measured.

(b) Results

Results are given in Table IX.

Table IX. Effect of Adding Sodium Thioglycollate, after Rennet Action at 4°C, on Gelation and Syneresis of Calcium Caseinate - Calcium Phosphate Systems at pH 6.8 and 38°C.

Samples	A	B	C	D
Sodium thioglycollate added (mg)	Control	10	30	100
pH of the "milk" system	6.8	6.8	6.8	6.8
Gelation time (minutes) at 38°C	< 5	< 5	< 5	< 5
Syneresis, mls. whey/40 mls. curd after:				
60 minutes	3	6	4	2
80 minutes	4	9	6	4
100 minutes	5	12	8	7
120 minutes	7	14	11	9
17 hours	26	29	28	28
20 hours	30	32	31	31
Turbidity of whey	clear	+	+	++

Results from Table IX indicate that the presence of sodium thioglycollate does not inhibit gelation of the "artificial milks". The extent of syneresis, after 20 hours, is about the same for all samples but the initial rate of syneresis appeared to be faster for samples 'B' and 'C' containing 10 and 30 mg of sodium thioglycollate respectively, compared to the control and to sample 'D' containing 100 mg sodium thioglycollate. Because of the high levels of sodium thioglycollate used a sulphhydryl-disulphide interchange reaction does not appear to be involved in gelling or syneresis in this system. It has been found to be important in the gelling of egg albumin and serum albumin⁽¹⁶⁶⁾.

The wheys from samples B, C and D were noticeably more opaque than the whey from the control and the opacity increased with increasing levels of added sodium thioglycollate. This observation suggested that the wheys from samples to which sodium thioglycollate had been added contained increasing levels of protein which had not been bound by the curd. This was confirmed by the precipitation of protein from these wheys when the pH was adjusted to 4.6 with dilute acetic acid.

2. Effect of adding Sodium Thioglycollate on Gelation and Syneresis of Calcium Caseinate - Calcium Phosphate Systems under conditions of Gradual Reduction of pH and Increase in Temperature.

(a) Experimental

In this experiment an attempt was made to simulate conditions occurring during the cheesemaking process in regard to pH and temperature change.

"Milks" containing 3g casein, 100 mg% calcium and 55 mg% phosphorus as H_3PO_4 were prepared in bulk at pH 6.8 and dispensed in 40 ml. quantities as before. Sodium thioglycollate was added in somewhat greater quantities than those in experiment 1, (200, 300 and 400 mg). The addition was done at room temperature and prior to adding rennet. To one sample was added 200 mg NaCl for comparison. After a reaction time of 30 minutes, 160 mg glucono-acid-lactone was added to all samples, followed by 2 drops of neat commercial rennet at 30°C. When the "milks" had gelled the temperature was gradually raised from 30°C to 38°C in about 35 minutes. Whey expulsion was then measured.

Table X. **Effect of Sodium Thioglycollate on Gelation and Syneresis of Calcium Caseinate - Calcium Phosphate Systems under conditions of Gradual Reduction in pH and Increase in Temperature.**

Samples	A	B	C	D	E	F
Sodium thioglycollate added (mg)	Control	200	300	400	300 (pH control)	-
Glucono-acid-lactone (mg)	160	160	160	160	160	160
Sodium chloride (mg)	-	-	-	-	-	200
Initial pH of "milks"	6.8	6.8	6.8	6.8	6.8	6.8
Gelation time (minutes)	< 5	-	-	-	(no rennet)	-
Syneresis, mls whey/40 mls curd after:	Samples B, C, D and F did not form a gel. B, C and D gave precipitates in decreasing quantities after one hour. Supernatants were very milky.					
35 minutes	15				pH 5.9	
55 minutes	28				pH 5.7	
65 minutes	33				pH 5.5	
100 minutes	36				pH 5.4 (no ppt.)	
Turbidity of whey	clear	+	+	++	-	+

A control sample containing 160 mg glucono-acid-lactone and 300 mg sodium thioglycollate and treated similarly to others showed that the pH dropped gradually from 6.8 to 5.4 at the end of 100 minutes.

(b) Results

Results are given in Table X. (Page 82)

Results given in Table X indicate that sodium thioglycollate in the high concentrations used (4.4, 6.6 and 8.8 mM)* did not allow a gel structure to form and instead precipitates in decreasing quantities were formed after 1 hour at pH 5.4 with increasing levels of sodium thioglycollate. Correspondingly the turbidity of the supernatants increased. Sodium chloride at an equivalent concentration to the sodium thioglycollate level used in Sample D produced a similar, although slightly smaller, quantity of precipitate (F in Table X). The precipitates in all cases became noticeable after the pH had dropped to about 5.4. In Sample E, which contained no rennet but had both sodium thioglycollate and glucono-acid-lactone, there was no formation of a precipitate at pH 5.4. In a separate experiment samples were first reacted with rennet at 4°C for one hour and then 400 mg sodium thioglycollate per 40 mls. (8.8 mM)** was added. To one sample was added glucono-acid-lactone while the other was examined for syneresis at pH 6.8 and 30°C. Results showed that at pH 6.8 and 30°C no coagulation occurred in the absence of glucono-acid-lactone and after 60 hours only slight destabilisation of the caseinate appeared to have taken place. The pH of the

* Read as 44, 66 and 88 mM

** Read as 88 mM.

sample containing glucone-acid-lactone had dropped to 5.5 within 60 minutes and this sample had then formed an appreciable precipitate. This suggests that the formation of the precipitate was the consequence of a pH effect. The isoelectric point of para-casein (rennet treated casein) is around pH 5.2 and may explain this observation. In another experiment 40 mls. commercial rennet was treated with 400 mg sodium thioglycollate for 1 hour at 30°C and then 2 drops were added to a "milk" sample. The "milk" clotted in normal time indicating that sodium thioglycollate had no effect on the rennet itself.

3. The Effect of Sodium Thioglycollate on the Gelation and Syneresis of Skim Milk at pH 6.7 and 38°C.

(a) Experimental

Skim milk was obtained from the local milk treatment station and dispensed into 100 ml. beakers in 40 ml. quantities. The samples were reacted with the required quantity of sodium thioglycollate for 30 minutes at 38°C and then 1 drop of commercial rennet was added. Two hours later the amount of whey expelled was measured.

(b) Results

Results are indicated in Table XI.

Table XI. The Effect of Sodium Thioglycollate on the Gelation and Syneresis of Skin Gels at pH 6.8 and 38°C.

Samples	A	B	C	D
Sodium thioglycollate added (mg)	Control	200	300	400
pH of skin milks	6.7	6.7	6.7	6.7
Gelation time (minutes)	< 10	< 15	< 15	< 15
Syneresis, mls.whey/40 mls. curd after 120 minutes	23	21	21	22
Turbidity of whey	clear	+	+	++

Results given in Table XI indicate that addition of sodium thioglycollate (4.4, 6.6 and 8.8 mM)* had little effect on the extent of syneresis at 38°C though the rate appeared to be a little slower. The gels appeared to be weaker with increasing concentration of sodium thioglycollate. Interestingly, at the 400 mg addition, sample D had gelled though admittedly appearing weaker than the rest. At this concentration of sodium thioglycollate "artificial milks" had not gelled at 30°C. This may be a temperature effect similar to Cheeseman's⁽⁷⁵⁾ observations wherein it was indicated that at an addition 17.1 mM of sodium chloride the system did not gel at room temperature but did so at 50°C.

* Read as 44, 66, 88 mM

SECTION D: EFFECT OF SULPHYDRYL BLOCKING AND DISULPHIDE SPLITTING AGENTS ON GELATION AND SYNERESIS OF "ARTIFICIAL MILK" SYSTEMS AT pH 6.0 AND 38°C.

1. Experimental

"Artificial milk" at pH 7.0, containing 100 mg% calcium but no phosphate and 3% casein, was prepared in bulk without finally adjusting to volume. It was divided into equal portions and each portion was treated separately with ^N-ethyl maleimide, methyl maleimide (sulphydryl blocking agents) and dithiothreitol (disulphide splitting agent)⁽¹³⁶⁾ for 30 minutes at room temperature. Later, the pH of all samples was adjusted to 6.0 by the addition of 0.1 N HCl. The volumes were then adjusted so that the "milks" contained 3% casein and 100 mg% calcium. 40 ml. lots of variously treated samples were then examined for gelation and syneresis as before, using 1 drop of 1:3 diluted commercial rennet for setting the "milks". None of the "milks" showed signs of destabilisation after reaction with the reagents.

2. Results

Results are given in Table XII.

Table XII. Effect of Sulphydryl Blocking and Disulphide Splitting Agents on Gelation and Syneresis of Calcium Caseinate Systems at pH 6.0 and 38°C.

Samples	A	B	C	D	E
Initial pH	7.0	7.0	7.0	7.0	7.0
Dithiothreitol added (mg)	Control	20	N11	N11	N11
Ethyl maleimide added (mg)	N11	N11	20	40	N11
Methyl maleimide added (mg)	N11	N11	N11	N11	20
Reaction time at room temperature (minutes)	30	30	30	30	30
Final adjusted pH	6.0	6.0	6.0	6.0	6.0
Gelation time (minutes)	< 3	< 3	< 3	< 3	< 3
Gel type	firm	precipitate	firm	firm	firm
Syneresis, mls whey/40 mls curd, after:					
15 minutes	10	milky	9	5	2
25 minutes	29	supernatant	28	29	26
35 minutes	33		32	33	32
90 minutes	36		36	36	36
120 minutes	36	37	36	36	36
Turbidity of whey	clear	milky (+++)	clear	clear	clear

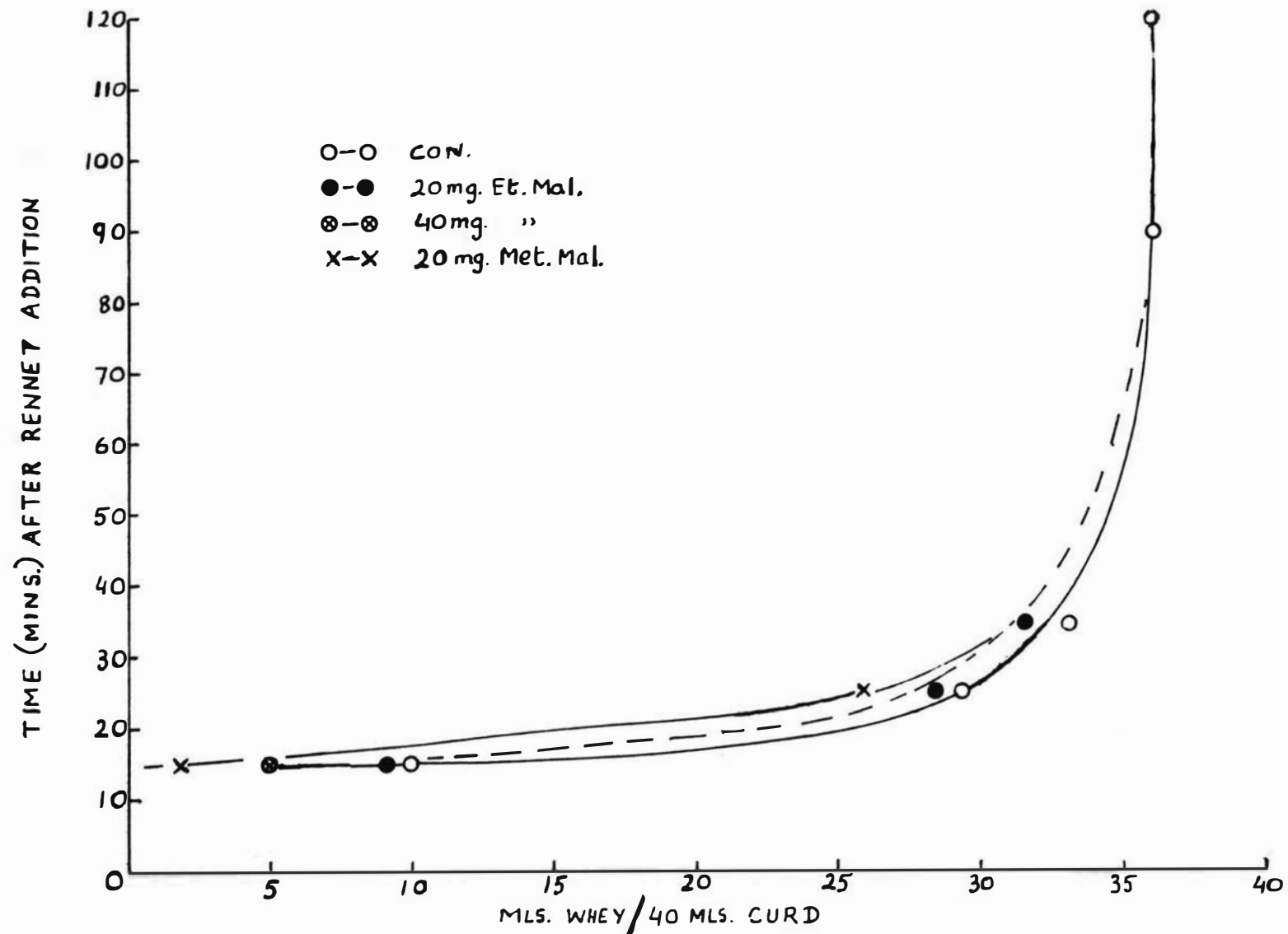


FIG. 6: SYNERESIS OF RENNET-CASEIN GELS IN THE PRESENCE OF -SH BLOCKING AND DISULPHIDE SPLITTING AGENTS, pH 6.0/38°C

Results given in Table XII and plotted in Figure 6 show that addition of sulphydryl blocking and disulphide splitting agents does not inhibit coagulation, that is, the primary phase of rennet action on casein micelles. They also do not appear to affect the overall rate and extent of syneresis of the calcium caseinate gels to any great extent. Samples containing the additives appear to start syneresizing at a slightly slower rate but this rate increases so that at the end of thirty five minutes the extent of syneresis was the same for all samples. Under the conditions of the experiment, the possibility of sulphydryl-disulphide interchange reactions occurring during gelation, as postulated by Rege⁽⁶³⁾, or during syneresis appears to be ruled out. There is no inhibitory action on clotting by the additives, in particular, by ethyl maleimide and methyl maleimide. It also appears that a sulphydryl group is not involved in the active centre of the enzyme.

It is significant to note that dithiothreitol did not destabilise the casein micelles in the "artificial milks". However, the renneted samples were unable to form a gel, that is, it interfered in the formation of a gel structure while allowing a precipitate to form almost immediately on renneting. The supernatant whey was very milky. This observation was similar to that observed with sodium thioglycollate (experiment 2), except that dithiothreitol appears to be much more effective than sodium thioglycollate at equivalent concentrations in the prevention of gel formation.

SECTION E: DISCUSSION ON THE EFFECT OF THIOL BLOCKING AND DISULPHIDE SPLITTING AGENTS ON THE GELATION AND SYNERESIS OF RENNET-CASEIN GELS.

Results from the preceding two sections do not bear out the postulates by Chuan⁽²²⁾ and Rege⁽⁶³⁾ that milk clotting was similar to blood clotting in which a sulphhydryl-disulphide chain reaction is involved. The sulphur amino acids, cystine and cysteine, are concentrated in kappa-casein⁽¹³⁷⁾ which is primarily associated with the surface of the micelle. It was shown earlier in this work (Electron microscopy - Plates 4 - 11) that gelation was essentially a surface phenomena of the casein micelles, but it is difficult to see sulphhydryl-disulphide interchange reaction occurring in the presence of sulphhydryl blocking agents such as N-ethylmaleimide or methyl maleimide. Also, the sulphhydryl group is well protected in whole casein⁽⁸²⁾⁽⁸³⁾ and becomes available only under the most drastic disaggregating conditions⁽⁸³⁾. On the other hand, Hill's work⁽⁸³⁾ suggests that the disulphide groups on whole casein are more readily available for reaction with external agents. It is unlikely, however, that a disulphide exchange reaction, as distinct from sulphhydryl-disulphide exchange reaction, would take place at about neutral pH or at pH 6.0, as this normally occurs only under strongly acid conditions⁽⁸²⁾. For similar reasons, it is unlikely that sulphhydryl-disulphide or disulphide exchange reactions play any significant role in the syneresis of rennet-casein gels as proposed by Christ⁽⁷³⁾ and Schober et al⁽¹¹³⁾.

The present work indicated that the clotting activity of rennet was not inhibited in the presence of sulphydryl blocking or disulphide splitting agents. This suggests that the sulphydryl group in rennin was unreactive and/or not involved in the active centre of the enzyme. Cheeseman⁽¹³⁸⁾ has recently come to a similar conclusion.

At low concentrations (10 - 30 mg), sodium thioglycollate appeared to increase the rate of syneresis at pH 6.8 and 38°C, but higher concentrations (excess of 100 mg) proved inhibitory and at a sufficiently high concentration (excess of 200 mg) gelation was inhibited. This may be attributed to the dispersive action of the sodium ions from sodium thioglycollate similar to that found for sodium chloride by Cheeseman⁽⁷⁵⁾ and Stoll⁽¹⁰⁴⁾. This possibly occurs by the displacement of calcium from the curd by the sodium ions through competition for binding sites. This dispersive action would also account for the turbid wheys obtained from sodium thioglycollate treated samples.

As noted in Section D, dithiothreitol was much more effective than sodium thioglycollate in low concentrations in preventing a gel from developing. Instead, rennet action gave a quickly settling precipitate and a milky supernatant within minutes after its addition. Dithiothreitol is an effective disulphide bond splitting agent⁽¹³⁶⁾ and would be more effective than sodium thioglycollate particularly since it possesses two -SH groups. The glycomacropeptide and the sulphur amino acid cystine appear to be located at distant portions of the kappa-casein molecule⁽³⁾⁽⁹¹⁾. If sodium thioglycollate or dithiothreitol split the disulphide bond prior to rennet action, this would not necessarily lead to micelle destabilisation. Destabilisation was not, in fact, observed in this work, the "milks" being quite stable hours after the addition of the reagents. The highly hydrophilic

moiety of the kappa-casein could still be attached to the micelle, conferring stability to the system despite the disulphide bond scission.

On the other hand, with para-kappa-casein, scission of the disulphide bond may lead to the release of a fraction of the para-kappa-casein into solution or it may lead to its modification. In either case, the casein micellar system loses its ability to form a gel. This might suggest that the para-kappa-casein on the micelle surface takes part in the formation of a gel structure.

In summary, disulphide splitting agents (1) do not destabilise unrenneted "milks", (2) inhibit gel formation in renneted "milks" but allow the formation of precipitates with appreciable release of acid precipitable protein into solution, (3) at lower levels (below 100 mg sodium thioglycollate) have no effect on the ultimate extent of syneresis when gels can be formed. They do modify the initial rates of syneresis.

They, therefore, appear to be without observed effect prior to rennet action. They also appear to modify the ability of the micelle surface to form the open network structure necessary for gel formation. Sulphydryl and disulphide groups on the caseinate do not appear to be directly involved in syneresis.

CHAPTER V

A Study of the Effect of Calcium on the Gelation and Syneresis of Caseinate Systems

SECTION A: THE EFFECT OF CALCIUM ON THE GELATION AND SYNERESIS OF CASEIN SYSTEMS IN THE PRESENCE OF DEVELOPING ACIDITY BUT IN THE ABSENCE OF RENNET ACTION ON CASEIN.

1. Experimental

"Artificial milk" at pH 6.8 containing 3g casein, 100 mg% calcium and 55 mg% 'P' as H_3PO_4 was prepared as described earlier. A sodium caseinate solution at pH 6.8 was prepared in a similar manner. To 40 mls. of each at $30^{\circ}C$ was added 400 mg glucono-acid-lactone and the hydrolysis of the lactone was allowed to proceed under quiescent conditions. Temperature was maintained at $30^{\circ}C$ by means of a water bath.

Two extra samples for each solution were also incubated, one for determining the rate of pH change with time and the other for determining the gel point. The gel point was found by carefully picking up a small quantity of the liquid at the edge by means of a spatula and letting it run down the sides of the beaker. The gel point was taken as the time of first appearance of specks of curd and the pH of the control sample was immediately checked.

2. Results

Results are indicated in Table XIII.

Table XIII.

The Effect of Calcium on the Gelation and Syneresis of Casein Systems in the Presence of Developing Acidity and in the Absence of Rennet Action.

Sample	Time (minutes)										Syneresis mls. whey/ 40 mls. curd, after 48 hours at 30°C and pH 4.0
	0	10	25	30	50	70	75	105	120	330	
	Change of pH with time										
"Artificial milk" (Ca + P)											
+ 400mg G.A.L.	6.8	6.0	5.6	5.4	5.3	5.2	5.1	<u>4.9</u> *	4.8	4.0	Nil
Sodium caseinate											
+ 400mg G.A.L.	6.8	6.2	5.7	5.5	5.3	5.1	<u>4.9</u> **	4.6	4.4	4.0	Nil

Note: Figures underlined indicate gel point.

* weak gel.

** firm gel.

Results given in Table XIII show that, under the conditions of the experiment, there is no syneresis of isoelectric casein gels, irrespective of the presence or absence of calcium. Even after 48 hours at 30°C there was no signs of exudation of whey. A separate experiment in which "artificial milks" containing only calcium (100 mg%) and no phosphate were used gave similar results. The sodium caseinate solution which was greyish-opaque to start with gradually attained a "milky" appearance as the acidity developed in the system until below pH 5.0 when, visually, it matched the whiteness of the "artificial milk". This indicated the development of fine particles throughout the body of the system, which induced greater light scattering, (Plate 24, No.1 and 2). Both the "artificial milk" and the sodium caseinate system gelled at the same pH (4.9) but the sodium caseinate system reached this point much earlier at 75 minutes as against 105 minutes. This was probably due to the greater buffering ability of the "artificial milk" containing both calcium and phosphate. The final pH of 4.0 was, however, similar in both cases. It was noticeable that at the gel point of pH 4.9, the sodium caseinate system appeared to have a firmer gel than the "artificial milk" system, although at the lower pH's the difference was not so obvious. Both gels could be easily disaggregated after which they collapsed. This could be induced either by shaking the beaker or by dipping a spatula or glass rod into it.

The results indicate that it is not necessary for calcium to be present for the formation of an isoelectric casein gel, that is, a 3-dimensional network can form in the absence of calcium. Also, acidity by itself or in the presence of calcium and/or phosphate does not cause syneresis of casein gels even at low pH.

The forces of attraction between the protein particles thus appear to be only just sufficiently strong to "immobilise" the liquid phase within the 3-dimensional network but are not strong enough to shrink the gel and expel the whey.

SECTION B: THE EFFECT OF CALCIUM ON THE GELATION AND SYNERESIS
OF RENNET-TREATED CASEIN SYSTEMS IN THE PRESENCE OF
DEVELOPING ACIDITY.

1. Experimental

"Artificial milks" at pH 6.8, containing 3g casein, 100 mg% 'Ca', 55 mg% 'P', and sodium caseinate solution (pH 6.8) was prepared as before. 40 ml. portions were taken in 100 ml. beakers and placed in water bath at 30°C. 400 mg glucono-acid-lactone followed by 1 drop of 1:1 diluted commercial rennet was then added to the samples, which were examined for gel formation and syneresis. As before, duplicate controls were set up for each sample, one for measuring pH at various time intervals and the other for determining the gel point.

2. Results

Results are indicated in Table XIV.

Table XIV. The Effect of Calcium on the Gelation and Syneresis of Rennet-treated Casein Systems in the presence of Developing Acidity.

Sample	TIME (minutes)								Syneresis mls. whey/40 mls. curd at 30°C and pH 4.0	
	0	3	8	25	60	80	105	180	2 hours	48 hours
Change of pH with time										
"Artificial milk" (Ca + P) + 400 mg G.A.L. + rennet/30°C	6.8	firm gel	6.0	5.6*	5.0	4.7	4.5**	4.0	36	-
Sodium caseinate + 400 mg G.A.L. rennet/30°C	6.8	-	6.1	5.7	<u>5.2</u>	4.8	4.6	4.1	nil	nil

Note: Figures underlined indicate gel point.

* syneresis well under way.

** syneresis virtually completed.

"Artificial milks" containing only calcium behaved similarly although they syneresed at a faster rate than when phosphate was also present.

Results given in Table XIV show that rennet conversion of casein to para-casein alone is not sufficient to give syneresis even if the pH is lowered well below the isoelectric point. Both rennet action and the presence of calcium appear to be necessary for syneresis, (Plate 24, No. 3 and 4)*. Proteins are regarded as having a minimum water holding capacity at their isoelectric points. However, the absence of syneresis in the gels, observed in the previous section (Table XIII) and in the rennet-treated sodium caseinate system observed here, indicates that the type of gel formed and the forces involved in the gel structure are more important in syneresis than the adjustment to the isoelectric point by the development of acidity. The fact that renneted "artificial milks" containing calcium as distinct from the sodium caseinate solutions clot at near neutrality indicates that the divalent calcium is involved in the cross links. Rennet-treated sodium caseinate, on the other hand, only gelled at a pH of 5.2 which is near the isoelectric point of para-casein indicating that weak van der Waal's or electrostatic forces of attraction are in operation giving a flexible 3-dimensional network, as appears to be the case also with isoelectric non-rennet-treated casein gels.

Results show that in rennet-treated caseinate systems calcium is not necessary to give a gel structure at low pH values, but that calcium is necessary for syneresis to occur and that pH is not a significant factor in the absence of calcium in syneresis. It is also significant to note that even in the presence of calcium there is no syneresis except after rennet action (Plate 24, No. 1 and 3). Thus both calcium and rennet modification of casein are essential for syneresis to occur.

* after page 94.

SECTION C: THE EFFECT OF CALCIUM ON THE GELATION AND SYNERESIS
OF RENNET-TREATED CASEIN SYSTEMS AT NEUTRAL pH.

1. Experimental

"Artificial milk" at pH 6.8 and containing 100 mg% 'Ca' and 55 mg% 'P' as H_3PO_4 and sodium caseinate solution at pH 6.8 was prepared and dispensed in the usual way. A drop of 1:1 diluted commercial rennet was added to the samples which were then examined for gel formation and syneresis.

2. Results

Results are indicated in Table XV.

Table XV. The Effect of Calcium on the Gelation and Syneresis of
Rennet-treated casein systems at neutral pH.

	TIME (minutes)				
	0	3	100	180	1200
Sample	Syneresis, mls. whey/40 mls. curd, with time				
"Artificial milk"					
(Ca + P) pH 6.8					
+ rennet/30°C	-	gel point	2	5	35 (completed)
Sodium caseinate					
pH 6.8					
+ rennet/30°C	_____ no gel formation _____				

Results given in Table XV (Plate 24, No.5 and 6) ^{*} show that in the absence of calcium, rennet-treated sodium caseinate does not gel at near neutral pH. In the absence of acidity the sodium caseinate system remains in suspension for long periods. The only change is the development of slight opacity after rennet has reacted with sodium caseinate, indicating that rennet action has not been inhibited by the lack of calcium. Addition of calcium ions to such a system, causes it to gel.

The "artificial milk" on the other hand gave a firm gel within 3 minutes of rennet addition. As compared to rennet-treated "milks" in presence of developing acidity (Table XIV) syneresis of the neutral "milk" gel is very much slower but it does occur and becomes complete in 20 hours as against less than 2 hours. Syneresis is thus essentially associated with the presence of calcium and the modification of casein by rennet action. This confirms the results of the previous section.

The results from these studies indicate that:

1. Gels will form but will not synerese when
 - (a) pH reaches isoelectric point for both non rennet-treated casein and rennet-treated casein in the absence of calcium,
 - (b) when pH reaches isoelectric point for non rennet-treated casein even in the presence of calcium.
2. Gels will not form
 - (a) at neutral pH with non rennet-treated casein in the presence or absence of calcium,
 - (b) at neutral pH with rennet-treated casein in the absence of calcium.
3. Gels will form and show syneretic behaviour
 - (a) at neutral or acid pH for rennet treated casein in the presence of calcium.

Therefore for gels to form and show syneretic behaviour the casein must have undergone a change induced by rennet enzymes, and calcium must be present. Acidity hastens the syneretic process as does temperature. Syneresis appears to be an inherent property of a gel formed under certain conditions. Gel formation in itself does not automatically infer that syneresis is to follow.

SECTION D: THE EFFECT OF VARIOUS LEVELS OF CALCIUM ON THE GELATION
AND SYNERESIS OF RENNET-CASEIN GELS AT pH 6.8 AND 30°C.

1. Experimental

(a) Preparation of calcium caseinate "artificial milks"
containing 60 - 200 mg% (w/v) calcium.

"Artificial milks" containing up to 125 mg% (w/v) calcium were prepared directly as described earlier at a pH of 6.8. For additions of calcium beyond the level of 125 mg% (w/v) the following procedure was evolved:

"Artificial milk" containing an equivalent of 125 mg% calcium was first prepared, without making to final volume. After complete solubilization of the caseinate the "milk" was held below 5° for at least 12 hours.

The "milk" was then warmed to 30°C and a calcium chloride solution containing 5 mg/ml calcium was added very slowly until the desired calcium level was reached. Concurrently, 0.2N NaOH was added to maintain the pH at 6.8. The "milk" was then made up to volume to contain 3% (w/v) casein, and the required percentage of calcium. After making to volume the "artificial milk" was allowed to equilibrate for at least an hour before syneretic studies were carried out. The addition of extra calcium, particularly in excess of 150 mg had to be very carefully carried out to avoid destabilisation of the caseinate. In many instances, such precipitation could have been avoided by simply building up the viscosity of the system to that of ordinary milk by the addition of gelatin or sodium alginate. However, these

substances might have affected gel characteristic and syneresis and were, therefore, not added.

(b) Adjustment of the electrical resistance (conductance) of the "artificial milks".

Since different levels of calcium were being used in the various "milks" differences in the ionic strengths might have affected the results. The ionic strength of the "artificial milks" was difficult to calculate since the amount of calcium bound to the caseinate could not be precisely evaluated. The electrical resistance of the systems was therefore measured using a Phillips conductivity meter. The values for the higher resistance "milks" were adjusted by the addition of small quantities of sodium chloride so that all the "artificial milks" were comparable with respect to their electrical conductivity. Thus to a 60 mg% (w/v) calcium "milk", 65 mg NaCl had to be added per 100 mls "milk" to adjust the electrical resistance to a value near that of a 150 mg% (w/v) calcium "milk".

(c) Measurement of syneresis.

The method followed was similar to that given under Chapter IV:A:3. The "milks" were incubated at 30°C, and one drop of a 1:10 diluted commercial rennet was added at staggered intervals. Duplicates were set up for each sample for measurement of the gel point.

2. Results

Results are indicated in Table XVI for two different experiments.

Table XVI. Effect of Various Levels of Calcium on Gelation and Syneresis of "Artificial Milks".

Sample No.	Calcium mg% (w/v)	Gelling time (minutes)	Gel type	Extent of Syneresis mls. whey/40 mls. curd at 30°C.			Turbidity of whey
				<u>90 minutes</u>		<u>180 minutes</u>	
				(a)	(b)	(Av)	
1	60	40	fragile	18	18	30	++
2	75	30	fragile	13	15	33	+
3	100	21	firmer than 1 and 2	11	13	33	+
4	125	18	firm	19	20	35	clear
5	150	26	firm	10	9	33	clear
6	175	26	less firm than 4 and 5	8	6	32	clear
7	200	28	fragile	14	13	35	clear

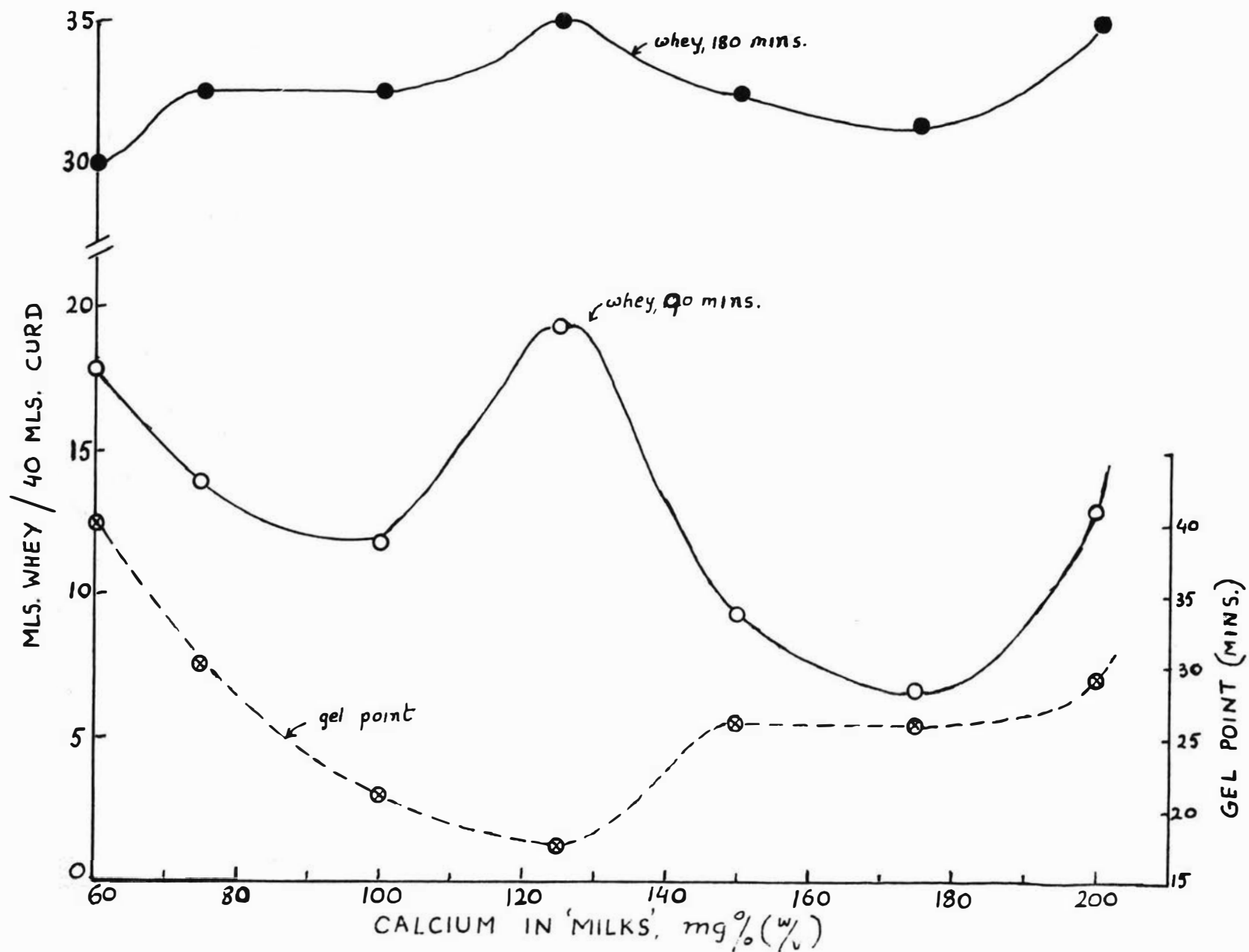


FIG. 7: EFFECT OF LEVEL OF CALCIUM ON GELATION AND SYNERESIS OF 'MILKS', pH 6.8/30°C

Results given in Table XVI and plotted in Figure 7 show that the amount of calcium in an "artificial milk" system affects the whey expulsion from the curd. At 90 minutes and 30°C, increasing the calcium from 60 mg% to 100 mg% reduces extent of syneresis by about 33% (average of a and b) taking the 60 mg% whey expulsion value of 18 mls. as equal to 100. Raising the calcium to 125 mg% (No.4) raises the extent of syneresis by 8% while a further increase in the amount of calcium to 175 mg% causes a sharp drop in the extent of syneresis by about 61%. Beyond 175 mg% calcium, the drop in the extent of syneresis is not as great being only about 22%.

After 180 minutes at 30°C the differences in effects due to calcium are very much reduced (Figure 7) but the general trend beyond the 100 mg% calcium level appears to be the same as when examined at 90 minutes. Both the 75 mg% and the 100 mg% calcium "milks", however, have synerised to a greater extent at 180 minutes than the 60 mg% calcium "milk" thus reversing the earlier trend.

The time to reach gel point drops by more than half when calcium is raised from 60 mg% to 125 mg%, thereafter increasing calcium raises the time to gel point. Minimum time to gel point occurs at 125 mg% calcium. Ernstrom⁽⁶⁰⁾ has reported that the minimum time to gel point with skim milk occurred at 142 mg% added calcium chloride.

SECTION E: DISCUSSION ON THE EFFECT OF CALCIUM ON THE GELATION
AND SYNERESIS OF CASEINATE SYSTEMS.

From the foregoing sections, it would appear that certain essentials are necessary in the casein gel for it to have syneretic properties. Thus, development of acidity to, and below, the isoelectric point to give a clot or 3-dimensional network under quiescent conditions was not sufficient in itself to give the curd syneretic properties. Such an acid developed gel, whether from normal or rennet-treated casein, was able to hold its structure for a long time in the absence of calcium unless deliberately broken. Even when broken, the curd had little tendency to mat. Such acid-developed gels showed little syneretic tendency even at higher temperatures such as 38°C.

Acid developed casein gels in the presence of calcium also showed little syneretic tendency unless the casein was first reacted with rennet. Rennet modified "artificial milks" containing calcium were able to synerese, although at a slower rate, at near neutral pH. This indicated that rennet modification of the caseinate and the presence of calcium were both necessary for syneresis to occur, but the role of acidity appears, in the main, to be one of hastening up the process of syneresis.

The forces involved in the cross links to give the 3-dimensional network in the two systems, that is rennet-calcium-gels and the isoelectric gels, must obviously be different.

In isoelectric gels, the developed acidity would reduce the negative character of the caseinate until at the gel point the system is electrically neutral. It does not necessarily follow that all the

proteins in the casein complex each have an equal number of positive and negative charges. Some may still be negative in character, and some possibly positive in character, depending on individual isoelectric points. The gel point simply signifies that overall balance has been achieved between the positive and negative charges, and gelation may occur at this point. As Katchalsky⁽¹¹¹⁾ has pointed out, the spatial arrangement of these charges on the molecules is important to give a 3-dimensional network, otherwise a precipitate rather than a gel would result. Little or no calcium is bound to casein below pH 5.0⁽⁶⁰⁾⁽⁶⁹⁾, therefore both the "artificial milks" containing calcium and the sodium caseinate systems would be expected to give similar types of gels at the isoelectric point. The caseins in the "artificial milks" at the isoelectric point would not be expected to be in their normal calcium stabilised micellar form. They would be in a dissociated state to a large extent. It would be reasonable to expect the presence, under low pH conditions, of large numbers of either monomers or small aggregates, which, when the system has become electrically neutral, would interact by weak van der Waals or electrostatic attractive forces to form a gel structure and thereby immobilise the whole liquid. Because of the very nature of these van der Waal's forces, the gel network would acquire a certain amount of flexibility. There would be little tendency to shrink with consequent whey expulsion. Such isoelectric gels would be termed fine under Ferry and Morrison's⁽¹⁰³⁾⁽¹⁰⁵⁾ classification, with the cohesive forces not much greater than is just necessary to maintain the network structure. Stoll⁽¹⁰⁴⁾ found that rennet-gels in the presence of calcium at and below pH 5.4 tended to be very weak, which is to be expected on the above hypothesis. Cheeseman's⁽⁷⁵⁾ results

indicate that rennet milk gels set below pH 5.3 retained more moisture than gels set at higher pH. This would be expected as below pH 5.3 little calcium would be bound to the network, the gel would resemble an isoelectric gel having little syneretic tendencies.

On the other hand, coagulation of the rennet-treated "artificial milks" at pH 6.8 appear to involve calcium in the cross links which give the 3-dimensional network, since rennet-treated sodium caseinate solutions do not gel at neutral pH. At near neutral pH, gelation would essentially occur by cross-linking between micelles, as was shown earlier in the Electron Micrographs (Plates 4 - 11). It is probable that, at the gel point, not all micelles or micellar strands are involved in the network, some would still be free. Flory⁽¹⁷⁶⁾ in a theoretical treatment of gelation proposes that there is minimal linking at the gel point but the extent of linking increases thereafter with time. It is possible that this increased linking brings about the syneretic behaviour at neutral pH of rennet-casein gels in the presence of calcium.

Development of acidity evidently acts as an aid in enhancing or speeding up the process possibly in such a way that the mobile structural elements of the gel are able to react with each other and with the rigid elements more profusely and quickly and in the process give rise to faster shrinkage. If acidity or low pH was absolutely essential for syneresis, then little or no whey expulsion should have occurred at the near neutral pH. In a rennet-casein system where acid is continually being formed, the micellar structure is probably gradually broken down and, as well, some of the calcium links involved in the original 3-dimensional network would disappear if they are ionic in character. The breakdown of the micellar nature of the system to smaller aggregates would lead to greater protein sub-unit concentration per unit volume and to creation

within the network of new attractive forces, both electrostatic, as repulsion would be reduced at the lower pH, and perhaps hydrogen and hydrophobic bonding. In such a case the gel system would be subjected to a very strong internal attractive force. This might explain the greatly increased rate of syneresis in the presence of developing acidity as against syneresis at pH 6.8; where syneretic forces are not as well developed. Increased temperature increases syneresis probably by increasing the rate of contacts of the mobile elements with each other and with the structural elements of the gel; and from possibly increased hydrophobic interactions within the gel network.

The results obtained on the effect of the various levels of calcium on the syneresis of rennet-casein gels at near neutral pH and 30°C is rather more difficult to interpret. It might be explained on the basis of modification of the charge on the caseinate through interaction of the protein with the calcium ions.

At low levels of calcium (62.5 mg%) the gels were noted to be fragile and collapsed at an early stage giving a false indication of greater syneresis. In such a system, a large proportion of the added calcium would be taken up in the formation of the micelles. Less, therefore, would be available for interaction between the rennet-treated micelles for gel formation. This might explain the observed fragility of the low calcium gels. The wheys obtained from such gels were opaque indicating that large amounts of protein were not enveloped within the network. When the calcium level was raised progressively to 125 mg%, the rigidity of the gels also increased noticeably and the clarity of the whey was considerably improved.

Raising the level of calcium in the "milks" up to the 125 mg% level maximises the rate of syneresis probably by allowing for greater

interaction between the elements of the gel structure. This may occur through the calcium acting as counter ion to the repulsive negative charges on the protein, reducing net charge to a minimum at near neutral pH.

Further increase in calcium up to the 175 mg% level was observed to give rise to a sharp decrease in syneresis. The gels were also noted to become increasingly fragile, although the wheys obtained remained clear. In this case it is possible that most, if not all, of the available negative charges on the caseinate complex have been neutralised by the added excess of calcium ions. The calcium ions themselves, however, would not be fully neutralised and would still bear a positive charge. If this assumption is valid, then the gel network would tend to acquire a net positive charge. This would induce swelling of the gel as opposed to the forces giving rise to shrinking. Syneresis, therefore, would be reduced. Katchalsky⁽¹¹¹⁾ in discussing the mechano-chemistry of myosin has stated that in the presence of polyvalent ions, especially calcium and magnesium, the cataphoretic picture of this protein is transformed. The molecular potential becomes positive at all pH values up to 9.0.

A concentration as low as 0.03 M of the divalent ions was stated to be sufficient to change the sign of the mobility at alkaline pH from negative to positive. The combination with divalent cations was proposed⁽¹¹¹⁾ to take place as follows:



Such a reaction may play a role in the caseinate systems above the 125 mg% level of added calcium in regulating the bonding possibilities by determining its molecular potential. A concentration of 0.03 M is equivalent to 120 mg% calcium. As noted earlier, syneresis was drastically reduced between the 125 mg% and 175 mg% added calcium levels.

A possible alternative explanation for the observed reduction in syneresis may be that it is due to osmotic pressure difference between ions within the gel and those outside. Katchalsky⁽¹¹¹⁾ has pointed out that in a non-isoelectric network the surplus of charges of one sign requires in the imbibed solvent an equivalent number of small free ions of the opposite sign to maintain electro-neutrality. The large number of small ions within the gel may give rise to a higher osmotic pressure relative to the gel environment. If this were the case, there would be a tendency for the gel to swell and thereby oppose syneretic forces. The rate of syneresis would then tend to be lower.

The fact that syneresis does occur in all rennet-casein systems containing various levels of calcium indicates that syneretic forces are stronger than forces resisting syneresis. Beyond the 175 mg% addition of calcium the gels were observed to be fragile and collapsed readily. Excess of added calcium probably caused a general destabilisation of the system and reduced water holding capacity of the gels.

Results obtained with disulphide splitting agents, reported earlier, had indicated that para-kappa-casein on the micelle surface may be involved in the development of a 3-dimensional network. This probably involves also calcium since it has been shown in this study that gel formation does not occur in the absence of calcium. Hill and Craker⁽⁴³⁾ had shown that modification of the epsilon amino group of lysine on rennin-altered kappa-casein prevented aggregation of the caseinate. Thus it seems possible that the epsilon amino group on para-kappa-casein on the micelle surface may be involved in the interactions with calcium to give rise to a gel structure. The

interaction with calcium may be similar to that postulated for e-amino groups or for imino groups by Baker and Saroff⁽⁷¹⁾ which involved the formation of co-ordinated covalent links.

The specific part that rennin plays in the syneretic process can only be conjectured at this point, but in view of the dehydrating effect of rennet demonstrated earlier, one possible explanation is that rennet action has disturbed sufficient of the stabilising "immobilised" water envelope around the casein micelles to permit the micellar strands to approach close enough to one another for some form of crosslinking through the calcium ions to be established.

At gel point, depending on conditions of gelation, it is possible that greater or lesser numbers of casein micelles remain which have yet to react with rennin. It is also possible that partially coagulated micelles in the form of "beaded" strands form the mobile structural elements within the gel at the gel point. Electron micrographs shown in Plates 4 - 11 and 17 - 21 provide some evidence that this is probable. The unsatisfied bonding potential of the mobile elements within the gel network might be expected to influence the rate and extent of syneresis. The greater the unsatisfied bonding potential the greater would be the tendency for the gel elements to increase their crosslinking and in doing so to contract or synerese given the right conditions.

CHAPTER VI

A Study of the Effect on the Gelation and Syneresis of Calcium-Caseinate "Milks" of the Addition of Different Amino Acids, of 1-fluoro- 2:4 Dinitrobenzene (F.D.N.B.), and of Heating in the Presence of Reducing Sugars.

SECTION A: THE EFFECT OF THE ADDITION OF DIFFERENT AMINO ACIDS ON THE GELATION AND SYNERESIS OF CALCIUM CASEINATE "MILKS".

1. Introduction

The effect of the addition of some amino acids on gelation and syneresis of rennet-casein systems was studied to find out whether modification of the dielectric constant of the medium affected these phenomena. Studies on water binding in sausages show that addition of glycine helps enormously to increase the water retention during processing, possibly through the increase in the dielectric constant of the medium by the added glycine⁽¹⁷⁵⁾. It was of interest, therefore, to study the effect of such additions of amino acids on the water retention property of rennet-casein gels. The quantities chosen for the addition were based upon doubling the content normally present in kappa-casein of the selected amino acid. It was assumed that the kappa-casein content was 15% of the weight of whole casein⁽¹³⁾. The amino acid composition used was that given by Jolles et al⁽⁴⁴⁾.

2. Experimental

"Artificial milk" at pH 6.8 containing an equivalent of 3% casein and 100 mg% w/v calcium was prepared in bulk as before, but without adjustment to volume. The "milk" was dispensed into beakers in equal quantities. One of the following additives was then added to each of the samples: L-lysine hydrochloride 13 mg ($\bar{=}$ 10.4 mg lysine), e-(2:4-dinitrophenyl) -L-lysine hydrochloride 26 mg ($\bar{=}$ 10.4 mg lysine), L-glutamic acid 33 mg, F.D.N.B.* - 30 mg. The pH of all the "milks" was adjusted to 6.8. All samples were then adjusted to volume (40 mls.). To 40 mls. "milk" at 30°C one drop of 1:10 diluted rennet was added and gel point and syneresis measured as before.

3. Results

Results are indicated in Table XVII.

Table XVII. Effect of Added Amino Acids on the Gelation and Syneresis of Calcium-caseinate "milks".

No.	Treatment	Final pH	Gel Point (min.)	Extent of Syneresis. mls. whey/40 mls. curd at 30°C/90 mins.
1	"Artificial milk" (CONTROL)	6.8	< 5	25
2	"Artificial milk" + 13 mg lys.HCl	6.8	< 5	22
3	"Artificial milk" + 26 mg e-D.N.P.-lysine	6.8	< 5	26
4	"Artificial milk" + 33 mg glutamic acid	6.8	< 5	25

* F.D.N.B. = 1-fluoro, 2:4 dinitrobenzene.

Results given in Table XVII indicate that, in general, addition of small quantities of the amino acids used do not significantly affect the water retention property of the curd, except perhaps for lysine-HCl which appears to have given a somewhat lower extent of syneresis. The addition of e-D.N.P. lysine has, however, had no inhibitory effect on syneresis. MacInnes⁽¹¹⁰⁾ has pointed out that addition of most non-amphoteric compounds to a medium decreases the dielectric constant. However, the addition of amphoteric compounds such as the amino acids which have a large dipole moment increases the dielectric constant of the medium. Even amongst the amphoteric compounds, the increase in the dielectric constant produced by one mole of the solute, that is the dielectric increment, increases linearly with the number of carbon atoms separating the amino and carboxylate groups. Thus lysine would have a much higher dielectric increment than glycine, or glutamic acid and so on. e-D.N.P. lysine, on the other hand, has its epsilon-NH₂ group blocked and would be expected to give lower dielectric increments than lysine itself. Increase in the dielectric constant would probably tend to keep a gel in a "swollen" state, as forces of attraction would be weakened. Thus, there is a possibility that changes have occurred in the dielectric constant of the water as a result of these small additions of amino acids and this explains the difference in extent of syneresis (15 - 20%) in the presence of added lysine HCl. Presumably the increment from the addition of the glutamic acid was too small to be significant.

SECTION B: THE EFFECT OF DINITROPHENYLATION ON THE GELATION AND SYNERESIS OF CALCIUM-CASEINATE SYSTEMS: THE RATE OF REACTION OF 1-FLUORO-2:4 DINITROBENZENE (F.D.N.B.) WITH CASEIN MICELLES IN CALCIUM-CASEINATE "MILKS" AT 30°C.

1. Introduction

It has been reported⁽⁴³⁾ that modification of the lysine side chain on kappa-casein inhibits coagulation of casein after reaction with rennet. It was, therefore, of interest to establish the effects of limited dinitrophenylation of the free amino groups on the gelling characteristics and syneresis of calcium-caseinate systems. The dinitrophenylation was carried out under conditions whereby the casein components were not expected to exist as separate entities but in the form of calcium induced micelles. The aim of using only small quantities of F.D.N.B. for the dinitrophenylation reaction was to limit the reaction specifically to the free amino groups of the caseinate complex. Preliminary experiments indicated that limited dinitrophenylation of the "milks" gave an unexpectedly low extent of syneresis when compared with controls. This was evident even at pH values below 6.0. Dinitrophenylation also appeared to be without observed effect on the rigidity of the rennet-casein gels.

2. Experimental

"Artificial milks" containing an equivalent of 3% casein and 100 mg% (w/v) calcium were prepared as before at pH 6.8.

After holding for 12 hours at 4°C, the "milks" were warmed to and held at 30°C for 30 minutes. The pH was then gradually taken to 8.4 and volumes adjusted. 24 mg F.D.N.B. per 100 mls. "milk" were then mixed in. The solution was kept stirred at a temperature of 30°C and pH measurements made at regular intervals. In a separate experiment "artificial milks" were treated with different levels of F.D.N.B. and the pH measured at the end of 1 hour.

3. Results

Results are indicated in Table XVIII.

Table XVIII. Rate of Reaction of F.D.N.B. with Calcium Caseinate in "Artificial Milks" at 30°C.

Time (mins.) after F.D.N.B. addition (24 mg/100 mls. milk)	Observed pH	Observed pH after 1 hour, using different levels of F.D.N.B. (mg/100 mls. "milk")					
		0	8	16	24	32	64(mg)
0	8.40						
10	8.20						
20	7.95						
30	7.70						
40	7.55						
50	7.45						
60	7.40	8.0	7.7	7.45	7.3	7.05	6.5
70	7.40						

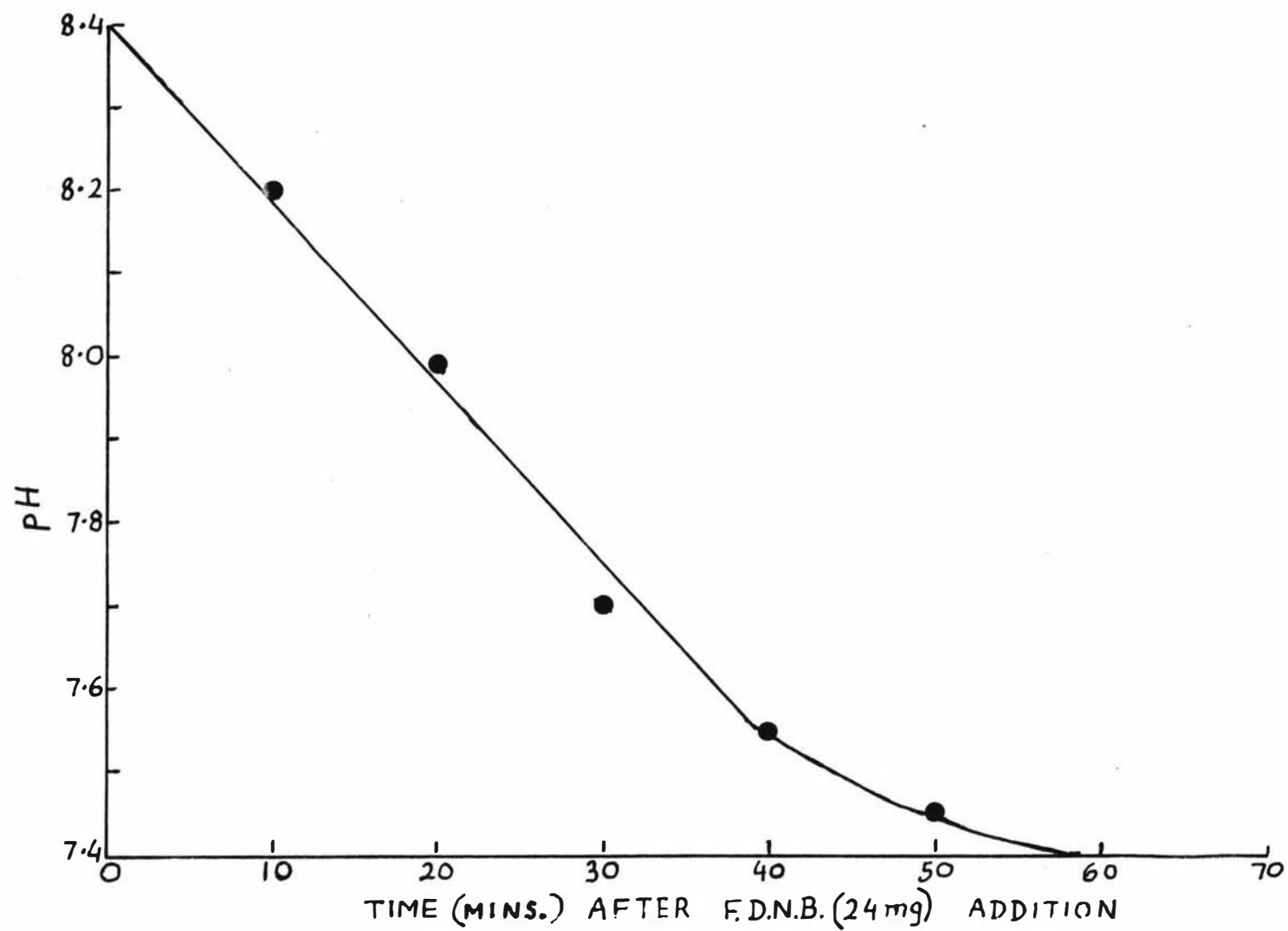


FIG. 3: RATE OF REACTION OF F.D.N.B. WITH CASEIN MICELLES IN 'ARTIFICIAL MILKS'

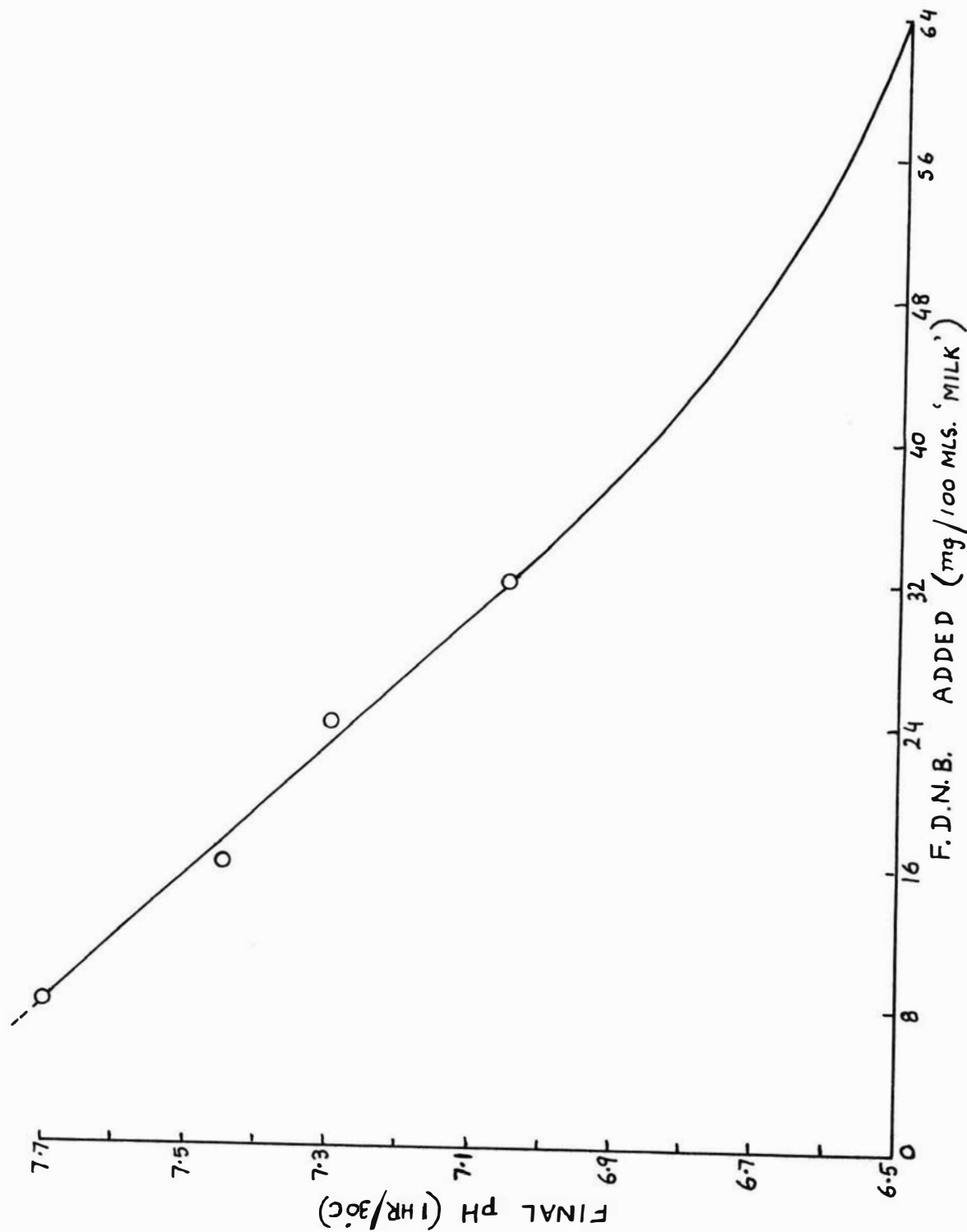


FIG. 9 : RELATION BETWEEN QUANTITY OF F.D.N.B. ADDED AND FINAL PH OF 'ARTIFICIAL MILKS'

Results from Table XVIII, plotted in Figure 8 show that there is a linear relation in the reaction between the calcium-caseinate and F.D.N.B. up to 40 minutes after F.D.N.B. addition. The rate then rapidly falls off. After about an hour, there is no further drop in pH indicating the termination of the reaction. There thus appears to be a very fast initial reaction between F.D.N.B. and the caseinate.

The relation between quantity of F.D.N.B. and the final pH after 1 hour reaction is shown in Figure 9. Up to 32 mg addition of F.D.N.B. there appears to be linear relation, possibly indicating a specific reaction between the F.D.N.B. and amino groups on one of the casein components. Beyond 32 mg addition the straight line curves away indicating a slower and perhaps a more non-specific or general reaction with amino groups. It seems unlikely that the slowing up of the reaction is due to unfavourable pH conditions for, in the presence of higher concentrations of F.D.N.B., the pH reached considerably lower levels. Preliminary work had shown that the reaction with F.D.N.B. was possible to a pH of 5.8.

The initial fast reaction of F.D.N.B. with the caseinate (Figure 8) and the linear relationship between F.D.N.B. concentration and pH up to 32 mg F.D.N.B. addition (Figure 9) are probably due, in the main, to its reaction with $\epsilon\text{-NH}_2$ groups of lysine (discussed later).

SECTION C: EFFECT OF DIFFERENT LEVELS OF ADDED F.D.N.B. ON THE
GELATION AND SYNERESIS OF CALCIUM-CASEINATE SYSTEMS
AT pH 6.8 AND 30°C.

1. Experimental

"Artificial milk" at pH 6.8 and containing 100 mg% (w/v) calcium was prepared and treated as before. The pH was gradually taken to 8.20. The "milk" was then divided into equal portions and treated with 0, 8 mg, 16 mg, 24 mg, 32 mg, and 64 mg F.D.N.B. for 1 hour at 30°C. At the end of the hour, the pH was adjusted to 6.8 with 0.1N HCl. After adjustment of volume, gel point, using 1:1 diluted rennet, and extent of syneresis on 40 ml. aliquots was determined as before. Electron micrographs were taken of the 24 mg F.D.N.B. treated sample to observe the effect on the casein micelles. Polyacrylamide gel electrophoresis was carried out on the "milks" treated with F.D.N.B. to see which protein component was affected most.

2. Results

Results are given in Table XIX.

Table XIX.

Effect of Different Levels of F.D.N.B. on the Gelation and Syneresis
of Calcium-Caseinate Systems at pH 6.8 and 30°.

Sample No.	F.D.N.B. (mg/100 mls. "milks")	Final adjusted pH	Gel point (mins.)	Gel type	Extent of Syneresis mls. whey/40 mls. curd, 30° C/90 mins.
1	Nil	6.8	< 5	Firm	24
2	8	6.8	< 5	Firm	12
3	16	6.8	< 5	Firm	5
4	24	6.8	< 5	Firm	3
5	32	6.8	< 10	Firm	0
6	64	6.8	< 15	Firm	2

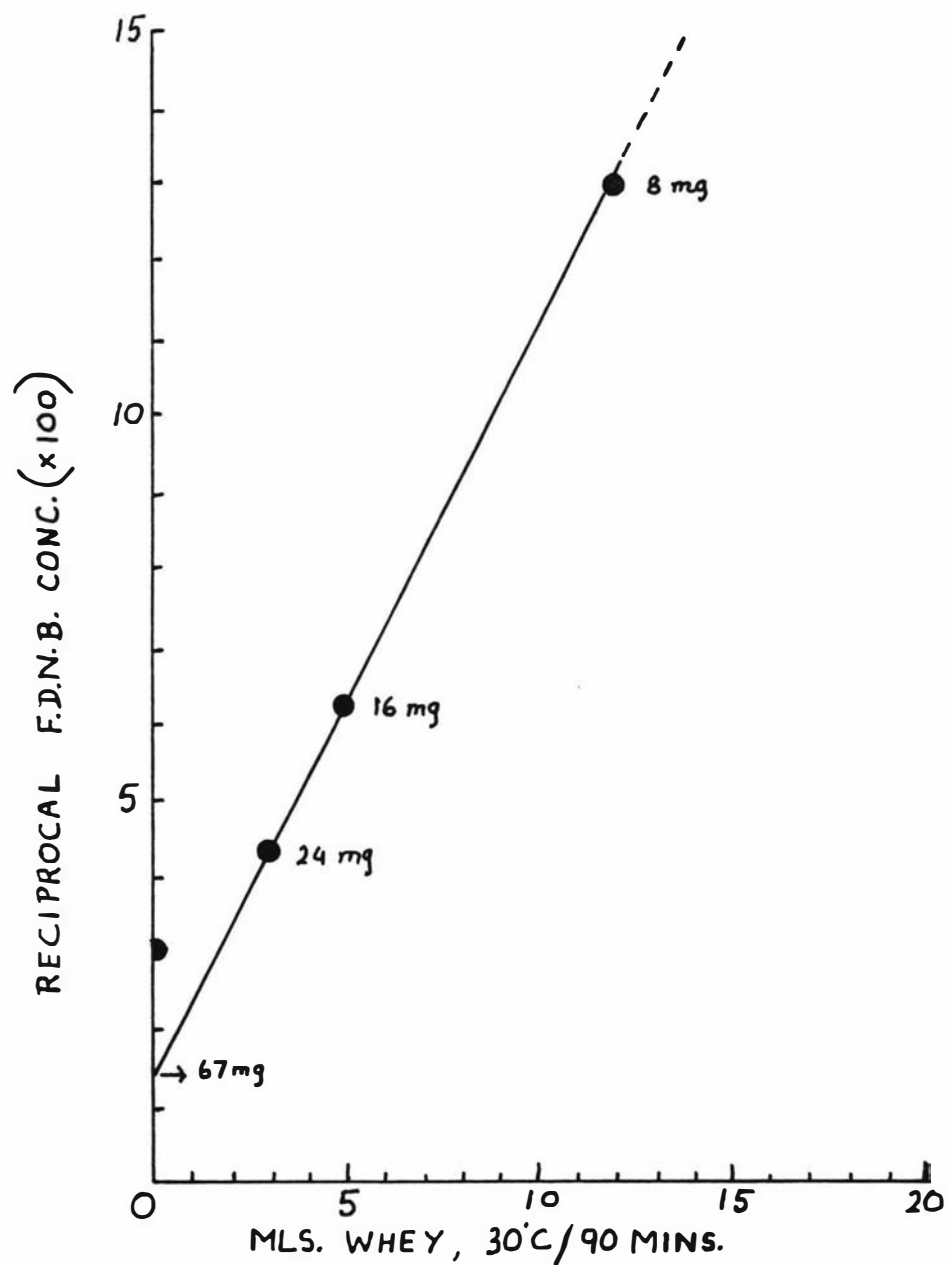


FIG. 10: RELATION BETWEEN F.D.N.B. CONCENTRATION AND EXTENT OF SYNERESIS OF CALCIUM-CASEINATE GELS.

Results from Table XIX show that while gel point was little affected at the lower F.D.N.B. levels (8mg - 24mg), it was delayed at the higher levels. The extent of syneresis was drastically reduced as the F.D.N.B. concentration in "milk" was increased. Beyond 24 mg F.D.N.B. addition, little or no syneresis was evident. A plot of the reciprocal of F.D.N.B. concentration ($\times 100$) against the quantity of whey expelled in 90 minutes at 30°C showed a linear relationship (Figure 10), thus indicating a definite relationship between the blocking of amino groups and the reduction in the syneretic properties of the gel. Extrapolation of the line in Figure 10 gives a reciprocal value of 1.5. This is equivalent to a concentration of 67 mg F.D.N.B. giving zero syneresis. This concentration of F.D.N.B. is approximately equivalent to the amount of lysine residues present in β casein⁽¹²²⁾, assuming the latter to comprise 30% of whole casein⁽¹³⁾⁽¹⁴⁾ in the "milks". Calculations show that 0.9 g β casein in the "milks" would contain about 60 mg lysine. For complete saturation of these lysine residues 76 mg F.D.N.B. would be required. This suggested that β casein may be involved in syneresis with all its amino groups participating.

Polyacrylamide gel electrophoresis of the treated "artificial milks" (Plate 25) clearly showed that the reaction with F.D.N.B., particularly up to the 24 mg F.D.N.B. addition, was primarily with the β casein. β Plate 25 shows β casein to be gradually spreading on its front towards the α_s casein with increasing levels of F.D.N.B. treatment. At the 64 mg level, the gap between α_s and β has almost disappeared. The minor components and kappa-casein seem to be little affected by the F.D.N.B. treatment. Thus a decreasing extent of syneresis of the rennet-casein gels seems to be associated with an increasing modification of

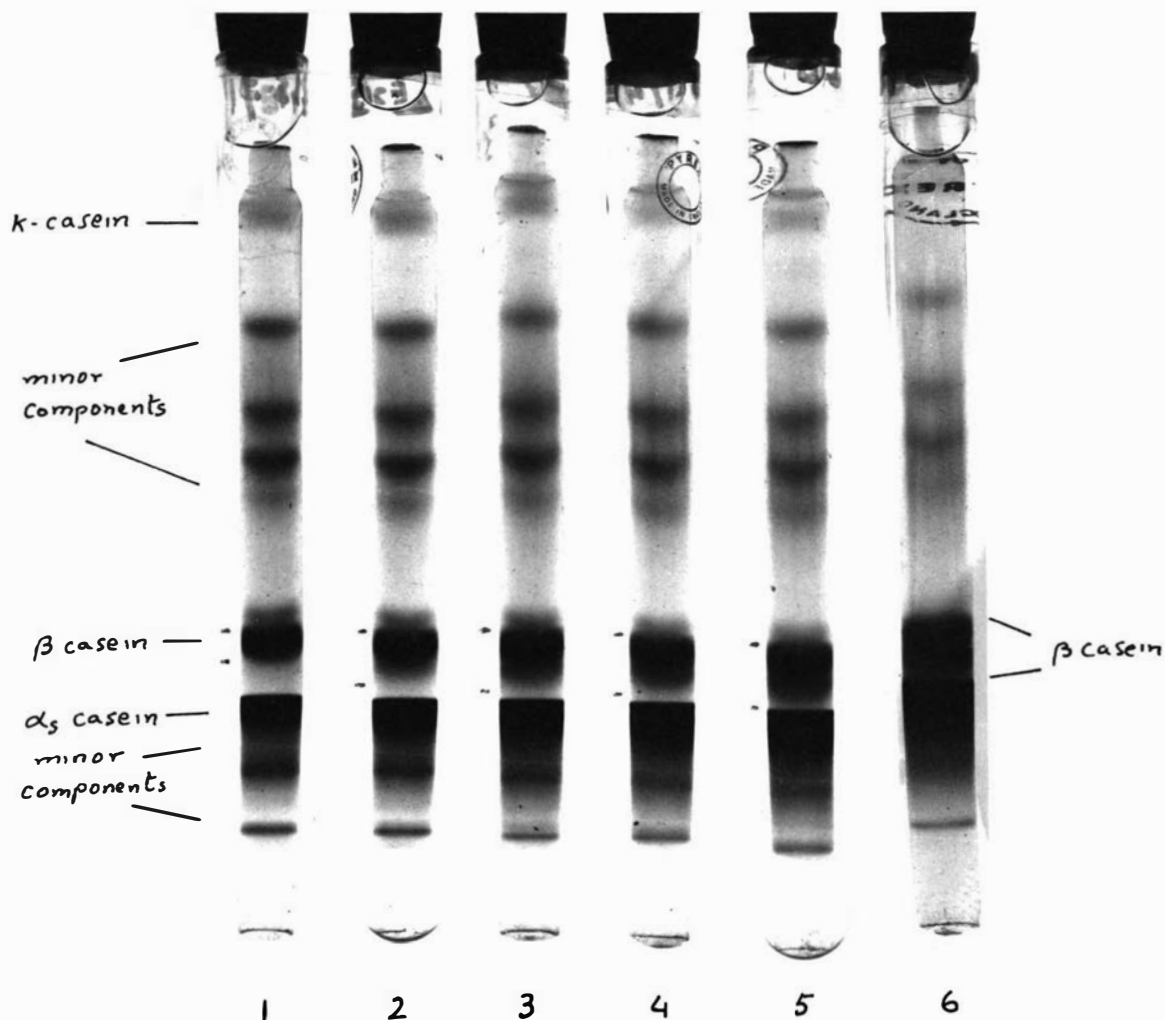


PLATE 25:

Polyacrylamide gel electrophoresis of 'artificial milks' (100 Mg % 'Ca') after reaction with 1-fluoro 2 : 4 dinitro benzene for 1 hour at 30°C

1. Control
2. + 8 mg FDNB
3. + 16 mg FDNB
4. + 24 mg FDNB
5. + 32 mg FDNB
6. + 64 mg FDNB

Note increased mobility of β casein with increased level of F.D.N.B.

the β casein component of the casein micelles. β casein seems to be more affected by reaction with F.D.N.B. when compared with the other micellar components under the conditions of the experiment.

The electron micrograph (Plate 26) of an "artificial milk" sample treated at the 24 mg level of F.D.N.B. shows hazy spherical micelles. The micelles themselves do not appear to be affected as far as their physical organisation is concerned. They are still able to maintain their spherical form although their outline is less distinct. It is possible that the organisation of the micelle is such that only the amino groups of β casein are able to react with the F.D.N.B. It would thus appear that β casein plays an important role in syneresis, possibly through its free amino group providing sites for farther cross-linking after gel formation has occurred. The action of F.D.N.B. in inhibiting syneresis may be due to:

- (a) the provision of steric hindrance to the shrinking of the gel network,
- (b) through blocking of the -NH_2 groups and thus increasing the negative character of the gel, and
- (c) by possibly preventing the positively charged -NH_2 groups from taking a direct part in the cross-linking within the 3-dimensional network.

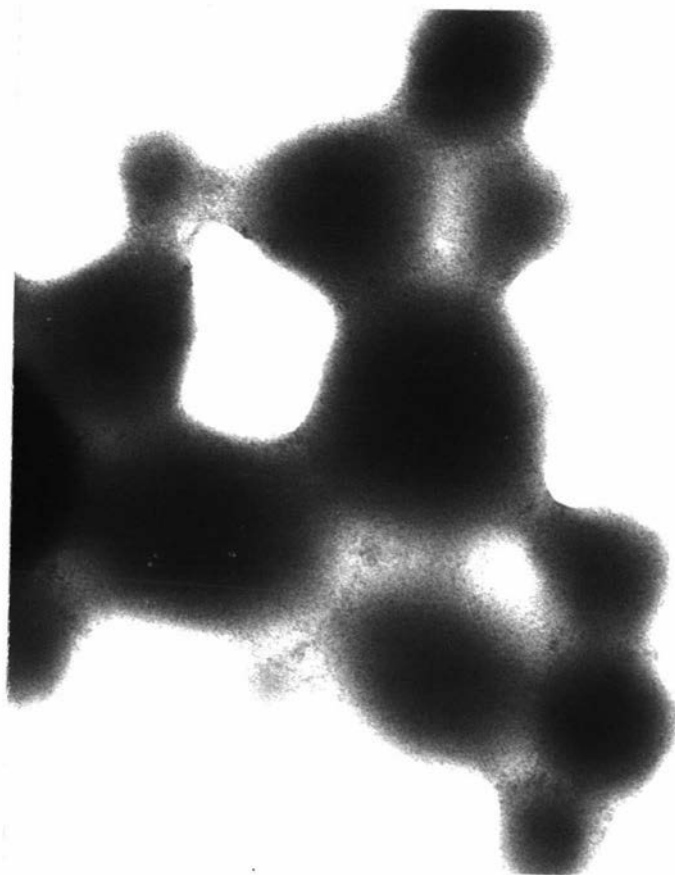


PLATE 26: Electron micrograph of casein micelles in an
'artificial milk' system (100 mg % 'Ca') after
reaction with F.D.N.B. (24 mg) for 1 hour at
30°C ——— x 35000.

SECTION D: EFFECT OF DINITROPHENYLATION ON THE RATE AND EXTENT OF
SYNERESIS OF CALCIUM-CASEINATE SYSTEMS AT pH 5.4 AND 30°C.

Results reported in Section C had shown that dinitrophenylation had caused a drastic reduction in syneresis of the rennet-casein gels at pH 6.8 and 30°C. It was of interest to examine whether such an extent of inhibition of syneresis was possible at lower pH values. A pH value of 5.4 was selected as this was close to the value normally found in cheesemaking practice, by which time most of the whey expulsion from the curd has already taken place. It was also the lowest value at which a stable suspension of casein could be maintained in the "artificial milks".

1. Experimental

"Artificial milk" at pH 6.8 and containing 100 mg% (w/v) calcium was prepared and treated as described earlier, prior to reaction with 24 mg F.D.N.B./100 mls. for one hour at pH 8.2. The control was treated similarly but without addition of F.D.N.B. The quantity of 24 mg F.D.N.B. was chosen as this had almost completely inhibited syneresis at pH 6.8 (Table XIX) and electrophoretic patterns (Plate 25) had shown that at this level of treatment β casein was the main protein affected. A 24 mg treatment represented about 30% conversion of the lysine residues in β casein in 100 mls. of "artificial milk" containing 3 g casein, assuming complete and specific reaction.

At the end of 1 hour the pH for both control and treated sample was adjusted to 5.4 and the rate and extent of syneresis determined on 40 ml. aliquots at 30°C using 1:3 diluted rennet.

2. Results

Results are given in Table XX.

Table XX. Effect of Dinitrophenylation on the Rate and Extent of Syneresis of Calcium-Caseinate Systems at pH 5.4 and 30°.

Sample	CONTROL	F.D.N.B. Treated
Gel point (minutes)	< 3	< 3
Gel type	Firm	Firm
Time after rennet addition (minutes)	Syneresis, mls. whey/ 40 mls. curd, at 30°C.	
10	3	-
15	10	-
20	18	-
25	26	3
30	30	7
35	32	10
40	33	14
45	-	16
60	-	22
75	-	27
90	36	29
120	36	30
Turbidity of whey	clear	clear (slightly yellow)

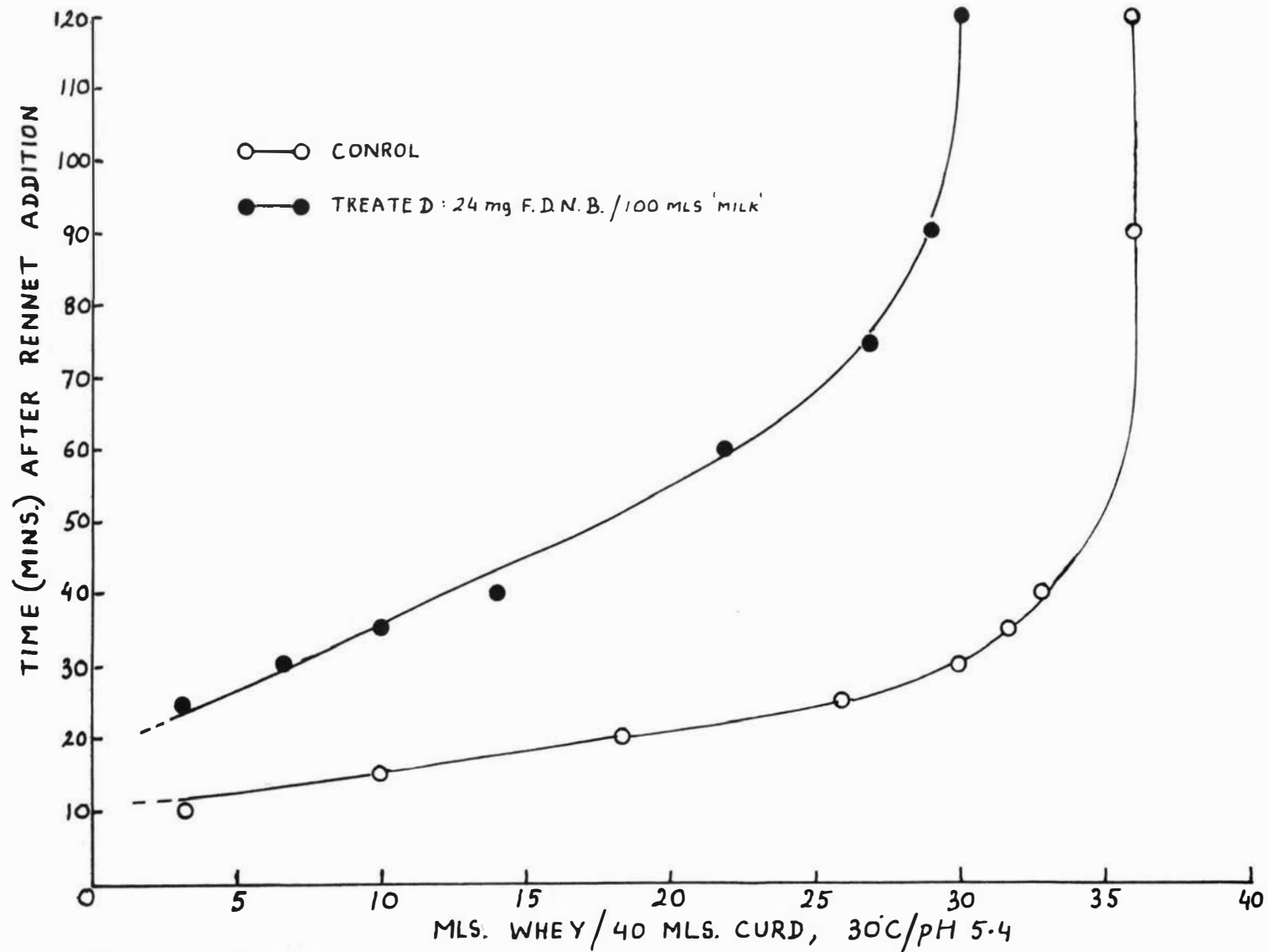


FIG. 11 : EFFECT OF DINITROPHENYLATION ON RATE AND EXTENT OF SYNERESIS OF CALCIUM-CASEINATE GELS, pH 5.4/30°C

Results from Table XX, plotted in Figure 11, show that F.D.N.B. treatment has greatly reduced both the rate and extent of syneresis compared to the control under the conditions of the experiment.

At the end of 120 minutes the amount of whey given by the treated sample was 30 mls. which the control had expelled within 30 minutes. The 24 mg F.D.N.B. treatment has not, however, completely inhibited syneresis at pH 5.4 as it nearly did at pH 6.8 (Table XIX). This indicated that part of the effect at least of F.D.N.B. treatment was probably due to increasing the electro-negative character of the gel through blocking of the positive amino groups. In this study this has been counterbalanced to some extent by lowering the pH. Increase in electro-negativity would give rise to increased repulsion between the mobile structural elements and the rigid structural elements of the gel network and would allow the network to remain in a partially swollen condition. Dinitrophenylation may also inhibit syneresis by preventing van der Waals or electrostatic forces of attraction, in which -NH_2 groups may be involved, to operate. Structural hindrance provided by the F.D.N.B. may play a very limited part, if at all, since if it was important the difference in the extent of syneresis at pH 6.8 and at pH 5.4 would not be expected to be so high.

It also appears from the lack of inhibition of gelation by F.D.N.B. that the syneretic phenomena may not be a continuation of the gelation phenomena although the syneretic properties may be dependent on the nature of the gel formed initially and indirectly, on the conditions which affect the nature of the gel.

SECTION E: EFFECT OF DINITROPHENYLATION ON THE EXTENT OF SYNERESIS
OF CALCIUM-CASEINATE SYSTEMS AT DIFFERENT pH LEVELS AND
30°C.

1. Experimental

To examine more closely the part played by F.D.N.B. in the inhibition of syneresis of rennet-casein gels, the effect of F.D.N.B. treatment was examined at four different pH levels. "Artificial milk" at pH 6.8 and containing 100 mg% (w/v) calcium was prepared in bulk and treated as before with 24 mg F.D.N.B./100 mls. at pH 8.2 for one hour. The treated samples were adjusted to the following pH values: 6.8, 6.0, 5.8 and 5.4. Appropriate controls were set up for each pH value. After adjustment of volume, 40 ml. aliquot of the samples were examined for syneresis as before, using 1:1 diluted rennet for pH 6.8 samples, 1:3 for pH 6.0 and 5.8 samples, and 1:4 for the pH 5.4 sample, to give roughly equivalent coagulation times.

2. Results

Results are given in Table XXI.

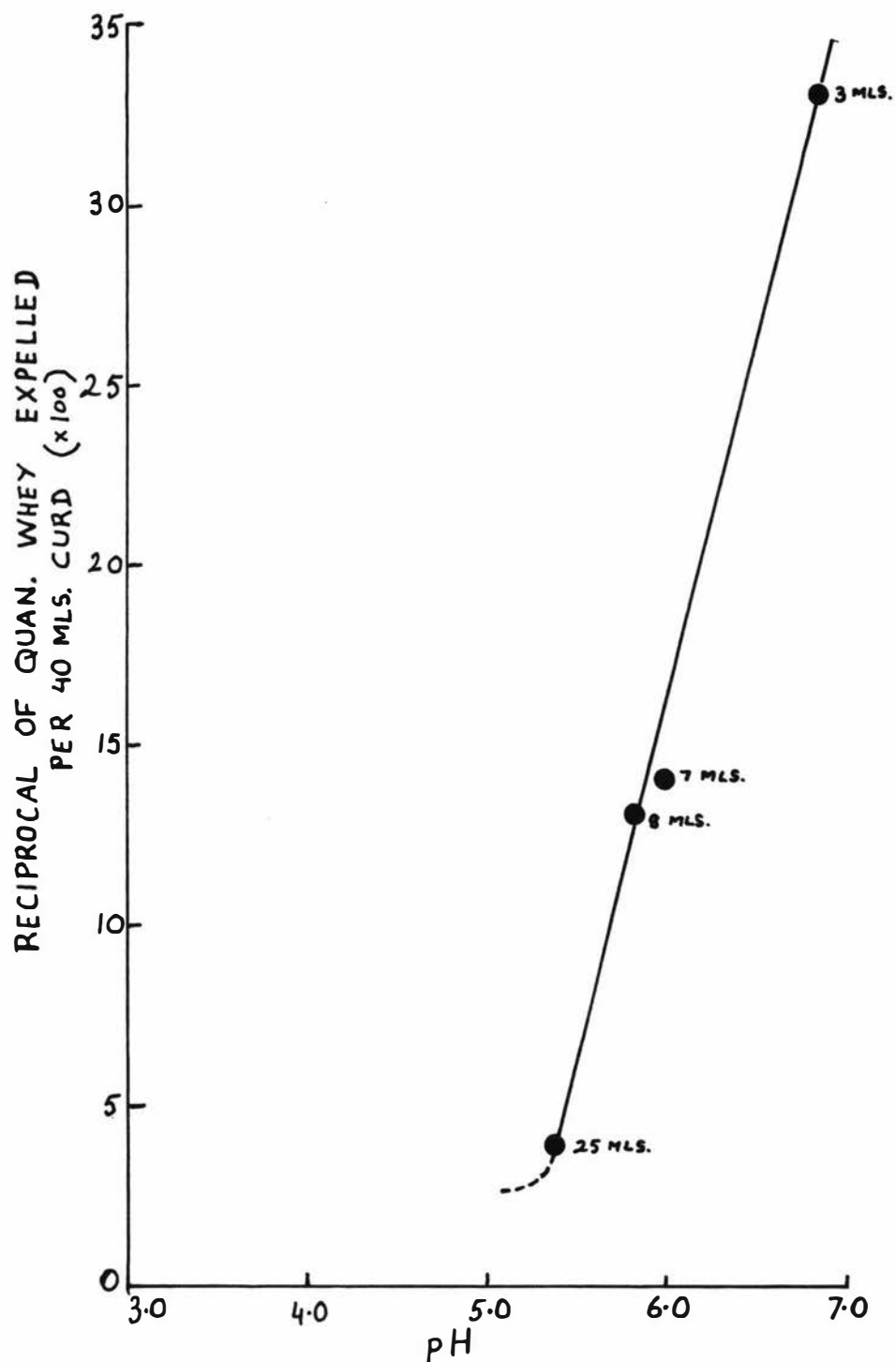


FIG. 12: EFFECT OF F.D.N.B. TREATMENT (24 mg/100 MLS.) ON SYNERESIS OF CALCIUM-CASEINATE GELS AT DIFFERENT pH VALUES AND 30°C/90 MINS.

Results shown in Table XXI indicate that F.D.N.B. treatment greatly reduces the extent of syneresis at all observed pH values, when compared to control samples at similar pH values. However, a reduction in pH of the system, in other words, a reduction in the electro-negativity of the gel system, gives rise to an increase in the syneresis, this increase being particularly evident below pH 5.8. A plot of the reciprocal of the quantity of whey expelled and the pH of the gel system as shown in Figure 12 gives a straight line showing the proportionality of the whey expelled to the pH of the system. This suggested that the inhibitory effect of F.D.N.B. treatment on syneresis was due either purely to increasing the electro-negative character of the gel elements by blocking of the positive $-NH_2$ groups, thereby creating more repulsive forces, and/or possibly reducing the number of amino groups ($e-NH_2$) which take part directly in the cross-linking to give syneresis. Extending the incubation beyond 120 minutes at pH 5.4 did not greatly increase syneresis.

There is a straight line relationship indicated in Figure 12 for the reciprocal of quantity of whey expelled and the pH. From this it would appear that F.D.N.B. in itself provides little, if any, steric or structural hindrance within the gel network to "inhibit" syneresis. The effect appears to be largely due to modification of the amino groups of β casein, possibly only the $e-NH_2$ groups.

SECTION F: EFFECT OF DINITROPHENYLATION ON THE RATE AND EXTENT
OF SYNERESIS OF CALCIUM-CASEINATE SYSTEMS AT pH 6.0
AND 38°C.

It had been shown earlier that treatment with 24 mg F.D.N.B. virtually halted syneresis at pH 6.8 and 30°C. It also significantly reduced syneresis at low pH values. In general cheesemaking practice, most of the whey expulsion occurs by the time the pH has dropped to 6.0 and at a temperature of about 38 - 40°C. The syneretic study after dinitrophenylation was therefore carried out at a pH of 6.0 and at 38°C.

1. Experimental

"Artificial milk" at pH 6.8 and containing an equivalent of 100 mg% (w/v) calcium was prepared and treated in bulk, as described earlier, and reacted with 24 mg F.D.N.B. per 100 mls. at pH 8.2 for one hour. At the end of the reaction the pH was adjusted to 6.0 with 0.1 N HCl and the system made to volume to contain 3% casein and 100 mg% w/v of calcium.

40 ml. aliquots of the samples were then examined for syneresis at 38°C using a 1:3 diluted commercial rennet.

2. Results

Results are given in Table XXII.

Table XXII.

Effect of Dinitrophenylation on the Rate and Extent of Syneresis of
Calcium-Caseinate Gels at pH 6.0 and 38°C.

Sample	Gel point (mins.)	Gel type	Syneresis at 38°C mls. whey/40 mls. curd, after rennet addition										Appearance of whey
			-	15	20	30	40	45	50	60	75	90	120(mins)
CONTROL	< 5	firm	10	17	32	-	34	-	35	-	35	36	clear
Treated (24 mg F.D.N.B./ 100 mls./1 hour)	< 5	firm	-	-	8	12	-	18	-	30	31	32	clear (slightly yellow)

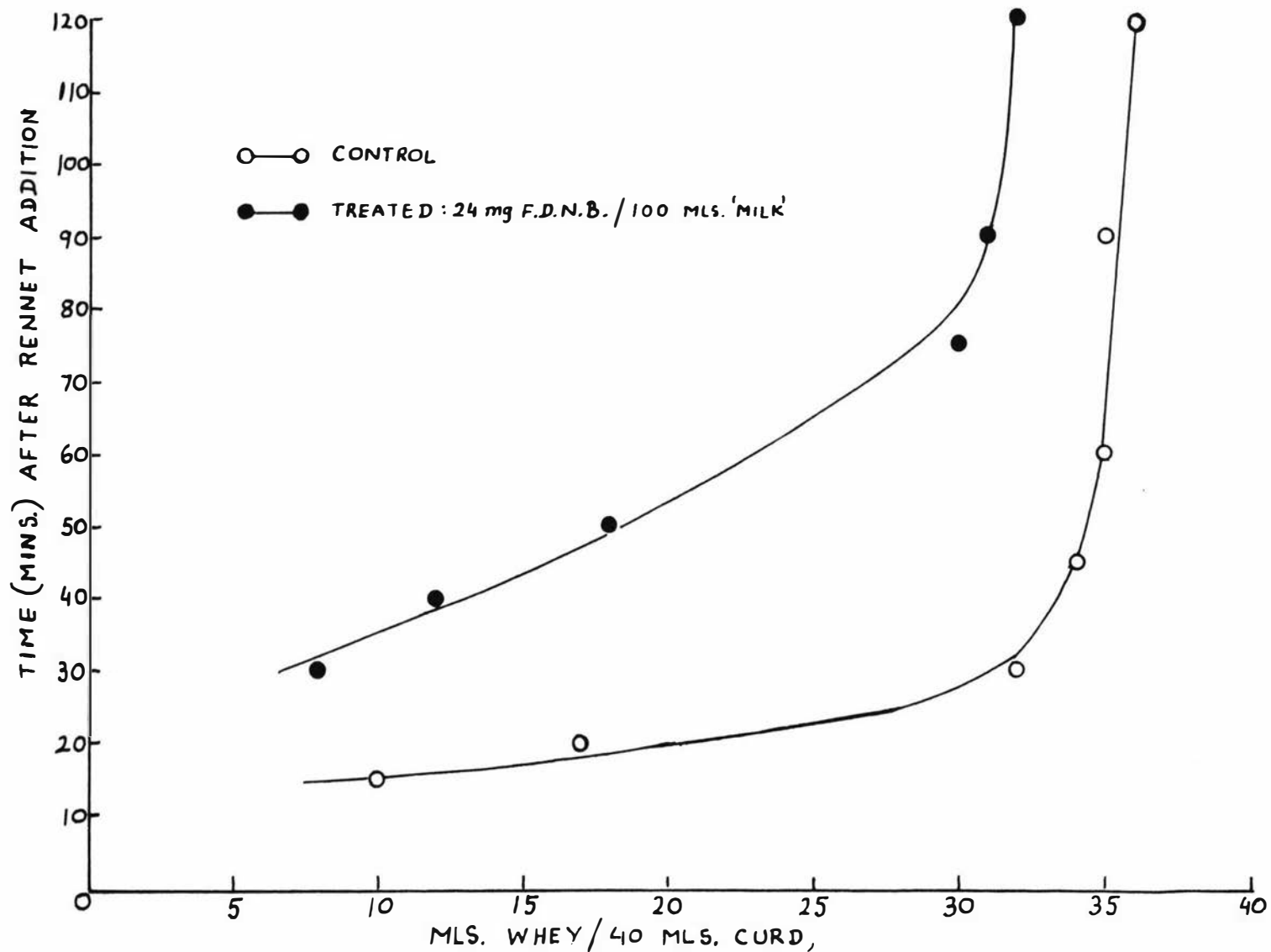


FIG. 13 : EFFECT OF DINITROPHENYLATION ON RATE AND EXTENT OF SYNERESIS OF CALCIUM-CASEINATE GELS, PH 6.0/38°C

Results from Table XXII, plotted in Figure 13, show the marked inhibitory effect of F.D.N.B. treatment on the syneretic properties of a calcium-caseinate gel, even at a relatively high temperature of 38°C . The general pattern shown in Figure 13 resembles closely the one shown in Figure 11. This shows that at the higher pH of 6.0 the higher temperature of 38°C compensates for the effect of a lower pH (5.4) used at a lower temperature (30°C).

The control sample had almost fully synerised by 30 minutes at which point only 25% syneresis, relative to the control, was shown by the treated sample. Thus, F.D.N.B. has an inhibitory effect under all observed conditions of gel formation and syneresis, the effect being lessened by use of lower pH values and by use of higher temperatures during syneresis.

SECTION G: EFFECT OF DINITROPHENYLATION ON THE RATE AND EXTENT OF SYNERESIS OF CALCIUM-CASEINATE SYSTEMS UNDER CONDITIONS OF CHANGING pH AND TEMPERATURE.

1. Experimental

The effect of dinitrophenylation on syneresis was studied after adding glucono-acid-lactone to the samples, setting the "milks" at 30°C and raising the temperature gradually to 38°C.

"Artificial milk" at pH 6.8 and containing 100 mg% (w/v) calcium was prepared and treated as before and reacted with 24 mg F.D.N.B./100 mls./1 hour.

The pH was adjusted to 7.2 with 0.1 N HCl and the "milk" then made up to volume.

To 40 ml. aliquots of the "milks" at 30°C, pH 7.2, was added 300 mg glucono-acid-lactone with stirring. This immediately brought the pH down to 6.7 - 6.8. A 1:1 dilution of commercial rennet was then added and after setting (5 minutes) the temperature of the water bath was raised gradually by 1°C every 4 minutes to reach a final temperature of 38°C in 30 minutes. This rate of increase in temperature is about the same as used in commercial cheesemaking practice. Syneresis was measured at various time intervals as before.

2. Results

Results are shown in Table XIII.

Table XXIII. Effect of Dinitrophenylation on the Rate and Extent of Syneresis of
Calcium-Caseinate Gels under conditions of Changing pH and Temperature.

TIME (minutes after rennet addition)	Rate of pH drop		Gelation time(minutes)		Syneresis, mls.whey/40 mls.curd	
	Control	Treated	Control	Treated	Control	Treated
0	6.5	6.5	< 5	< 5	-	-
10	6.0	6.0			-	-
20	5.5	5.4			18	-
25	-	-			32	12
30	5.2	5.2			34	18
35	-	-			-	24
40	4.8	4.8			36	30
90	4.0	4.0			-	32
120	4.0	4.0			37	33

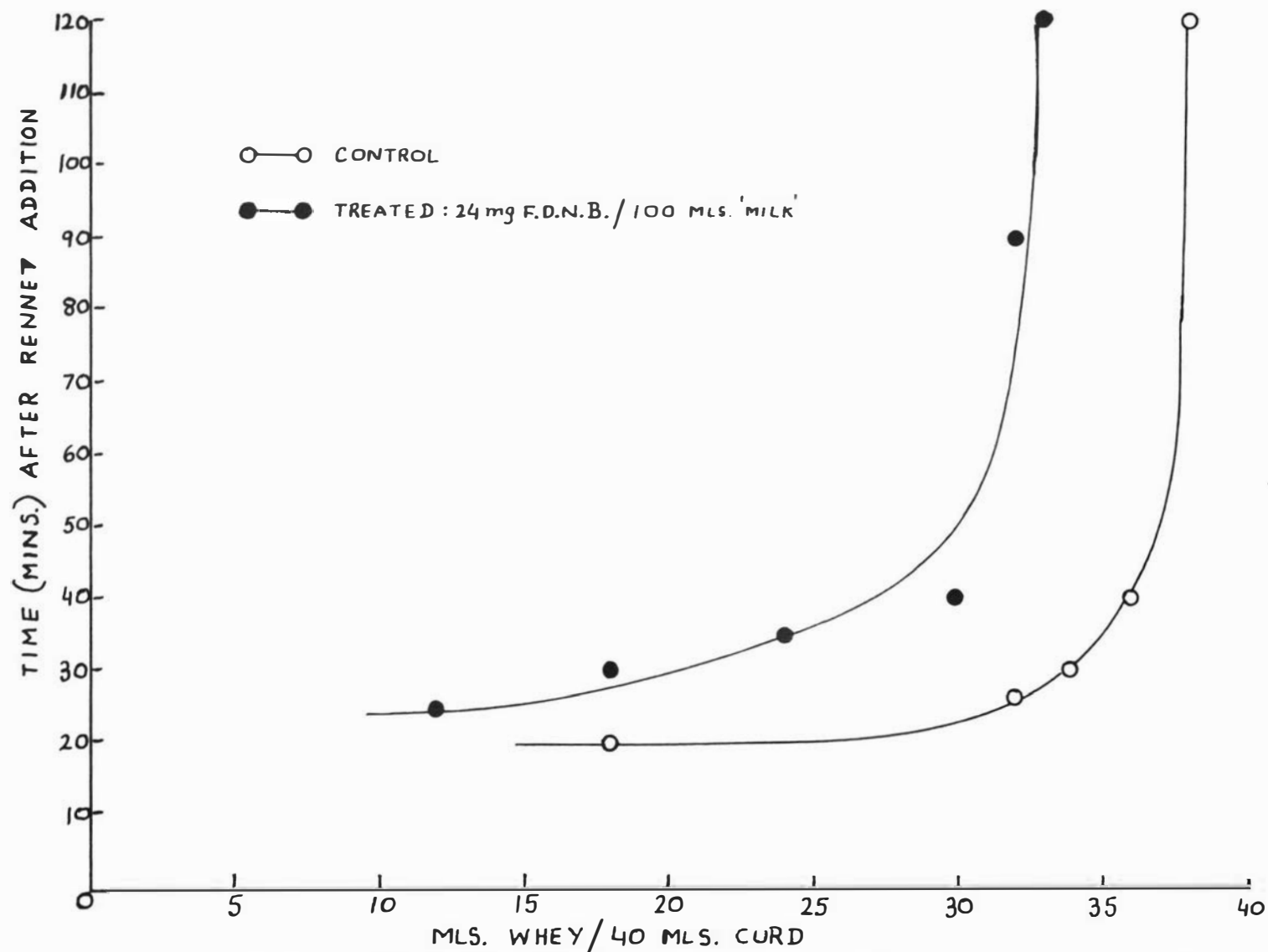


FIG. 14 : EFFECT OF DINITROPHENYLATION ON RATE AND EXTENT OF SYNERESIS OF CALCIUM-CASEINATE GELS (PH 6.5 → 4.0, TEMP. 30 → 38°C)

Results from Table XXIII, plotted in Figure 14, show that even under conditions of developing acidity within the curd and a rise in temperature from 30°C to 38°C in 30 minutes after setting, F.D.N.B. has retarded the rate and extent of syneresis of rennet-casein gels. It will be noticed from columns 2 and 3 that the rate of pH drop was faster than was intended. Also the final pH values of 4.0 obtained in the gels was very much lower than usually encountered in practice (about 5.0 in "green" cheeses). Nevertheless, despite this higher acidity, the F.D.N.B. treated gel has not released all the whey even after 120 minutes at a final temperature of 38°C .

SECTION H: EFFECT OF POTASSIUM CYANATE (CARBAMYLATION) ON THE
SYNERESIS OF CALCIUM-CASEINATE SYSTEMS AT pH 6.8
AND 30°C.

Since dinitrophenylation was shown to inhibit both rate and extent of syneresis, it was of interest to determine whether modification of the amino groups by other means, would also modify the syneretic properties of the calcium-caseinate gel. For this purpose potassium cyanate was used to modify the amino groups by the carbamylation reaction^(140 - 142), the reaction with casein being attempted when the latter was in micellar form.

1. Experimental

"Artificial milk" containing 100 mg% calcium was prepared in bulk at pH 6.8 as before. After holding for 12 hours at 4°C, the temperature was raised to 30°C and the pH to 7.5. The samples were treated with various levels of KCNO (0-24 mg/100 mls. "milk") for 3 hours at 30°C. At the end of the period, the pH was brought down to 6.8 using 0.1 N HCl and extent of syneresis measured as before at 30°C, using 1:1 dilution of commercial rennet for setting.

2. Results

Results are indicated in Table XXIV.

Table XXIV. **Effect of Carbamylation on the Syneresis of Calcium-Caseinate gels at pH 6.8 and 30°C.**

No.	Treatment with KCNO (mg/100 mls "milk")	Gel point	Gel type	Extent of syneresis, mls. whey/40 mls. curd, at 30°C/90 minutes	Nature of whey (turbidity)
1	Control	< 5	firm	24	clear
2	4	< 5	firm	18	clear
3	8	< 5	firm	16	clear
4	12	< 7	less firm	14	+
5	16	< 10	less firm	12	++
6	24	< 20	less firm	7	+++

Results from Table XXIV show that potassium cyanate also retards the extent of syneresis of the gels at 30°C and pH 6.8. The inhibitory effect, however, appears to be somewhat less than that produced by F.D.N.B. treatment at equivalent concentrations. About 12 mg KCNO and 24 mg F.D.N.B. would modify similar numbers of free amino groups on the β casein, assuming this was the only reaction taking place. Addition of potassium cyanate is, however, complicated by the possible displacement of calcium by the potassium from the curd, thereby affecting gel formation and structure. This is indicated by the fact that at the higher levels of cyanate addition, gel point is increased considerably. Furthermore, at the higher concentrations of KCNO, the turbidity of the whey is greatly increased indicating displacement of casein from the curd network. Beyond 24 mg KCNO addition (32 and 64 mg) the system did not gel within 90 minutes. However, the lower levels of added KCNO appear to definitely inhibit syneresis and confirm results obtained from F.D.N.B. treatments.

SECTION I: EFFECT OF GUANIDINATION OF CASEIN IN CALCIUM-CASEINATE SYSTEMS WITH O-METHYL-ISO UREA ON THE SYNERESIS OF CALCIUM-CASEINATE SYSTEMS AT pH 6.8 AND 30°C.

1. Introduction

1-fluoro, 2:4 dinitrobenzene and potassium cyanate were both shown to retard syneresis of rennet-caseinate gels. Although it was likely that at the small concentrations used the reaction between the F.D.N.B. and KCNO was mainly with the lysine side chain, it was felt that more definite information might be obtained by the use of a reagent specific for the ϵ -NH₂ group of lysine. A search of the literature⁽¹⁴³⁾⁽¹⁴⁴⁾ indicated that the compound o-methyl-iso urea was essentially specific for the ϵ -NH₂ group of lysine, converting this to a guanidino group (homoarginine). The advantage of such a conversion was that there would be no radical change in the nature of the chemical group resulting in loss of a charged or ionised group. The conversion of the lysine -NH₂ to a guanidino group would not produce a drastic change in the charge pattern, as a basic amino group would be replaced by a somewhat more basic guanidino group. Furthermore, the introduction of a guanidino group in a protein does not involve introduction of a chemical group foreign to biological systems⁽¹⁴³⁾.

2. Experimental

(a) Preparation of calibration curve for the determination of guanidino groups.

The technique followed for the preparation of the standard

curve for arginine in the presence of protein was similar to that described by Aiyar and Wallace⁽²¹⁾ (publication attached)* with some modifications based on the method described by Rosenberg et al⁽¹⁴⁵⁾.

To a series of test tubes were added in turn,

- (i) 0.3 mls. "artificial milk" (3% casein, 100 mg% (w/v) calcium, pH 6.8)
- (ii) 0.1 ml. to 0.5 mls. of arginine hydrochloride solution in distilled water (containing 1 μ m/ml. arginine)
- (iii) 1 ml. 3-N NaOH
- (iv) 2 mls. development solution (prepared from 20 mls. of 25% w/v α -naphthol in n-propanol + 2.5 mls. 1% diacetyl in water, made to 100 mls. with propanol)
- (v) Distilled water to a final volume of 10 mls.

The control "milk" sample was treated similarly, except that the addition of the arginine solution was omitted.

The red colour was developed for 30 minutes at room temperature.

Optical density measurements were made in a Hitachi spectrophotometer at 470 m μ , being the wavelength of maximum absorption under the conditions of the experiment.

The plot of optical density versus arginine concentration was linear and passed through the origin (Figure 15).

(b) Guanidination of calcium-caseinate systems and determination of the newly formed guanidino groups.

"Artificial milks" at pH 6.8 and containing an equivalent of 100 mg% (w/v) calcium was prepared and treated as before without making to final volume. The pH was gradually raised to 10.3 with 0.2 N NaOH. The temperature was maintained at 4°C.

* see appendix

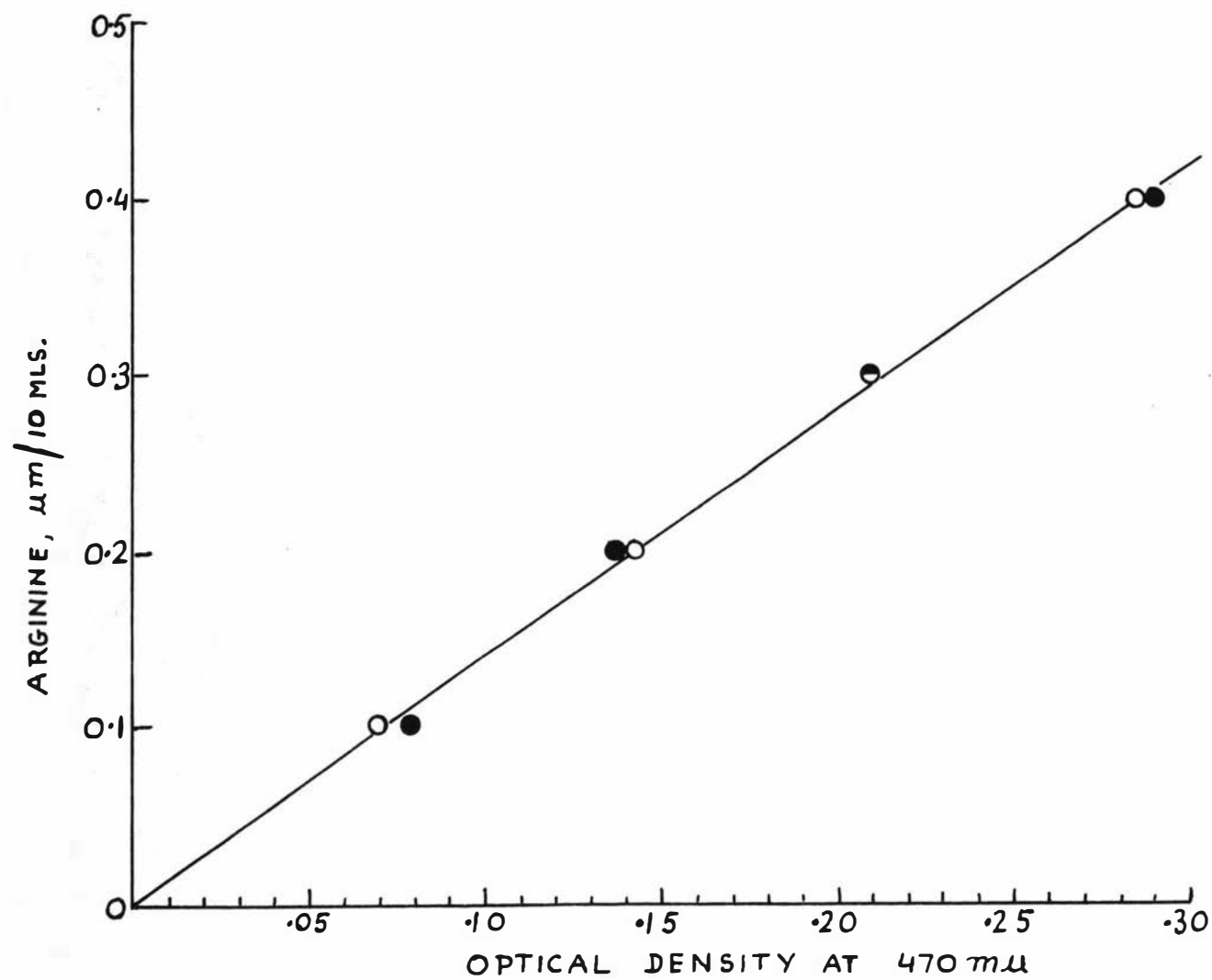


FIG. 15 : CALIBRATION CURVE FOR THE DETERMINATION OF GUANIDINO GROUPS

To the "milk" was added 35 mg methyl-iso urea ($\text{CH}_3\text{OCNH.NH}_2$) and the mixture held with stirring for 24 hours at 4°C , at the end of which the guanidination reaction was assumed to be completed. The pH was then lowered to 6.8 using cold 0.2 N HCl. The temperature of the "artificial milk" was allowed to come up to ambient temperature and the pH readjusted to 6.8. The volume was then adjusted to give a "milk" containing 3 g casein and 100 mg% calcium. Hughes et al⁽¹⁴⁴⁾ have pointed out that the chemical modification of a protein can be carried out safely at low temperatures because of the much larger temperature coefficient of denaturation when compared to that of chemical reactions. A control sample was treated similarly except that the addition of methyl-iso urea was omitted. A second control was also prepared to which methyl-iso urea had been added at pH 6.8. This sample was examined soon after the addition of methyl-iso urea, for guanidino groups and for syneresis. Results showed that the addition of o-methyl-iso urea in itself was not causing any changes in either optical density readings or in syneresis.

The determination of the newly formed guanidino groups was carried out on the unhydrolysed protein itself. Aiyar and Wallace⁽²¹⁾ had indicated^{*} that guanidino groups could be estimated directly on the unhydrolysed protein and more recently, Luigi⁽¹⁴⁶⁾, using a modified Sakaguchi method, has also been able to estimate arginine directly on the unhydrolysed protein. The technique followed was similar to that used for determination of the calibration curve for arginine.

* see appendix

To 0.3 mls. of the o-methyl-iso urea treated "artificial milks" were added, in turn, the following solutions:

- (i) 1 ml. 3 N NaOH
- (ii) 2 mls. development solution (Section I;2:a)
- (iii) distilled water to a final volume of 10 mls.

The control in this case was either an untreated "milk" sample as used for the calibration curve, or a sample treated with o-methyl-iso urea at pH 6.8 and used immediately. Similar results were obtained with both controls.

Results of the guanidination process are given in Table XXV.

Table XXV. The Extent of Guanidination in Calcium-Caseinate Solution after Methyl-iso Urea Treatment.

Sample	Optical Density at 470 mμ	Arginine equivalent from standard curve (μm)	Extent of guanidination (% lysine converted in β casein)
Control	Blank	-	
Treated sample	0.16	0.23	19

Table XXV shows that under the conditions of the experiment, about 19% of the lysine residues in β casein may have had their e-NH₂ groups converted to guanidino groups, assuming that only β casein was affected by o-methyl-iso urea.*

*Note: The amino acid composition of β casein taken for the calculation was that given by Jeness and Patton⁽¹²²⁾.

A 0.3 ml. sample of "artificial milk" was calculated to contain 1.23 μm lysine assuming β casein to be

30% of whole casein⁽¹⁴⁾. A production of 0.23 μ m homoarginine from lysine therefore represents about 19% conversion.

(c) Measurement of syneresis

Extent of syneresis was measured as before, using a 1:1 diluted rennet for setting at 30°C, the whey being measured after 120 minutes incubation.

(d) Polyacrylamide gel electrophoresis

The method described under Chapter IV:A:4 was followed.

3. Results

Results are indicated in Table XXVI.

Table XXVI. Effect of Guanidination on the Syneresis of Rennet-Casein Gels at pH 6.8 and 30°C.

No.	Treatment	Final pH	Gel point (minutes)	Extent of syneresis, mls. whey/40 mls. curd, at 30°C/120 minutes	
				A	B
1	Control (no methyl-iso urea)	6.8	10	34	33
2	Control + 35 mg methyl-iso urea added at pH 6.8	6.8	10	29	27
3	Guanidinated "milk"	6.8	10	24	22

Results given in Table XXVI indicate that guanidination of the caseinate with o-methyl-iso urea greatly retarded the extent of syneresis at 30°C and 120 minutes. However, when compared to F.D.N.B. treatment at an equivalent level (Figure 10) this retardation of syneresis is much lower. This lesser inhibition of syneresis may, in part, be due to the high pH conditions used in the conversion of ϵ -NH₂ groups of lysine to guanidino groups, which might have modified the protein. It will be noted that even the control sample (No.1) has given greater syneresis than normal (usually 25 - 30 mls.) whereas control No.2 whose pH never exceeded neutrality has behaved in the usual manner. It is also possible that a pH of 10.3 used for guanidination would have had dissociative effects on the casein micelles allowing for more general guanidination rather than being specific for β casein. A more likely explanation, however, for the lesser inhibition of syneresis is that the guanidination reaction has merely substituted a basic guanidino group for the basic epsilon amino group. The charge pattern on the protein is therefore little changed. Thus if electrostatic forces are important in syneresis, the new guanidino group would still be able to take part in syneresis, although perhaps less effectively for steric reasons.

Polyacrylamide gel electrophoresis (Plate 27) showed no difference in the relative mobilities of the different caseins when compared to the control. This indicated that methyl-iso urea treatment had not greatly modified the caseins and that guanidination of lysine on β casein to the extent of about 19% did not affect its mobility to any noticeable extent. The overall charge pattern thus appears similar to the control sample which gave a similar electrophoretic pattern. This suggests again that the lysine ϵ -NH₂ groups on β casein probably take a direct part in syneresis and that electrostatic forces may be important.

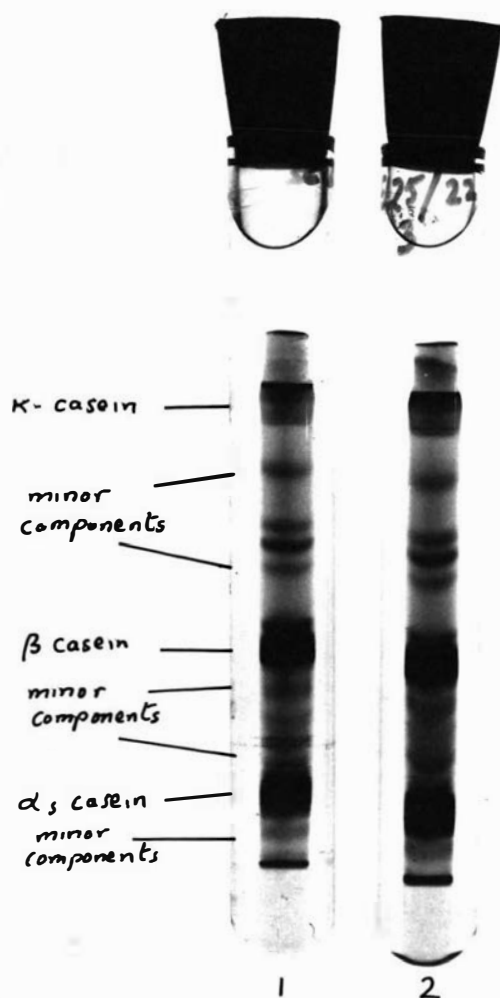


PLATE 27: Effect of guanidination of casein by methyl-isourea on its electrophoretic patterns.

1. Control
2. Guanidinated 'artificial milk' (100 mg % 'Ca')

SECTION J: EFFECT OF HEATING IN THE PRESENCE OF ALDOPENTOSE AND ALDOHEXOSE ON THE RATE AND EXTENT OF SYNERESIS OF CALCIUM-CASEINATE SYSTEMS : EFFECT OF HEATING TO 86°C FOR 40 MINUTES IN THE PRESENCE OF XYLOSE ON THE RATE AND EXTENT OF SYNERESIS AT 38°C AND pH 6.8.

1. Introduction

The idea of studying the effect of heating in the presence of aldoses on syneresis of rennet-casein gels arose from earlier observations that F.D.N.B. treatment of "milks" inhibited the syneresis of rennet-casein gels. This was believed to be due to a reaction with the ϵ -NH₂ groups of lysine in β casein. The browning reaction between proteins and reducing sugars is generally believed⁽¹²²⁾⁽¹⁵⁸⁾ to be between the ϵ -NH₂ group of lysine and the reducing sugar. It was of interest, therefore, to study the extent syneresis would be inhibited by the browning reaction.

2. Experimental

(a) Treatment of "artificial milks"

"Artificial milk" at pH 7.20 and containing an equivalent of 3% casein and 100 mg% w/v calcium was prepared and treated as before. The "milk" was divided into 40 ml. aliquots. Two samples served as unheated controls, with and without the addition of 4% xylose, and one as a heated control without added sugar. Two other samples contained 10 mg (0.02%) and 2 g (4%) xylose respectively and these along with one control

without added sugar were heated to 86°C for 40 minutes.

At the end of the period, the pH of all samples was adjusted to 6.8 with 0.1 N HCl. The samples were then made up to volume.

(b) Measurement of browning reaction and calculation of % loss of reflectance values

After adjustment of pH and volume, light reflectance values were determined for all samples in a UNIGALVO TYPE 20 Reflectance Spectrophotometer using magnesium carbonate as standard ($\bar{\bar{=}}$ 100% reflectance) and No. 609 filter.

In certain experiments, the samples were heated for different time intervals at 86°C and reflectance values measured.

There was no direct linear relationship between Reflectance values and the time of heating at 86°C. However, it was found that a linear relationship held, under the conditions of the experiment, between % loss of reflectance and the time of heating. Specimen calculations are shown below:

<u>Sample</u>	<u>Reflectance value</u>
1. MgCO_3	100
2. Heated control (86°/25 mins) without sugar	75
3. Heated sample (86°/25 mins) + 4% xylose	62
Difference in reflectance values (2 - 3)	13
Therefore % loss of reflectance relative to heated control =	
$\frac{13}{75} \times 100 =$	17.3

Thus, this sample would have a % loss of reflectance value of 17.3% relative to the control.

(c) Measurement of syneresis

The measurement was carried out as detailed under Chapter IV:A:3, using a 1:1 diluted commercial rennet at pH 6.8 and 1:3 diluted rennet at pH 6.0.

(d) Polyacrylamide Gel Electrophoresis

Electrophoresis was carried out under conditions detailed in Chapter IV:A:4.

3. Results

Results are given in Table XXVII.

Results given in Table XXVII and plotted in Figure 16 show that there is a marked decrease in both rate and extent of syneresis of samples containing 4% xylose and heated to 86°C for 40 minutes. Heating alone has caused a reduction in the extent of syneresis at pH 6.8 and 30°C although its initial rate appeared to be higher than that for the untreated control. This observation is similar to that of Grigorov⁽¹⁵⁷⁾, who, however, worked with yoghurt made from cows' milk. He reported that heating to 85°C for 20 to 30 minutes prevented a rapid and abundant separation of milk serum during storage. Dimov and Minerva⁽¹²⁰⁾ have reported on a similar reduction in syneresis of rennet-milk gels due to heating of the milks. The presence of a small amount of xylose (0.02%) during heating has had only slightly more inhibitory effect than heating alone. The presence of 4% xylose in the unheated control gives initially a much slower rate of syneresis. This increases with time and the final extent of syneresis is about the same as for the unheated sugar-free control. This effect may partly be due to increased viscosity of the system through the added xylose

Table XXVII. Effect of Heating to 86°C for 40 minutes in the Presence of Xylose, on the Rate and Extent of Syneresis of Calcium-Caseinate Gels at 38°C and pH 6.8.

Sample No.	Treatment	pH			Reflectance value ($\text{MgCO}_3=100$)	Gel point (mins)	Syneresis, mls. whey/40 mls. curd, after rennet addition			
		Initial	After Heating	Final adj.			25	45	90	120 (mins)
1	CONTROL (unheated)	7.20	-	6.8	63	< 5	8	28	34	35
2	CONTROL + 4% xylose (unheated)	7.20	-	6.8	70	< 5	5	15	32	34
3	CONTROL (heated)	7.20	7.25	6.8	63	< 7	10	17	25	28
4	+ 0.02% xylose (heated)	7.20	7.20	6.8	61	< 7	9	15	23	27
5	+ 4.0% xylose (heated)	7.20	6.75	6.8	43	< 8	3	10	20	24

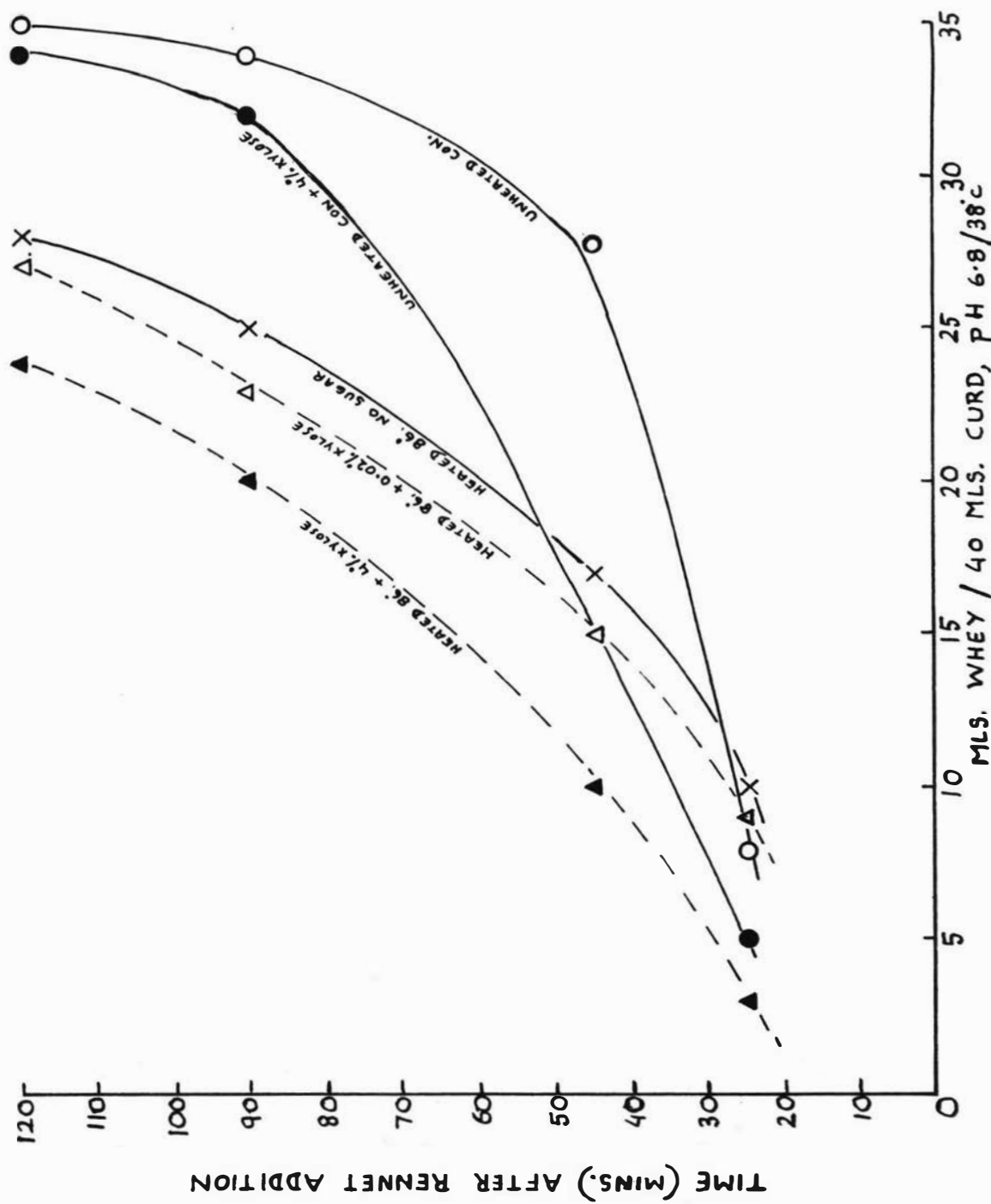


FIG. 16: SYNERESIS OF RENNET-CASEIN GELS AFTER PRE-HEATING TO 86°C/40 MINS. IN THE PRESENCE OF AN ALDOPENTOSE.

which would reduce mobility of the mobile structural elements within the gel structure. The effect observed with xylose is in contrast to Cheeseman's⁽⁷⁵⁾ observations that syneresis increased with added lactose.

The results clearly show that heating in the presence of 4% xylose greatly reduces rate and extent of syneresis at pH 6.8 and 30°C.

SECTION K: EFFECT OF HEATING TO 86°C FOR 40 MINUTES IN THE PRESENCE OF XYLOSE AND LACTOSE, ON THE RATE AND EXTENT OF SYNERESIS OF CALCIUM-CASEINATE SYSTEMS AT pH 6.0 AND 38°C.

As reaction with xylose had greatly reduced the extent of syneresis of rennet-casein gels at pH 6.8, it was of interest to study the effect of a lower pH on the inhibition of syneresis.

1. Experimental

"Artificial milk" at pH 7.1 and containing an equivalent of 3% casein and 100 mg% w/v calcium was prepared and treated in bulk as before. The samples were later heated to 86°C for 40 minutes in the presence of 4% xylose and 4% lactose at pH 7.1. The pH was then reduced with 0.1 N HCl to 6.0 and the samples made up to volume. Suitable heated and unheated controls were treated similarly. After adjustment of pH and volume, reflectance values relative to a MgCO_3 standard were measured in the Reflectance Spectrophotometer.

Syneresis was measured as before.

2. Results

Results are indicated in Table XXVIII.

Table XXVIII. Effect of Heating to 86°C for 40 minutes in the Presence of Xylose and Lactose on the Rate and Extent of Syneresis of Calcium-Caseinate Gels at pH 6.0 and 38°C.

No.	Treatment	pH			Reflectance value (MgCO ₃ =100)	Gel point (mins.)	Syneresis, mls.whey/40 mls.curd, after rennet addition				
		Initial	After heating	Final adj.			15	35	90	120	200 (mins)
1	CONTROL (unheated)	7.1	-	6.0	67	< 3	21	35	36	36	-
2	CONTROL + 4% xylose (unheated)	7.1	-	6.0	67	< 3	12	33	35	36	-
3	CONTROL + 4% lactose (unheated)	7.1	-	6.0	69	< 3	11	33	35	36	-
4	CONTROL (heated)	7.1	7.2	6.0	72	< 3	9	30	33	35	-
5	+ 4% xylose (heated)	7.1	6.7	6.0	49	< 3	1	3	15	22	27
6	+ 4% lactose (heated)	7.1	7.1	6.0	72	< 3	3	23	30	32	33

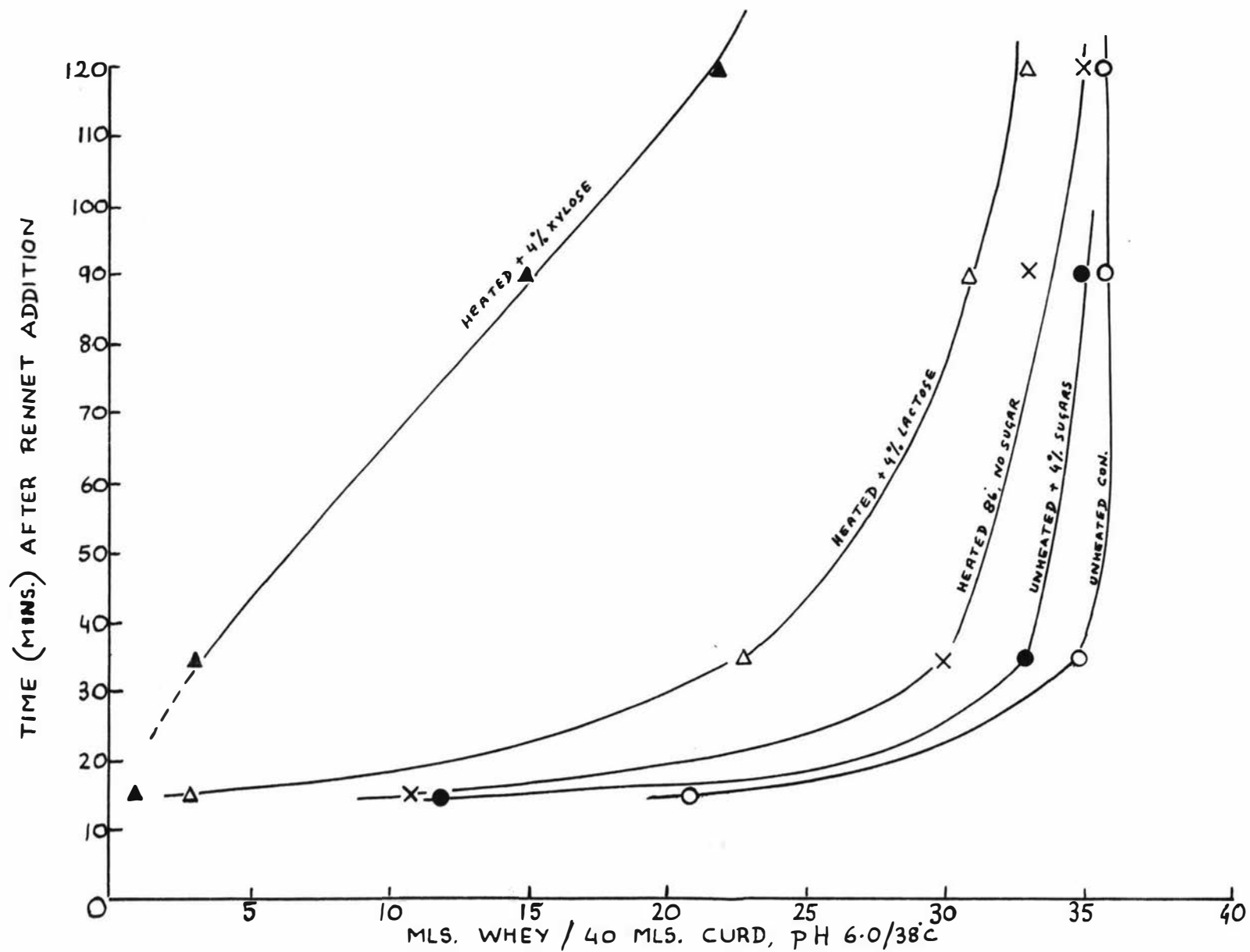


FIG. 17 : SYNERESIS OF RENNET-CASEIN GELS AFTER PRE-HEATING TO 86°C/40 MINS. IN THE PRESENCE OF ALDOSUGARS.

Results indicated in Table XXVIII and plotted in Figure 17 show that even at the high temperature of 38°C , and lower pH of 6.0 used in syneresis, the heated sample containing 4% xylose showed a greatly reduced tendency to synergise, when compared to the controls. A comparison with Figure 16 shows that the rate and extent of syneresis for the heated xylose "milk" is about the same despite the lower pH of 6.0 used in this study. This indicates that the free amino groups may take a direct part in the crosslinking during syneresis. The inhibitory effect may not be due solely to an increase in the electro-negative character of the protein.

Heating in the presence of lactose had much less effect on syneresis under the conditions of the experiment. However, the rate and extent of syneresis of the heated lactose treated "milk" was still less than the controls. From Table XXVIII it will be seen that there is no noticeable drop in the "milk" pH after heating in the presence of lactose. The reflectance value also has remained unaltered. If anything, it has shown a slight increase over the unheated control. This indicates that little browning reaction has occurred between the lactose and casein. However, Patton and Flipse⁽¹⁵⁹⁾ have isolated a colourless, labile complex of lactose and casein. They found that one-third of the lactose reacted with casein in one hour at 97°C . Such a reaction is possible in the heated lactose "milks" used in this study.

The browning reaction with xylose on the other hand, goes very much faster and to a greater extent. This is indicated by the drop in pH after heating and by greatly decreased reflectance values. This is in keeping with the observation that aldopentoses are more reactive towards casein than aldohexoses, with xylose being the most active⁽¹⁶⁰⁾.

The effects on syneresis of heating in the absence of sugar, has been reduced at the lower pH of 6.0. The rate of syneresis was slightly lower initially but this subsequently increased so that the extent of syneresis was about the same as the unheated control. Similarly, the somewhat slower rate of syneresis in the initial stages observed with unheated xylose-containing "milks" at pH 6.8 (Figure 16) have largely been eliminated at pH 6.0 (Figure 17).

All systems gelled within 3 minutes, indicating again that syneresis was not a continuation of the gelation phenomena although it may perhaps be dependent on the nature of the gel formed.

It is clear from the results obtained that sugar reaction with the free amino groups greatly reduces the syneretic properties of the rennet-casein gel. In the case of moist milk powders, the browning reaction has been attributed to a 1:1 interaction between the free amino groups of the milk proteins, largely the ϵ -amino groups of the lysine residues, and the potential aldehyde group of lactose⁽¹⁶¹⁾. A similar reaction between the aldoses and lysine may be occurring in the heated "milk" systems.

SECTION L: EFFECT OF HEATING TO 86°C FOR VARIOUS TIME INTERVALS
IN THE PRESENCE OF XYLOSE, ON THE RATE AND EXTENT OF
SYNERESIS OF CALCIUM-CASEINATE SYSTEMS AT pH 6.0 AND
38°C.

1. Experimental

"Artificial milks" at pH 7.1 and containing an equivalent of 3% casein and 100 mg% calcium were prepared and treated as before. The samples were heated to 86°C in the presence of 4% xylose for various time intervals. The pH was then reduced to 6.0 with 0.1 N HCl and the "milks" adjusted to volume.

Reflectance measurements were made to determine the extent of browning and the percentage loss in reflectance calculated.

Syneresis was measured as before at 38°C and pH 6.0, using 1:3 diluted commercial rennet.

Polyacrylamide gel electrophoresis was carried out as detailed in Chapter IV:A:4.

2. Results

Results are indicated in Table XXIX.

Table XXIX. Effect of Heating to 86°C for Various Time Intervals in the Presence of Xylose on the Rate and Extent of Syneresis of Calcium-Caseinate Gels at pH 6.0 and 38°C.

No.	Treatment	pH			Reflectance value (MgCO ₃ =100)	% loss in Reflectance	Gel Point (mins)	Syneresis, mls.whey/40 mls, curd, after rennet addition					
		Initial	After heating	Final adj.				15	35	60	90	120	200(mins)
1	CONTROL (unheated)	7.1	-	6.0	70	-	< 3	18	33	35	36	36	-
2	CONTROL (heated 10 minutes)	7.1	7.1	6.0	73	-	< 3	16	30	-	34	36	-
3	+ 4% xylose (heated 10 minutes)	7.1	6.8	6.0	68	6.9	< 3	0	27	31	33	34	-
4	CONTROL (heated 25 minutes)	7.1	7.1	6.0	75	-	< 3	12	27	32	33	35	-
5	+ 4% xylose (heated 25 minutes)	7.1	6.8	6.0	62	17.9	< 3	0	9	22	26	29	30
6*	CONTROL (heated 40 minutes)	7.1	7.2	6.0	72	-	< 3	9	30	-	33	35	-
7*	+ 4% xylose (heated 40 minutes)	7.1	6.7	6.0	49	31.5	< 3	1	3	-	15	22	27

* Values from Table XXVIII.

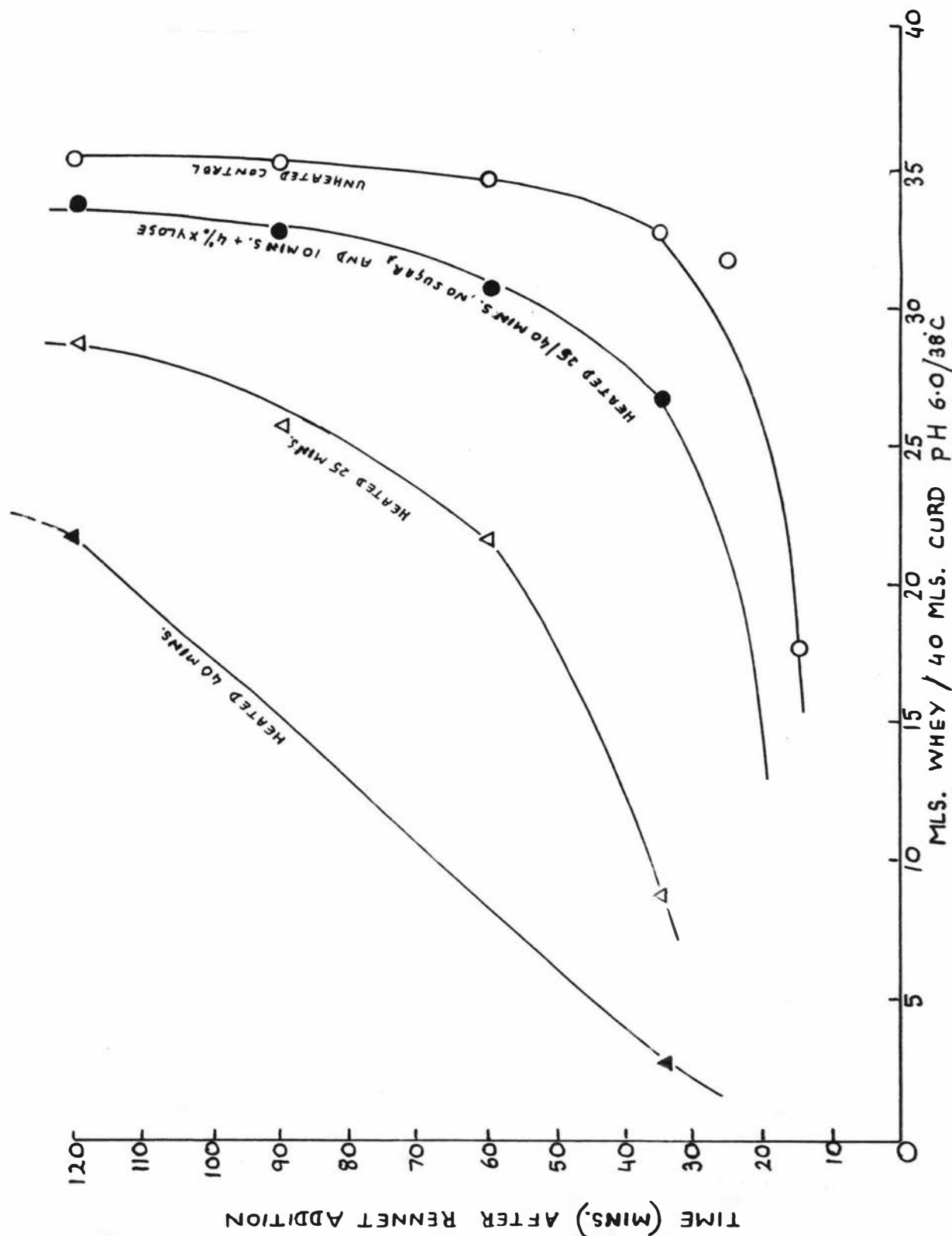


FIG. 18: SYNERESIS OF RENNET-CASEIN GELS AFTER PRE-HEATING TO 86°C IN THE PRESENCE OF 4% XYLOSE.

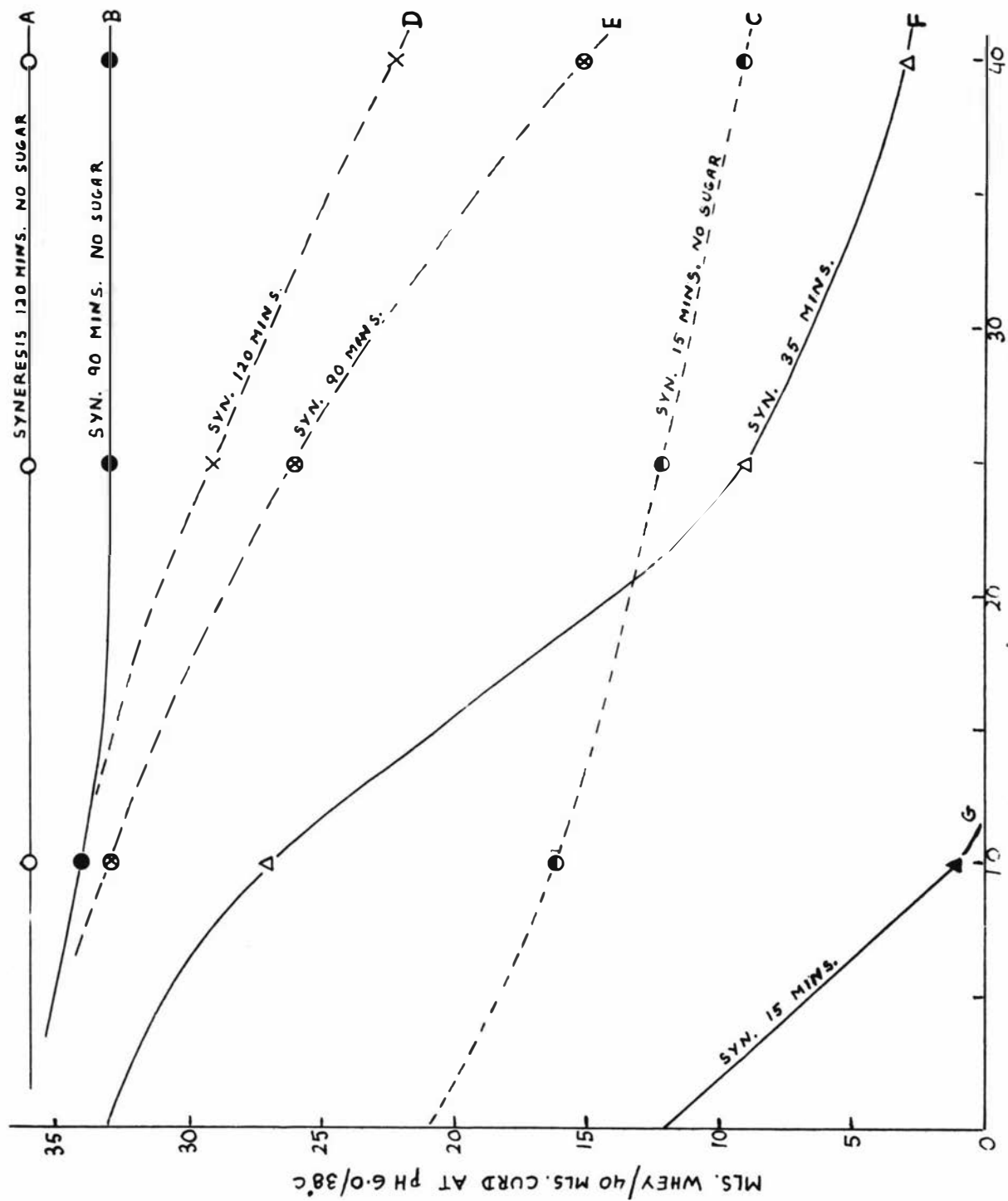


FIG. 19 : EFFECT OF HEATING TIME ON SYNERESIS OF RENNET-CASEIN GELS
TIME (MINS) AT 86°C IN PRESENCE OF 4% XYLOSE

Results given in Table XXIX and plotted in Figure 18 show the marked depressive effect on both rate and extent of syneresis, of increasing time of heating at 86°C , of the "artificial milks" containing 4% xylose. Heating for 10 minutes in the presence of the sugar had no greater effect than that of heating in the absence of sugar for 25 minutes or 40 minutes.

Data from Tables XXVIII and XXIX plotted in Figure 19 shows more clearly the effect of the time of heating at 86°C on the rate and extent of syneresis at the various time intervals during syneresis. Thus, when no sugar is present (curve A) the extent of syneresis after 120 minutes is not affected by the time of heating at 86°C . At the 90 minute level, (curve B), the effects due to heating alone have largely been neutralised under the conditions of the experiment, that is, at 33°C and pH 6.0. However, the rate of syneresis of sugar free "milks" becomes slower at the initial stages with increased time of heating at 86°C (curve C). This indicates that the curd from treated "milks" is slower in coming to an equilibrium during syneresis but speeds up so as to give the same extent of syneresis after 120 minutes as those curds from "milks" which have not been heated.

Heating of "milks" in the presence of sugar, however, gives a different picture. At 120 minutes (curve D), increased time of heating at 86°C continues to give a significant reduction in the extent of syneresis. So is the case at the 90 minute level (curve E). At the lower syneresis times, that is, 35 minutes and 15 minutes (curves F and G), there is a drastic inhibitory effect of heating time at 86°C on syneresis. This is particularly marked between 10 and 25 minutes heating at 86°C after which the effect lessens (curve F).

Figure 20 shows a linear relationship between % loss in reflectance

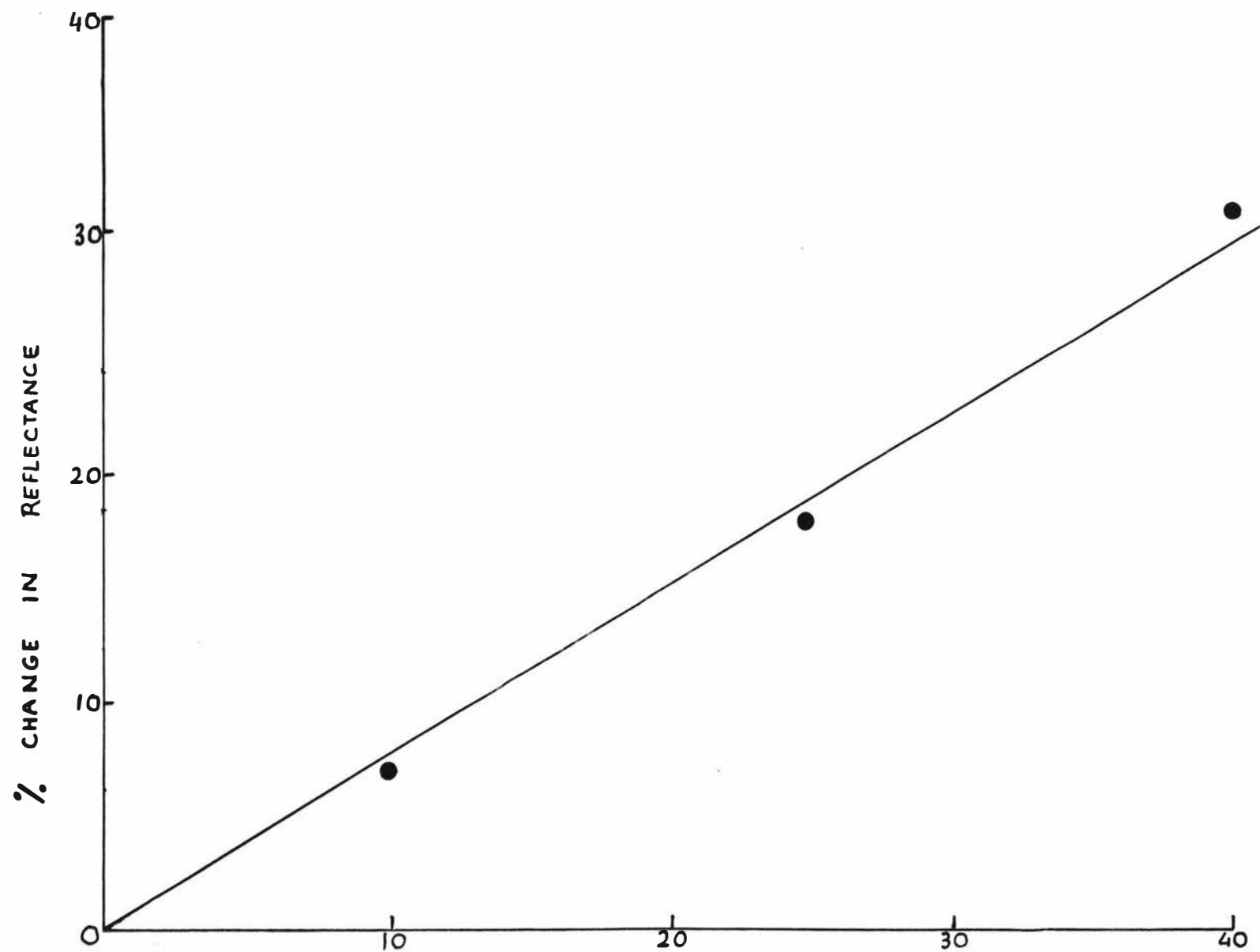


FIG. 20: RELATION BETWEEN % CHANGE IN REFLECTANCE (BROWNING) AND TIME OF HEATING AT 86°C.

and the time of heating at 86°C in the presence of 4% xylose. This indicates that browning reaction proceeds linearly with time, up to at least 40 minutes heating at 86°C .

It is clear, therefore, that modification of the free amino groups in the casein micelles in the "milks", through reaction with reducing sugars, greatly inhibits both rate and extent of syneresis of the rennet-treated calcium-caseinate gels.

To determine which of the casein components was most affected, polyacrylamide gel electrophoresis was carried out on the unheated and heated samples (25 minutes at 86°C). The result is shown in Plate 28.

It is clear from the electrophoretic patterns that there is little difference between sample 1 (unheated - no sugar) and sample 2 (heated $86^{\circ}\text{C}/25$ minutes) indicating that heating to 86°C has had little degrading effect on the caseinate. None of the major or minor proteins appear to be affected. Sample 3, however, shows that heating for 25 minutes to 86°C in the presence of xylose, greatly affects the pattern for β casein, giving a diffused tailing zone. Some of the minor protein components ahead of β casein also seem to be affected, but neither α_s nor kappa-casein, particularly the latter appear to be affected to any great extent. It appears reasonable to interpret the reaction between the casein micelles and xylose as being mainly one with β casein free amino groups, probably in the main with $\epsilon\text{-NH}_2$ groups of lysine.

The conclusion from the results obtained on the effects of heating in the presence of reducing sugars, on syneresis, is the same as that given for the effects of 1-fluoro 2:4 dinitrobenzene, that is, lysine $\epsilon\text{-NH}_2$ groups of β casein probably play an important role, possibly directly, in the syneresis of rennet-casein gels.

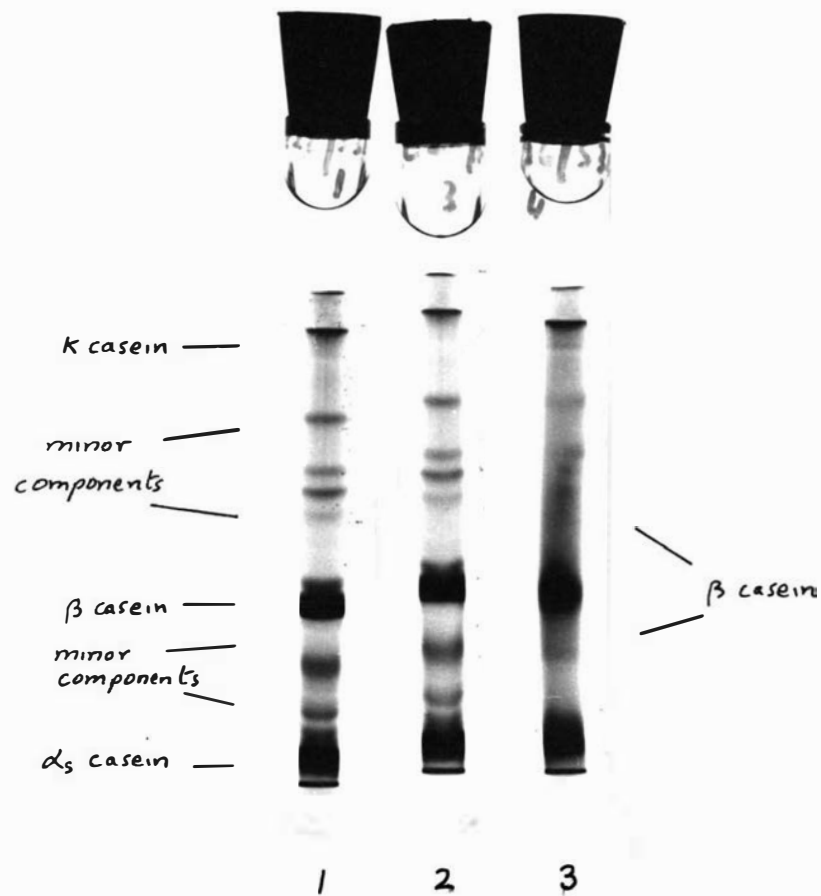


PLATE 28:

P.A.G.E. Patterns of heated 'artificial milks'
(100 mg % calcium, 86°C/25 mins., pH 7.1)

- 1. Unheated control - no sugar**
- 2. Heated control - no sugar**
- 3. Heated + 4% xylose**

Note: β -casein affected in the main in sample 3.

SECTION M: DISCUSSION ON THE EFFECT OF MODIFICATION OF THE FREE
AMINO GROUPS IN CALCIUM-CASEINATE SYSTEMS ON THE
SYNERETIC PROPERTIES OF RENNET-CASEIN GELS.

It has been usual to consider the effect of heat treatment of milk in terms of denaturation of whey proteins⁽¹⁰⁴⁾ and to think of casein micelle - fat globule interaction⁽¹¹⁸⁾ as being responsible for weak rennet-milk gels and the high whey retention of these gels. "Artificial milks" used in these studies were devoid both of fat and serum proteins. In addition colloidal calcium phosphate was absent so that heat effects were limited to modifications, if any, of the proteins themselves.

The modification of the amino groups in casein micelles has been clearly shown to retard syneresis of the calcium caseinate gels. In most cases involving chemical modification of the functional groups on casein, research workers have treated individual proteins in more or less pure form and then studied the effects of such treatment. This technique suffers from certain disadvantages. It involves extensive and often laborious procedures to isolate the protein in pure form, often by the use of denaturing agents such as urea, and the use of variations in pH and of various salts. During the process of isolation, there is no guarantee that the protein has not been modified so as to affect the results. Furthermore, in nature the proteins rarely occur as independent molecules or proteins but rather as complex aggregates as in the case of milk. It may, therefore, prove more useful to treat complex protein systems as they occur, and then attempt to find out which protein is affected to a greater or lesser extent.

A study of the effect of modification of the amino groups on the synergetic properties of rennet-casein gels was initiated by the finding of Hill and Craker⁽⁴³⁾ that modification to an extent of 20% of the lysine side chains on kappa-casein with dimethylaminonaphthalene sulphonyl chloride inhibited coagulation of the rennin-treated protein. This study was carried out on individual protein preparations. Earlier, Bargoni⁽¹⁴⁷⁾, using whole casein in non-micellar form, had found after extensive modification of the amino groups, that clotting was inhibited or prevented by such treatment. Woychik⁽¹⁴⁸⁾ reported that rennin-treated trifluoroacetylated kappa-casein would not clot.

It was, therefore, of interest to determine the effect of such specific agents on both gelation and syneresis, but after reaction with the caseinate in micellar form. To this end, therefore, "artificial milks" containing 100 mg% (w/v) calcium were used. For reactions with the amino groups, 1-fluoro-2,4 dinitrobenzene (F.D.N.B.), potassium cyanate, and o-methyl-iso urea, and also reducing sugars, were employed. Except for o-methyl-iso urea which reacts only with the epsilon amino group of lysine, these compounds are only broadly specific in the sense that all amino groups can react with them. Thus F.D.N.B. can react with all free amino groups as well as with the imidazole group of histidine, phenol group of tyrosine and the sulphhydryl group of cysteine⁽¹²²⁾. However, most of the α amino groups in the intact protein are involved in the peptide link and therefore are not available for reaction with the F.D.N.B. In β casein, almost 90% of the free amino groups are supplied by the lysine side chain⁽¹²²⁾. In this study, F.D.N.B. treatment of "artificial milks" appeared to affect only the β casein — gel electrophoresis, as shown in Plate 25, showed that the mobility of β casein was increased

substantially after F.D.N.B. treatment but no other fractions were affected by the 24 mg treatment. There is some indirect evidence to indicate that most reagents specific for amino groups, react primarily with the lysine side chain; although if the reaction is prolonged and/or the concentration of the reagent is high then other amino acid residues would also react. Thus Wormell and Kaye⁽¹⁴⁹⁾ reported that formaldehyde under neutral conditions first becomes attached to each lysine side chain. Kim et al⁽¹⁵⁰⁾ found that ammonium cyanate, present as an impurity in concentrated urea solutions, reacted primarily with lysine side chains to give homocitrulline derivatives. Stark et al⁽¹⁴⁰⁾ reported that carbamylation of ribonuclease with potassium cyanate produced in the main homocitrulline. Manson⁽¹⁴²⁾ has also reported similarly. Thus, under conditions where only small quantities of the reagent are used, it was assumed that the reaction would be limited to that chemical grouping on the protein with which the reagent preferentially reacts.

In the present work "artificial milks" were reacted with 0 - 64 mg F.D.N.B. for one hour at 30°C. Under these conditions, the reaction of the casein micelles with F.D.N.B. was found to have a linear relationship with time for the first 30 - 40 minutes at the 24 mg level of F.D.N.B. treatment (Figure 8). The relationship between quantity of F.D.N.B. and the final reaction pH (Figure 9) indicated that up to 32 mg F.D.N.B. a linear relationship held. This indicated that the reaction was probably specific to one chemical grouping on one protein. Beyond 32 mg addition of F.D.N.B., the reaction probably became more generalised. Treatment with 24 mg and less of F.D.N.B./ 100 mls. milk resulted in changes in electrophoretic mobility of only

one protein in the casein micelle, that is β casein (Plate 25). It appears, therefore, that the position and tertiary structure of β casein within the micelle is such that it is able to readily react with F.D.N.B. when compared with the other major proteins and that it plays an important role in syneresis. Extension of the straight line in Figure 10 indicates that at a concentration of 67 mg added F.D.N.B. no syneresis would have occurred. It is interesting to note that in "artificial milks" containing 3 g casein, the lysine content in β casein, 30% of whole casein⁽¹³⁾, approximates to 60 mg⁽¹²²⁾. For the modification of all ϵ -NH₂ groups of β casein, about 76 mg F.D.N.B. would be required on an equimolar basis. The figure of 67 mg F.D.N.B. obtained by extrapolation of the line in Figure 10 is close to this value. The similarity appears to be more than just coincidental, particularly if one refers to the electrophoretic pattern shown in Plate 25, where extensive modification of the β casein pattern after F.D.N.B. addition is obvious. It is, therefore, reasonable to presume that the ϵ -NH₂ group of lysine in β casein plays a major role in the syneresis of rennet-casein gels. Electron micrographs of "milks" treated with 24 mg F.D.N.B. (Plate 26) indicate that the physical organisation of the casein micelles are little affected by F.D.N.B. treatment. The F.D.N.B. appears to have reacted over the whole area of the micelle, giving a hazy appearance to the picture. Thus, the ϵ -NH₂ groups of β casein while appearing to readily react with F.D.N.B. do not appear to play an important role in the stabilisation of the spherical casein micelle.

The reaction of F.D.N.B. with ϵ -NH₂ groups of β casein would be expected to change the charge pattern of the system making the system, and in particular β casein, relatively more negative in character.

The reduction or inhibition of syneresis could have been due to this increased electro-negativity of the system. Another likely reason is the prevention of direct participation of the lysine side chain in β casein in crosslinking during syneresis. Syneretic studies at different pH levels (Figures 11, 12 and 14) indicate that the change in ionic character of the protein does play a part in the inhibition of syneresis, because reduction in pH does give increased syneresis, although the final extent of syneresis was well below the control values. The linear relationship between the reciprocal of the quantity of whey expelled with pH of the systems (Figure 12) suggests that steric hindrance from F.D.N.B. plays little or no part in the inhibition of syneresis. The extent of syneresis is, however, very much less when compared with the controls, even at low pH levels such as 5.4 and 4.0 (Figures 12 and 14). This suggests that increase in electro-negativity of the protein may not be the only reason for the reduction in syneresis. The ϵ -NH₂ group may take part directly in the crosslinking. That this may be the case is indicated by the fact that guanidination of the casein system with *o*-methyl-iso urea, which substitutes only a slightly more basic guanidino group in place of the ϵ -NH₂ group, does reduce syneresis, despite the fact that the charge pattern is little changed (Table XXVI, Plate 27). The extent of reduction in syneresis is, however, much less than in F.D.N.B. treated "milk".

Heating of calcium caseinate solutions in the presence of reducing sugars has also been found to give greatly reduced syneresis. The temperature and times of heating used in this study in the absence of reducing sugar have had in themselves little, if any, modifying effect on the structure of the various casein components. This is evident

from the electrophoretic patterns in Plate 28.

The effect of heating in the presence of aldopentose has been greater than that in the presence of an aldohexose on the reduction of syneresis. Syneresis is greatly inhibited even at pH 6.0 and 38°C.

Electropherograms (Plate 28) of treated and untreated samples, clearly show that β casein is the main protein effected by the reaction with the aldopentose. The reaction probably involves the ϵ -NH₂ groups and is again indicative of a micelle structure in which β casein is readily able to react with the aldoses under the conditions of the experiment, while α , kappa-casein, and the minor casein components remain relatively unaffected.

Results on syneresis obtained from heating "milks" in the presence of aldoses complement observations made with F.D.N.B. treated systems; that syneresis of rennet-casein gels is reduced in the main through increasing the electro-negative character and, therefore, repulsion of the gel structural elements, and/or preventing the direct participation of ϵ -NH₂ groups of β casein in the crosslinking.

It is difficult to see in what sort of forces or crosslinks the ϵ -NH₂ groups of lysine may be involved. The most simple explanation may be that they are electrostatic or ionic, in character. Rennin action on the casein micelles possibly permits the positive charges on the ϵ -NH₂ groups to come into juxtaposition with the negative charges on the carboxylate groups, so that new forces of attraction are created.

It is also possible that after rennet action cations such as calcium may be bound to β casein by chelation involving carboxyl and amino groups once coagulation has occurred. Calcium has been shown to be necessary for syneresis of rennet-casein gels (Chapter V:D). It was pointed out then that both rennet modification of the caseinate

and calcium were necessary for the syneresis to occur. Baker and Saroff⁽⁷¹⁾ have indicated that chelation of calcium between amino groups and carboxylate groups through the formation of a co-ordinate covalent bond is possible. There are several examples of metal chelate compounds⁽¹⁵¹⁾. Diamino ethane tetra acetic acid (E.D.T.A.) is a well known metal chelating compound which chelates calcium at an optimum pH of 7.5⁽¹⁵²⁾ by a similar system. Such metal chelates are generally unstable in acid conditions⁽¹⁵²⁾. Ter Horst⁽¹⁵³⁾ in proposing a new theory on the caseinate complex in milk presented evidence to indicate the binding of $(CaPO_4)$ to lysine $\epsilon-NH_2$ groups in casein in the form $R-NH_3PO_4Ca$. Hostettler and Ruegger⁽⁷²⁾ found that the pH of caseinate solutions dropped on the addition of calcium ions. They interpreted this as being due to exchange of protons on the amino groups on casein, for calcium. Thus, it is possible that the $\epsilon-NH_2$ groups of lysine in β casein may bond calcium at near neutral pH through a chelation phenomena involving carboxylate groups also.

The specific reaction of F.D.N.B. and aldoses with β casein indicates that the latter is readily available for reaction within the micelle. The structure of β casein would have to be an open one to permit such a rapid reaction with the lysine side chains, with little or no hindrance from either α_s or kappa-caseins. Payens and Markwijk⁽¹⁵⁴⁾ have, in fact, suggested that β casein molecules tend to associate by lyophobic forces into elongated thread-like structures, probably randomly coiled. Rose et al⁽¹⁵⁵⁾ reported that association of the protein sub-units to give primary particles was not α_s to α_s nor β to β but was an α_s to β inter-association in roughly a 3:2 ratio. The association was probably through weak hydrophobic forces. These facts,

along with others⁽⁹¹⁾⁽⁹²⁾⁽⁹³⁾, led Rose⁽¹⁵⁶⁾ to propose recently a new model for the casein micelle. In this model he represented β casein diagrammatically as randomly coiled elongated threads, with which α_s casein was associated. The highly charged kappa-casein molecules containing the hydrophilic glycomacropeptide was associated with widely dispersed α_s casein molecules. The elongated randomly coiled β casein polymers were shown to permeate the micelle and to be present over its whole surface. The model proposed by Rose⁽¹⁵⁷⁾ explains the ready reaction of β casein with F.D.N.B. and aldoses, with little hindrance from either α_s or kappa-casein, the reaction being particularly specific for β casein at low F.D.N.B. concentrations (see Plate 25). In this work, electromicrographs of F.D.N.B. treated casein micelles (Plate 26) had shown a uniformly diffused picture, and it was suggested that β casein is spread through and over the body of the micelle. Rose's model⁽¹⁵⁷⁾ for the casein micelle is quite in accord with this postulation. (The reference came to the author's notice after the electron micrographs had been taken and electrophoretic patterns had suggested that β casein was involved in syneresis.) It was shown earlier in electron micrographs (Plates 4 - 11) that gelation of rennet-casein gels was essentially a surface phenomena, probably involving para-kappa-casein on the different micelle surfaces, the crosslinking being probably through calcium. This could also bring β caseins in the neighbouring micelles (coagulated micelles) into closer juxta position, to enable further crosslinking, through calcium and/or by electrostatic forces. It now seems possible that β casein mainly is involved in the secondary crosslinks at neutral pH. Syneresis is a slower process than when acidity is developing. This is probably because fewer β casein molecules or aggregates are available for

interaction after gel formation, most of them probably being in micellar form. During developing acidity the negative repulsive charge would be neutralised. This may be accompanied by displacement of calcium from the curd structure as well as from within the micelles. The disintegrating micelles would then provide more mobile structural elements within the curd. Since elongated threads are more likely to participate in such interactions it seems likely that the β casein threads will be mainly involved. These would tend to react with each other and with the rigid structural elements of the network, probably by electrostatic, and also hydrophobic, forces so that syneresis is increased. High temperatures increase syneresis probably by increasing the rate of contact between the network elements and by formation of hydrophobic bonds which is an endothermic process⁽⁹⁷⁾.

The inhibitory action on syneresis of F.D.N.B. and of xylose in heated "milks" may be attributed to an increase in the electro-negativity of the β casein threads through blocking of the free ϵ -NH₂ groups, thereby creating repulsion within the gel system. This would be so at all pH levels. Blocking of the free amine groups would also reduce potential electrostatic forces of attraction in which the free amino groups may take part, and thereby reduce syneresis. Using Rose's model, Figure 21 depicts schematically the syneretic process as it may be occurring in rennet-casein gels.

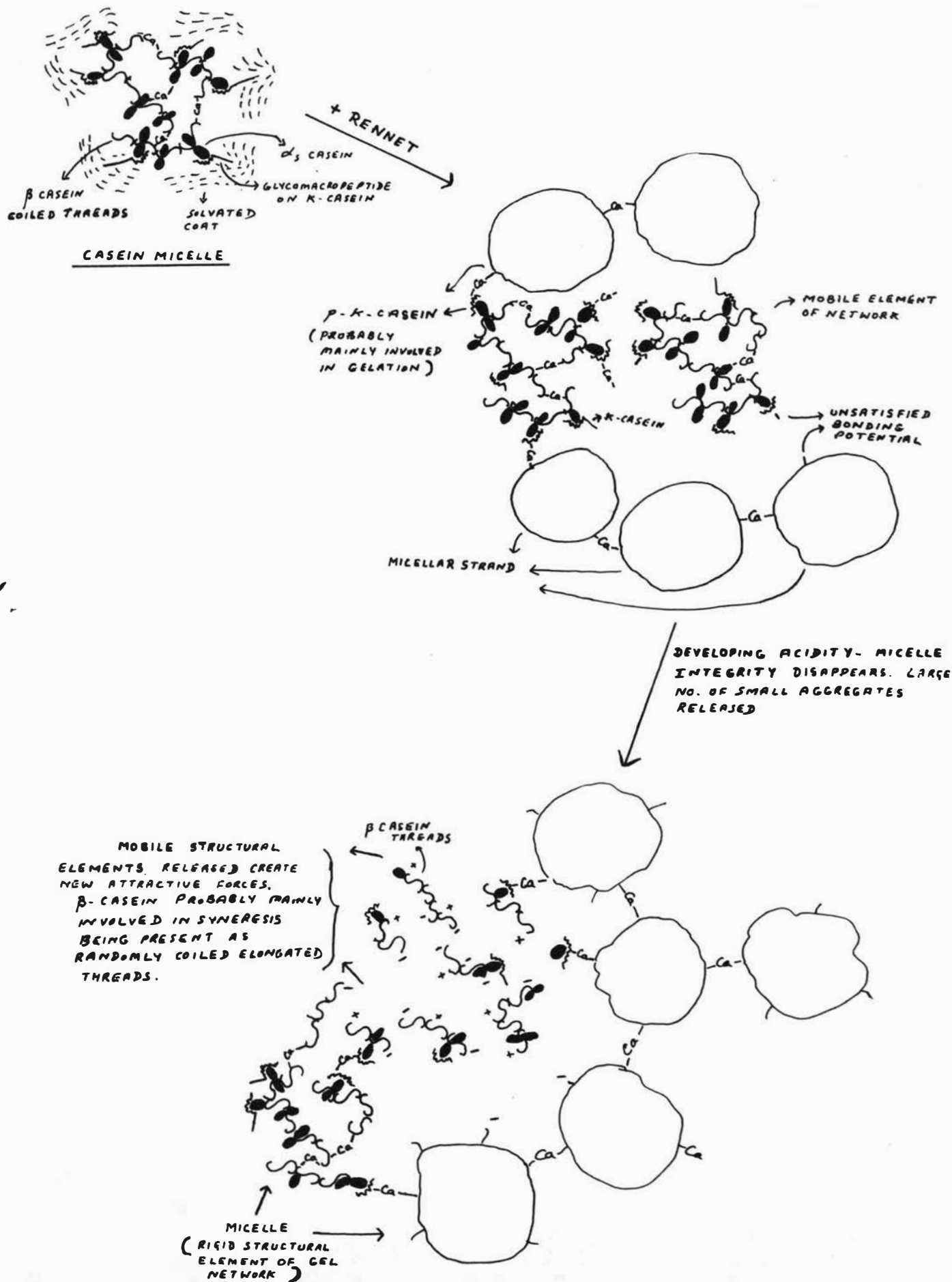


FIG. 21: SCHEMATIC REPRESENTATION OF THE PHENOMENA OF SYNERESIS AS OCCURRING IN RENNET-CASEIN GELS.

CHAPTER VII

Summary and Conclusions on the Study of Factors Contributing to Gel Formation and Syneresis of Rennet-Casein Gels

The study of factors contributing to gel formation and syneresis of rennet-casein gels was made on model systems or "artificial milk". The "milks" were, in essence, calcium caseinate systems prepared under specified and controlled conditions. The systems were stable even when heated to 90°C to 100°C for 1 hour. Since temperature plays an important part in the associations between the casein components as individual units⁽⁹¹⁾, and also between these units and calcium, and because casein micelles are formed under natural conditions at body temperatures, the "artificial milks" were prepared at 35°C rather than at refrigerator temperatures as has been done by others. In a limited number of experiments in this study calcium caseinate - calcium phosphate "milks" were used. However, the decision to discontinue using phosphate containing "milks" in the experiments stemmed from the fact that in the presence of disodium hydrogen phosphate or orthophosphoric acid it was observed that small irregular casein micelles were formed. These were quite unlike the physical organisation and size of casein micelles in natural skim milk. On the other hand, "milks" containing no added phosphate, particularly those containing 60 - 125 mg% w/v calcium, showed micelles very similar in size and general appearance to those in skim milk. The electron micrographs emphasise the important role of ionic calcium in acting as a cement for micelle integrity.

Viscosity studies at 0°C on both sodium caseinate and calcium caseinate "milks" indicated a significant drop in specific viscosity values during the initial phase of rennet action on the caseinate.

Since modification of molecular charge has been shown not to affect intrinsic viscosity in other proteins such as serum albumin⁽⁹⁶⁾, it was postulated that the fall in viscosity of rennetted-"artificial milks" was largely due to the release of "immobilised" water from casein micelles into the system. It is difficult to prove, nevertheless, that modification of the charge of the casein micelles, due to the release of the glyco-macro-peptide by rennet action, does not play a role in the drop in viscosity. Payens⁽⁹¹⁾ has stated that the zeta potential for casein particles is low, consequently electrical repulsion between the micelles would be low. Hydration or an "immobilised" water layer around the micelles could therefore assume an important stabilising role. An ultra-centrifugal technique was developed to measure the release of "immobilised" water, using haemoglobin as an indicator. It was found that rennet-treated micelles in "artificial milks" released into the system at, or about, gel point, a quantity of "immobilised" water equal to about 70% of the weight of the wet protein (caseinate). The term "immobilised" water is used to signify that the technique used for its determination does not discriminate between (i) true chemically bound water, which may not exceed 20-30% of the weight of the wet caseinate, (ii) water held within and on the surface of micelles in the form of icelike regions, and associated with hydrophobic amino acid residues, or (iii) mechanically trapped water within the micelle.

The electron micrographs of rennetted "artificial milks" and skim milks (Chapter II:D. Plates 4-11 and 17-21) clearly show that gelation is essentially a surface phenomenon between the "micelles". At gel point, the gel network is comprised mainly of "beaded" threads, made up of joined micelles. The micellar threads link up with others to

form a 3-dimensional network, enveloping the liquid in the process. It was demonstrated that fairly large concentrations of sodium thioglycollate, in excess of 200 mg/40 mls. "milk", or small concentrations of dithiothreitol (20 mg) prevented a gel structure from forming. Rather a precipitate formed and settled at the bottom of the beaker, at the same time giving rise to an opaque supernatant. Rennet action itself was not inhibited. It is recognised⁽⁹¹⁾⁽³⁾ that the glycomacropeptide and the sugar moiety of kappa-casein which rennin splits off, and the sulphur amino acid cystine are situated at the opposite and distant ends of the kappa-casein molecule. The splitting of the disulphide bond does not give rise to instability of the "milks", probably because the highly hydrophilic glycomacropeptide is still attached to the "micelle" surface. However, if this is followed by the release of the glycomacropeptide by rennin, a precipitate forms rather than a gel. One possible explanation of this observed fact is that the whole para-kappa-casein is needed on the micelle surface to give rise to a 3-dimensional network. The surface to surface interaction at gel point thus probably involves only the para-kappa-casein, which is probably still attached to the micelle surface, and ionic calcium.

The attendant release of "immobilised water" from casein micelles which seems to occur concurrently with the gelation phenomena as observed in this study, is in itself not a new idea. Lauffer⁽¹⁶²⁾ and Stevens and Lauffer⁽¹⁶³⁾ found that polymerisation of T.M.V. protein released water into the environment, the polymerisation itself being an endothermic process. One explanation offered by them⁽¹⁶³⁾ was the possible release of icelike domains of water normally associated with proteins in the disaggregated state about their hydrophobic groups.

In the "silk" systems one might liken the micelle to a huge protein molecule, stabilised by large domains of icelike water on the surface, and perhaps within. Rennin probably destroys the protective icelike domains enabling the micelles to link up through calcium and the para-kappa-casein segment. The preference for rennin to split bonds adjacent to hydrophobic residues is known⁽⁷⁾ and even in casein rennin is believed to attack a phenylalanine-methionine bond on the kappa-casein⁽³⁾. Apart from this, rennin action also removes the highly hydrophilic glycomacropeptide from the caseinate surface, thereby removing a highly hydrated portion of the coat.

After this work was completed, two recent references came to the author's notice which lend support to the contention that a solvated or an "immobilised water" layer around the casein micelles is a prominent factor stabilising the micelles against aggregation. Keyser⁽¹⁶⁴⁾ using 1 - ¹⁴C labelled lactose found that about 20% water on wet caseinate basis was released during the coagulation stage of rennet action. This was apparently a slow process and differs somewhat from the view presented in this work. Hill and Wake⁽¹⁶⁵⁾ in a detailed analysis of all available data on the caseinate complex conclude that kappa-casein is the first recognised example of a protein that has evolved to function as a detergent. The N- terminal, two-thirds of the kappa-casein molecule is hydrophobic and is said to remain attached to the micelle surface by hydrophobic interactions after rennet action. However, the C- terminal one-third portion of the kappa-casein molecule (glycomacropeptide) is hydrophilic and faces outward to give the micelles a negatively charged solvated surface envelope, which is removed by rennet action. The two regions probably function respectively to anchor the kappa-casein molecule to the hydrophobic calcium α_s - β caseinate core and to provide a solvated coat,

stabilising the micelles against aggregation. Because of its possessing two distinctly different portions on the same molecule, kappa-casein may be considered to be an amphiphile, which gives it a "detergent" function. Hill and Wake⁽¹⁶⁵⁾ state that casein micelles can be regarded as hydrophobic particles with a stabilising hydrophilic coat. Rennin removes this coat and the hydrophobic cores are no longer stable in solution. The surface hydrophilicity would account for a high degree of solvation on the surface, which rennet action would upset.

Gel formation does not appear to involve a sulphhydryl-disulphide nor a disulphide interchange reaction as has been postulated by others (22)(63). This is despite the fact that all cystine and cysteine⁽⁸²⁾ appear to be concentrated in the kappa-casein fraction. The concentration of both amino acids in casein are relatively much lower⁽⁸²⁾ than in other proteins where sulphhydryl-disulphide interchange reactions⁽¹⁶⁶⁾ do play a part in the gelation phenomena.

Calcium was found to be important both for gelation and for syneresis of rennet-treated systems. In its absence, neither phenomena occurs. In what way it provides crosslinks is still obscure. A link with calcium between the negatively charged carboxylate on one hand and the phosphoric groups on the other has never been satisfactorily proved. Attempts to correlate rennin clotting with changes in calcium binding by the para-casein have led to conflicting reports^(167 - 169). Instead of a straight calcium cross linkage, the aggregation may be induced by diminished solvation, as has been shown to be the case in this work, and to an ionic charge effect which would tend to neutralise the negative carboxylate groups and therefore reduce repulsion. Preliminary work by the author had

indicated that the rennet-casein gel was easily solubilised in urea solutions of greater than 4M concentration. It is difficult to explain how a gel with large scale cross links involving calcium could disperse so easily in a urea solution. The forces must be weak enough for urea to be effective. Hill and Craker⁽⁴³⁾ have shown that dansylation of kappa-casein lysine side chains prevented aggregation of the rennet-treated protein and they interpreted this to mean that the lysine side chain in kappa-casein plays a direct role in coagulation. Baker and Saroff⁽⁷¹⁾ have indicated the possibility of the imino groups of histidine or the ϵ -NH₂ groups of lysine participating in a chelating action involving calcium, with the carboxylate as the other reacting group. There are other instances of such chelate co-ordination compounds⁽¹⁵¹⁻¹⁵³⁾. It is possible, therefore, that in rennet-casein gel systems, the 3-dimensional network is formed by a reaction between lysine ϵ -NH₂ groups on para-kappa-casein and carboxylate groups on a different micelle, with calcium being chelated in the process. Such calcium links would be dissociated in the presence of acidity. It is also possible that calcium bridges of this type would not make the network insoluble in urea solutions.

Despite the fact that absence of calcium prevents both gelation and syneresis of rennet-casein gels, it does not necessarily follow that syneresis is a continuation of the gelation phenomenon. In fact it is clear from the present work that it is not so. Treatments which profoundly affect syneresis do not affect gelation. Syneresis, however, could be dependent on the type of gel formed, since subsequent interactions during syneresis may be affected by the number and type of crosslinks formed initially during gel formation. Studies on dinitrophenylation of the casein micelles and heating of "milks" in the

presence of aldo sugars and subsequent syneresis of the "artificial milk" gels indicates that β casein may be dominantly involved in this phenomena (Plate 25). Gelation was not observed to be affected by the limited dinitrophenylation. There was less inhibition of syneresis at lower pH values indicating that part of the effect of F.D.N.B. treatment was probably due to increasing the electro-negative character of the system. This, however, appears to be too simple an explanation. Even at the low pH of 5.4 and at higher temperatures of 38°C , the inhibition of syneresis was great compared to controls. In systems which had been allowed to gel at pH 6.8 and at 30°C , and then the conditions changed gradually (increase in temperature to 38°C and decrease in pH to 4.0), the F.D.N.B. systems did not synerese to the same extent as the controls. Conversion of the $\epsilon\text{-NH}_2$ group of lysine to a guanidino group by treatment with *o*-methyl-iso urea also gave a reduction in syneresis despite the fact that a basic $\epsilon\text{-NH}_2$ group had been replaced by the slightly more basic guanidino group. This indicated that the lysine side chain participates directly in the crosslinks or interactions to give syneresis of rennet-casein gels. The effect of heating "artificial milks" in the presence of reducing sugars has also been found to greatly decrease syneresis and electrophoretic patterns shown in Plate 28 again suggest that β casein is primarily involved.

Gel formation through lysine $\epsilon\text{-NH}_2$ groups from the para-kappa-casein and carboxylate and calcium interaction probably brings the surface β casein threads of different micelles into close juxta position for further interaction. This could occur through the same chelation phenomenon involving $\epsilon\text{-NH}_2$ groups, carboxylate groups and calcium at near neutral pH, as postulated for the initial gelation. Thus new bonds are established, the system contracts and syneresis results.

In the presence of developing acidity the chelation complex would become increasingly less stable. However, the possible reduction in syneresis anticipated as a result of this does not occur because of two independent modifying factors which also arise from the developing acidity. Increased hydrogen ion concentration would tend to neutralise the negative charges and thus reduce the repulsive forces between the individual casein components and, secondly, it will cause the micellar form to disaggregate thus releasing a large number of protein sub-units. The effective protein concentration, and hence bonding potential, would thus be increased through van der Waal and electrostatic forces, with β casein playing the major role in syneresis.

There is an apparent conflict in concept between the part played by ϵ -amino groups on the lysine in para-kappa-casein and calcium bridging in the formation of the gel and the similar reaction postulated for the syneretic behaviour based on the lysine groups in β casein. It is argued that the lysine groups in kappa-casein are not exposed and consequently do not react with F.D.N.B. under the conditions in the experiments reported here. Consequently, they are still available for complexing with calcium after rennet action has exposed them in the para-kappa-casein, whereas the lysine in the β casein is now unable to participate in a similar reaction. Hence gelation occurs but syneresis is reduced under these conditions.

The effect of increase in temperature is probably more simple; that of increasing movement and rate of contact and perhaps also in facilitating hydrophobic bond formation, which is an endothermic process.

β -Casein being in the form of elongated randomly coiled fibres,

is an ideal protein for these various interactions compared with the globular α_s casein, since the functional groups (e.g. ϵ -NH₂) would tend to be more exposed. Since it also comprises about 30% of the total caseinate, its apparently dominant role in syneresis is therefore not too surprising.

CHAPTER VIII

Lines of Future Research

While some evidence has been provided in this work for the initial desolvating action of rennin on the caseinate micelles, more sensitive techniques need to be developed for the measurement of the release of "immobilised water". The possibility of para-kappa-casein on the micelle surface taking part in the initial gel structure needs to be further investigated. While it has been shown that there is little or no scope for the formation of disulphide links in the gel network of rennet-casein gels, and that calcium is necessary for both gelling and syneresis in rennet-casein systems, it has not been made clear as to what form the calcium crosslinks take in either phenomena. The "chelate" co-ordination compound theory involving the $\epsilon\text{-NH}_2$ group of lysine, the carboxylate group and calcium is speculative in character and based on limited evidence, although it does appear that both lysine side chains and calcium are involved in the two phenomena. This aspect merits detailed investigation. That the reaction of F.D.N.B. and the sugars with β casein in the heated "milks" occurs, in the main, with the $\epsilon\text{-NH}_2$ groups of lysine needs to be more thoroughly checked.

Selection of this topic for research was prompted by the general view held⁽¹⁷⁰⁾ that buffaloes' milk curd when coagulated with rennet gives excessive syneresis and consequently a tough rubbery body in both Gouda type and Cheddar type cheeses made from it. Buffaloes' milk has been reported⁽¹⁷¹⁾⁽¹⁷²⁾ to contain much more β casein than cows' milk, being 52.4% against 39.1% of whole casein. Buffalo milk β casein has

been reported to have a slightly slower mobility than cows' milk β casein. Buffaloes' milk has also been reported⁽¹⁷³⁾ to contain higher levels of calcium, ranging from 170 mg% to 220 mg%, and slightly higher levels of phosphate (100-137 mg% 'P') than cows' milk.

In view of these compositional features of buffalo milk and the results of this thesis, it would appear that a rational explanation of the observed synergetic behaviour is possibly available. It also indicates helpful ways to modify the making process such as cooking with an aldopentose to block the $-NH_2$ groups to a limited extent or possibly using phosphate or E.D.T.A. to complex calcium ions - sodium hexametaphosphate or one of the polyphosphates should be most valuable here.

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The phosphoamidase action of rennin on casein

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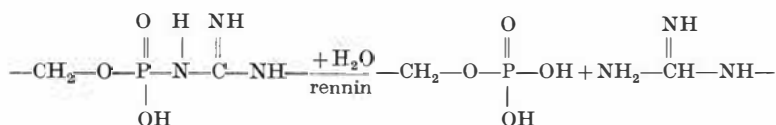
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SUMMARY. It is proposed that —N—P—N— bonds exist in casein and that these bonds can be hydrolysed probably at different rates by the action of rennin. It is proposed that the phosphorus is linked with the guanido group of arginine.

INTRODUCTION

Much interest has been shown in the action of rennin on casein and recently Lindquist (1963) reviewed the whole field. However, little interest has been shown in the phosphoamidase action of rennin.

Berridge (1945) inferred that rennin, in its action on casein, possessed such an activity. Holter & Li (1950) demonstrated that crystalline rennin had a hydrolytic action on a substituted amide of phosphoric acid and this prompted Nitschmann & Varin (1951) to suggest that such groups could exist in casein and could be attacked by rennin. Using electrometric titration data, Dyachenko (1959) showed that the isoelectric point of casein lay between pH 4·6 and 4·7, while that of paracasein (the product remaining after rennin action) was between pH 5·0 and 5·2. He also showed that the essential difference between the titration curves for the 2 caseins lay in the 'divergence in the strongly alkaline region starting from pH 10·2 when the ϵ -NH₂ groups of lysine and the guanidine groups of arginine exhibit their buffering effect'. Since lysine amino groups had been shown not to increase with rennin action (Inikhov, 1922; Koning & Stadhouders, 1962), the appearance of strongly alkaline groups in paracasein was attributed to arginine guanido groups. Dyachenko postulated that rennin splits a —P—N— linkage and proposed the following reaction.



Cheeseman, Rawitscher & Sturtevant (1963) have questioned the esterase activity proposed for rennin by Garnier, Mocquot & Brignon (1962).

It seemed desirable, therefore, to examine further the activity of rennin in the release of the bound phosphate in casein.

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EXPERIMENTAL

Preparation of rennin

Ernstrom's method (1958) was followed, except that the last 3 steps were eliminated and the rennin from step 5 was dissolved in a minimum of distilled water, freeze-dried and then stored at -15°C .

Starch-gel electrophoresis of the purified rennin indicated 2 major bands and 3 very minor bands (Aiyar, 1963). Ernstrom reported 4–5 electrophoretic peaks for his crystalline rennin using free-boundary electrophoresis. It is of interest to note that when the 2 major bands from the starch gel were eluted and re-examined by the same procedure they each gave 2 identical bands at positions corresponding to the sites of the major bands in the original run. The 2 bands thus appear to represent different states of aggregation of the enzyme, the extent of aggregation being controlled by an equilibrium condition.

Preparation of alkaline milk phosphomonoesterase

The method of Morton (1955) was followed up to and including the dialysis stage in step 4 after which the dialysed extract was freeze-dried and then stored at -15°C .

Starch-gel electrophoresis of the purified enzyme indicated 2 major and 4–5 minor bands.

Preparation of freeze-dried acid casein

The method followed was that of Hipp, Groves, Custer & McMeekin (1959) and Wake & Baldwin (1961) which gave a casein containing 2.0% ash and 0.74% phosphorus.

Urea starch-gel electrophoresis indicated 15–17 components (Aiyar, 1963), which was comparable with the results of Wake & Baldwin (1961) and Neelin, Rose & Tessier (1962).

Preparation of casein substrate

A smooth paste was first prepared by mixing the weighed quantity of the freeze-dried acid casein with a small quantity of distilled water and this was then dispersed with more distilled water. To the stirred casein suspension, 0.1 N-NaOH was added dropwise to adjust the pH to 6.75. Nisin was added to give a final concentration of 100 ppm. and the casein solution was then made up to the required volume (Nisin did not interfere with the enzyme actions of the rennin or alkaline phosphomonoesterase).

*(a) With rennin**Determination of phosphate liberation*

20 ml of a 3.5% (w/v) casein solution (pH 6.7) was held at 38°C in a temperature-controlled water bath and 5 ml of an aqueous solution of the enzyme was then added with mixing to give the desired enzyme/substrate ratio (w/w). After the desired time the reaction was stopped by pouring the mixture into a stoppered measuring cylinder containing 25 ml vanadium molybdate solution prepared according to Method Sheet no. 60, Unicam Instruments (1961). The vanadium molybdate precipitated the casein, inactivated the enzyme and reacted with liberated orthophosphate ions to give the soluble, orange coloured vanadium-phosphomolybdate complex. The tube was rinsed with distilled water and the combined reaction mixture and wash made up to 75 ml and filtered, through Whatman No. 42 paper on a Buchner funnel.

The filtered samples were set aside for at least 2 h to permit full colour development. Since there was a continuous slow aggregation of the reaction products it was found necessary to refilter the solutions before measuring transmissions. Repeated filtrations between 4 and 24 h after colour development did not significantly affect the final transmission reading.

Percentage transmission was measured on a Beckman DU Spectrophotometer at 420 m μ m.

(b) *With phosphomonoesterase*

(i) At pH 6.7—method as for rennin;

(ii) at pH 9.5—to 20 ml of a 3.5% (w/v) casein solution made up in the double strength Na₂CO₃/NaHCO₃ buffer of Tramer & Wright (1950) at pH 9.5 was added 5 ml of enzyme solution in a similar buffer. The reaction was stopped and phosphate liberation measured as in (a).

Using the synthetic substrate disodium *p*-nitrophenyl phosphate it was found that use of double strength Na₂CO₃/NaHCO₃ buffer did not affect the activity of the phosphomonoesterase.

(c) *With rennin then phosphomonoesterase*

To 20 ml of a 3.5% (w/v) casein solution (pH 6.7) the desired quantity of rennin was added as a powder. At the appropriate time the phosphomonoesterase was added as a solution in 5 ml of double strength Na₂CO₃/NaHCO₃ buffer. This increased the pH to 9.5, inactivating the rennin, but giving optimum conditions for phosphomonoesterase activity. The reaction was stopped and phosphate liberation measured as in (a).

(d) *With phosphomonoesterase then rennin*

To 15 ml 4.66% (w/v) casein solution in double strength Na₂CO₃/NaHCO₃ buffer at pH 9.5 was added 5 ml of the phosphomonoesterase in a similar buffer. At the appropriate time 5 ml of approximately 0.2 N-HCl was added to reduce the pH to 6.65–6.70 and the rennin was added as a powder. The reaction was stopped and phosphate liberation measured as in (a).

The final casein concentration was the same for all series.

Determination of exposure of guanido groups

The Sakaguchi (1925) test was used to detect the exposed guanido groups, the test being performed on a duplicate sample treated in a manner identical with that used for measuring phosphate liberation. The 2 tests were performed simultaneously. Two ml of the reaction mixture were removed at the appropriate time and transferred to a colorimeter tube containing 0.5 ml 1 N-NaOH (this stopped rennin action immediately); 1 ml of distilled water was added, followed by 0.5 ml of 0.1% (w/v) naphthol in 70% ethanol and 1 ml of 5% (w/v) sodium hypochlorite solution. The colour development was carried out with the tubes immersed in a water bath at 38 °C. The colour was developed for 2 min after the addition of reagents, the tubes were removed, allowed to cool in the dark, and percentage transmission read at 420 m μ m.

(maximum absorption) after a minimum of $\frac{1}{2}$ h had elapsed to permit full colour development. The colour was stable for at least 2 h.

A standard arginine solution was prepared by dissolving 120.92 mg of arginine monochloride (L.R. grade) in distilled water, the pH was adjusted to 7.0 with $N/20$ NaOH and the volume made up to 100 ml. The arginine content of this solution was taken as 100 mg % (w/v).

To prepare the calibration curve for the guanido group of arginine, measured quantities of the standard arginine solution were added to 2 ml of a 2.8 % (w/v) casein solution (i.e. the same concentration of casein as in the test samples). Distilled water was added to equalize volumes and this was followed by the Sakaguchi test reagents. Total volume was in all cases 5 ml.

The casein solutions used to measure the colour after the Sakaguchi reaction were opaque but control experiments in which casein samples were incubated with rennin for periods of up to 24 h and then treated with 0.5 ml 1 N -NaOH, as in the Sakaguchi reaction, showed no change in opacity. The increased adsorption that occurred after such periods of incubation and with the addition of all the Sakaguchi reagents was considered, therefore, to be due to increased colour production as the result of rennin action.

Since arginine was not released into solution by rennin action, but remained attached to the para-casein it was not possible to remove the casein by precipitation and then measure the colour in the clear supernatant. A filtered sample gave no colour test for soluble arginine. Control tests without enzyme addition were run with each experiment.

The results given in Table 2 are each the mean of 6 separate determinations and are based on the assumption that both free and bound arginine (free guanido group) have similar colour values. The differences between each succeeding mean value are, respectively, significant at the following values < 0.025 , < 0.01 , < 0.005 .

RESULTS

(a) *Phosphatase activity of rennin and alkaline phosphomonoesterase on 2 phosphomonoesterase substrates*

(i) *Disodium p-nitrophenyl phosphate*

The modified Aschaffenburg-Mullen phosphate test (Tramer & Wright, 1950) was carried out using the 2 enzymes. Two buffer systems were used, the $Na_2CO_3/NaHCO_3$ buffer with reaction pH 9.6 and Na_2HPO_4/KH_2PO_4 buffer pH 6.2 (Vogel, 1959).

Observation at 2 and 18 h indicated no activity with rennin at either pH, whereas the milk phosphomonoesterase was very active at the alkaline pH and still retained considerable activity at the acid pH.

(ii) *Glucose-1-phosphoric acid (di-potassium salt)*

Phosphate liberation was measured after incubation at pH 9.6. Milk phosphomonoesterase released all the phosphorus within 1 h.

It is interesting to note that commercial rennet showed limited phosphoesterase activity with both these substrates.

(b) *Liberation of phosphate from casein by rennin and phosphomonoesterase used individually or together*

Results are given in Table 1. These results are the average of 2 entirely independent experiments. Transmission values of duplicate samples differed by a maximum of 2 units (0.026 mg P_2O_5). Total phosphorus content in 700 mg casein used for each test amounted to 5.2 mg P or 12 mg P_2O_5 equivalent.

The extended reaction time (30 h) for phosphomonoesterase was based on Perlman's (1955) observation that phosphomonoesterase acted only slowly on unfractionated casein.

Table 1 clearly indicates that alkaline milk phosphomonoesterase under the conditions of the experiment does not release phosphorus from casein, whereas rennin liberated 3.1–3.4% of the total phosphorus in 1 h. Furthermore, alkaline phosphomonoesterase was not able to release any further phosphorus after the preliminary action of rennin on the substrate.

Table 1. *Liberation of phosphate from casein by rennin and alkaline phosphomonoesterase acting individually or together*

Sample	Reaction*	% transmission at 420 m μ	Phosphate released, mg P_2O_5	Phosphorus released, % total P
1	C 6.7/1 h + 5 ml D.S.B./30 h	105	Nil	Nil
2	C 9.5/30 h + 5 ml 0.2 N-HCl/1 h	105	Nil	Nil
3	R ^N + P 9.5/30 h	105	Nil	Nil
4	C 6.7/1 h + P 9.5/30 h	105	Nil	Nil
5	C 6.7 + R ^N /1 h + 5 ml D.S.B./30 h	74	0.40	3.4
6	C 6.7 + R ^N /1 h + P 9.5/30 h	75	0.39	3.3
7	C 9.5 + P/30 h + R ^N 6.7/1 h†	76	0.37	3.1

* C = 20 ml of 3.5% casein solution; 6.7 and 9.5 refer to reaction pH; D.S.B. = double strength carbonate/bicarbonate buffer pH 9.5; R^N = rennin; P = alkaline phosphomonoesterase; 1 h and 30 h refer to time in hours; enzyme/substrate ratios used: rennin, 1:87.5; phosphomonoesterase 1:116.7.

† Rennin was added as powder after 5 ml 0.2 N-HCl had been added to bring reaction back to pH 6.7.

(c) *Phosphatase activity of different rennin concentrations on casein at a fixed reaction time and of fixed rennin concentration over extended reaction times*

Enzyme/substrate ratios of 1:2188, 1:1094, 1:728, 1:364, 1:182, 1:87.5 were used and the phosphate liberated after 1 h was measured. It was found that with the most dilute system (1:2188) no detectable phosphate was liberated in 1 h, although a subsequent experiment gave 3.1% of phosphorus released after 52 h incubation. The quantities of phosphate released in 1 h with the remaining enzyme/substrate ratios were, respectively, 0.32, 0.50, 0.94, 1.9, 3.3%. The latter value agrees with values for the same enzyme/substrate ratio given in Table 1.

Fig. 1 shows the effect of different enzyme/substrate ratios on the rate of release of phosphate and on its extent over 24 h. Using an enzyme/substrate ratio of 1:87.5 and incubating for 24 h, 9.5% of the phosphorus was released, the slope of the curve indicated that further phosphate could be released given time.

Fig. 2 shows the effect of increasing the substrate concentration on the release of phosphorus over 24 h using the same concentration of enzyme. Since the plot of the

phosphorus released under these conditions shows no sign of flattening out it is assumed that saturation or limiting conditions of the enzyme-substrate relationship have not yet been reached, and in an endeavour to apply approximate quantitative relationships between the released phosphorus and the exposed guanido groups of

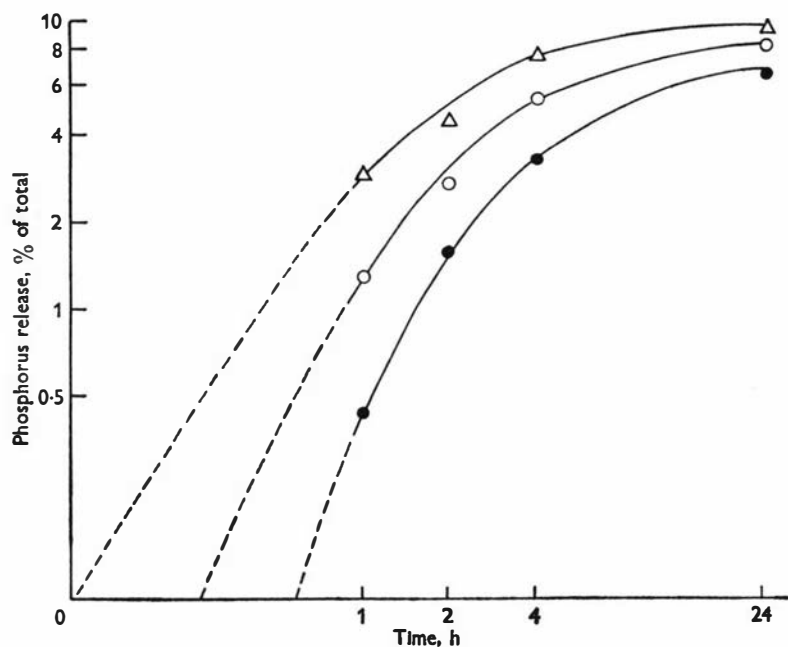


Fig. 1. The effect of various enzyme/substrate ratios on the release of phosphorus after different periods of incubation. Enzyme/substrate ratios: Δ , 1:87.5; $\circ$, 1:182; $\bullet$, 1:364. Scales: ordinate, log (1 cycle); abscissa, log (3 cycles).

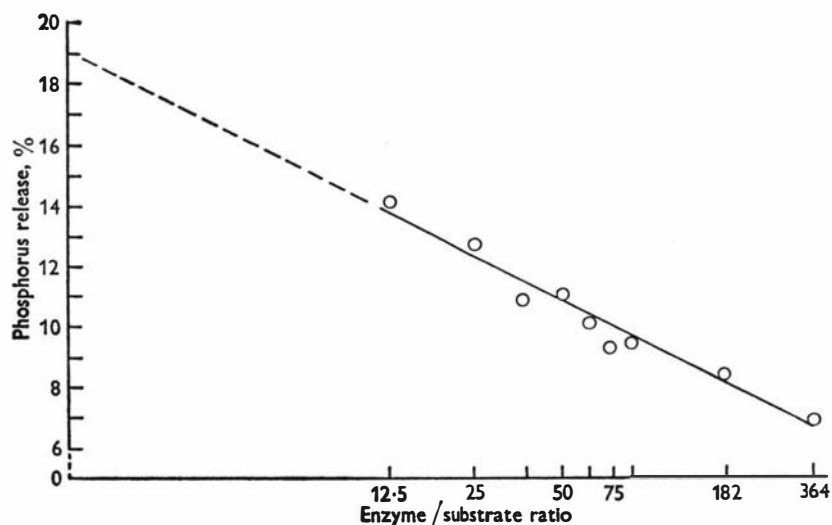


Fig. 2. The effect of various enzyme/substrate ratios on the total amount of phosphorus released during 24 h incubation. Scales: ordinate, linear; abscissa, log (1 cycle).

arginine the curve has been extrapolated to zero to obtain the probable maximum value for phosphorus released. By calculation the regression equation for the data depicted in Fig. 2 is

$$Y_c = 19.14 - 4.913x,$$

where x is the log value of the enzyme/substrate ratio, the variance is 0.24 and the standard error of the estimate is 0.49 % P. It would therefore appear that about 20 % of the total phosphorus of casein can be released by rennin.

The pH of the casein solutions during a 24 h experiment with added rennin generally dropped from 6.7 to 6.2–6.3 and during this time the solution became increasingly opaque and ultimately deposited a precipitate. The opaqueness was very obvious when compared with non-rennin treated controls after 4 h incubation. It is possible that as a result of rennin action on the dispersed casein, reactive groups are exposed which permit casein aggregates of greater than colloidal dimensions to develop or, alternatively, the casein molecule may, under the action of rennin, be unfolding to give a denatured product as proposed by Berridge (1942).

The drop in pH was found to have no measurable effect on rennin activity when a veronal-acetate buffer (Michaelis, 1931) at pH 6.7 was used to stabilize the reaction.

Table 2. *Exposure of guanido groups and liberation of phosphate from casein by rennin*

Sample	Reaction*	Phosphorus released		Arginine exposed		Moles arginine exposed Moles phosphorus released
		mg/100 g casein	moles $\times 10^5$ / 100 g casein	mg/100 g casein	moles $\times 10^5$ / 100 g casein	
1–4	C 6.7+5 ml distilled water	Nil	Nil	Nil	Nil	—
5	C 6.7+RN/1 h	20	65	730	420	6.5:1
6	C 6.7+RN/2 h	41	132	860	500	3.8:1
7	C 6.7+RN/4 h	56	180	980	560	3.1:1
8	C 6.7+RN/24 h	71	230	1210	700	3.0:1
9	C 6.7+RN/48 h	—	—	1160	670	—
10	C 6.7+6.25 ml N-NaOH+RN/24 h	—	—	Nil	Nil	—

* For explanation of abbreviations see footnote to Table 1; enzyme/substrate ratio, 1:87.5.

(d) *Release of phosphorus and exposure of guanido groups of arginine as a result of rennin action on casein*

Table 2 indicates that exposure of guanido groups as the result of rennin action on casein occurred much more quickly than the release of phosphorus. The release of phosphorus was comparable with that of earlier experiments. Thus, the ratio of moles of phosphorus released to moles of arginine exposed was 1:6.5 at the end of the first hour, but was only 1:3.0 at the end of 24 h as phosphate release continued and arginine exposure levelled off. No further guanido groups were detectable on incubating for a further 24 h. Results discussed earlier (Fig. 2) indicate that ultimately around 20 % of the total phosphorus appears to be capable of release by rennin. Twenty per cent of the total phosphorus in the casein used is equivalent to 148 mg P/100 g casein or 480×10^{-5} moles P/100 g casein. Thus, the ultimate ratio of moles of arginine exposed to moles phosphorus released approximates 1.5:1.

DISCUSSION

The present experiments have indicated that about 20 % of the phosphorus in casein may be released through the action of rennin, but no release of phosphorus was evident through phosphomonoesterase action. Purified rennin did not have phosphatase action on the synthetic substrate, di-sodium *p*-nitrophenyl phosphate, whereas the alkaline phosphomonoesterase was extremely active. Commercial rennet had some activity, which was not unexpected, since it is possible for contaminating enzymes from the calf mucosa to get into the rennet during its commercial extraction.

Kalan & Telka (1959) observed an 85 % dephosphorylation of whole casein, using calf intestinal mucosa acid phosphomonoesterase, which was in general agreement with Zittle & Bingham's finding (1959) based on the use of alkaline milk phosphomonoesterase which, however, had shown greatest activity on casein at neutral pH.

Sampathkumar, Sundararajan & Sarma (1957) reported 55 % dephosphorylation of whole casein using an acid pyrophosphatase from soyabean, and Sundararajan & Sarma (1957) dephosphorylated whole casein to the extent of 77 % using a phosphoprotein phosphatase (acid) from rat tissues. In general, results from the action of phosphatases on casein appear confusing. However, the results obtained by all the above workers indicate that about 15–20 % of the total 'phosphorus' in casein was not released by their enzyme preparations.

In this present study it has been deduced that rennin may be capable of liberating about 20 % of the total phosphorus in casein and in view of the quoted results of other workers with various phosphatases it is suggested that this phosphorus could arise from phosphate linkages which appear to resist the action of phosphatases in general.

Perlman (1955) indicated that 40 % of the total phosphorus in α -casein was bound through an —N—P—O— link and Dyachenko (1959) postulated a phosphoamidase action of rennin on casein, and suggested that the phosphoamidase action was on phosphorus linked to the guanido group of arginine.

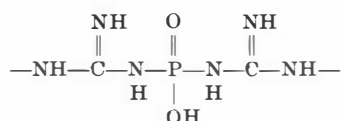
Perlman (1955) considered that the remaining phosphate linkages in casein were of the ester or pyrophosphate type. No consideration was given to a linkage of the type —N—P—N—.

If 40 % of the phosphate linkages in α -casein were of the —N—P—O— type, then it should be possible to liberate orthophosphate ions by the combined action of rennin and alkaline phosphomonoesterase. Table 1 (samples 4, 6 and 7) showed that rennin plus the alkaline phosphomonoesterase prepared for these studies released no more orthophosphate than was released by rennin alone. Since free orthophosphate ions only are capable of forming the phosphomolybdate complex (Durrant & Durrant, 1962; Pauling, 1952), rennin must be capable of completely hydrolysing some of the phosphate linkages present, and since the rennin used in this study did not possess measurable phosphoesterase activity, it is reasonable to conclude that some phosphorus in casein is linked —N—P—N—.

With regard to the exposure of guanido groups of the arginine residues in acid casein by rennin action the results indicate that ultimately for every mole of phosphorus released, more than 1 mole of arginine appeared to be exposed.

This exposure of arginine appeared to go at a very much faster rate initially than the release of phosphorus. Thus, the ratio of phosphorus released to arginine exposed

(at the guanido groups) was of the order of 1:6–1:7 after 1 h. The ultimate 1:1.5 ratio of phosphorus released to arginine exposed suggests an —N—P—N— link in the casein molecule, i.e. 2 arginine residues linked by their guanido groups through phosphorus as follows:



Half of the rennin-sensitive phosphorus is probably attached to only 1 guanido group.

A reasonable explanation for the observed changing ratio of phosphorus released to guanido groups exposed can be derived if one bears in mind that only phosphorus completely released as orthophosphate ions is measurable, whereas the guanido group exposed through the hydrolysis of only one bond of the —N—P—N— linkage becomes detectable. Since a 1:1 ratio of —N—P—N— linkages and —N—P linkages has been proposed, let us consider for mathematical convenience the hydrolysis of 66 of each of these linkages in casein by rennin. It is assumed that one phosphorus bond in —N—P—N— is more readily hydrolysed than either the remaining bond or the simple N—P bond and that this difference in hydrolysis rate is of the order 11:1. Table 3 indicates the gradual change in the ratio of guanido groups exposed to phosphorus released which would be expected with the passing of time.

Table 3. *Calculated change in the ratio moles arginine exposed: moles phosphorus released*

Time interval, h	...	t_0	$t_1 - x$	t_1	t_2	t_4	t_{24}	t_∞
No. of original —N—P—N— linkages remaining		66	55	0	—	—	—	—
No. of original —N—P linkages remaining		66	65	60	—	—	—	—
Total no. of —N—P linkages remaining		66	75	120	110.4	101.2	94.0	0
No. of 'P' released from all —N—P		—	2	12	21.6	30.8	38	132
No. of 'N' exposed from all —N—P		—	2	12	21.6	30.8	38	132
No. of 'N' exposed from all —N—P—N—		—	11	66	66	66	66	66
Total no. of 'N' exposed		—	13	78	87.6	96.8	104	198
Ratio 'N': 'P' $\left(\frac{\text{moles arginine exposed}}{\text{moles phosphorus released}} \right)$		—	6.5	6.5	4.05	3.1	2.7	1.5
Observed ratio 'N': 'P'		—	—	6.5	3.8	3.1	3.0	—

To have an equivalent time basis for comparison with the observed change in 'N': 'P' ratio, the number of moles of 'P' released for the various time intervals used in the calculation is the same as that observed for the release of phosphate with an enzyme/substrate ratio of 1:87.5 and since this is the same condition as used in determining the observed 'N': 'P' ratio the calculated and observed figures are comparable. The rates are found to agree very well.

No new α -amino end group has been demonstrated on the para-casein or para- κ -casein molecules formed during the initial fast reaction of rennin on casein. This would not be expected if, as suggested in this study, the phosphorus were attached to guanido groups at either end. Cleavage of either P—N bonds would only expose guanido groups and not produce a new α -amino end group.

The work of Dyachenko (1959) indicated that the basic groups exposed by rennin action on casein stem mainly from arginine, and results from this study support this view.

In conclusion, it appears that an —N—P—N— link is present in casein; the phosphorus links 2 arginine residues through their guanido groups; rennin is capable of hydrolysing N—P bonds and may hydrolyse the bonds of —N—P—N— at different rates.

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