"Functional Behaviour of Mixed Protein-Polysaccharide Systems"

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Thesis submitted to the University of Nottingham for the degree of Doctor of Philosophy, September 1995.

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Acknowledgements

I would like to firstly thank my supervisors, Professor John Mitchell and Dr. Steve Harding, for all their help and advice throughout my time at Nottingham.

I also thank all the members of the Consortium, within which this PhD was carried out, for making the project so friendly and workable. The financial support and interest of MAFF and the Industrial partners (Nestlé, Pedigree Petfoods, St. Ivel, Four Square, Amylum) is gratefully acknowledged. Thanks also go to the fellow academics at the Universities of Surrey and Cranfield; to Dr. Nazlin Howell for co-ordinating the project, Professor Ed. Morris and Dr. Stef. Kasapis for frequent helpful discussions and to the students, Sarah, Pratima and George. Especially thanks to Sarah Comfort for lengthy useful discussions both on the telephone and in the bar!

On a personal note I'd like to thank everyone with whom I shared the lab and kettle! It proved a really great environment to work and mix within. Special thanks go to the close friends I found in Kar-mun, Raj and Lorna. I would also like to thank Mr. Phil Glover for always seeming to have the solution to any technical problem!

Outside of Food Science thanks go to Andrea, Karen and Helen, decorators extraordinaire!

Thanks, too, to my family for their love and support especially to my late dear Nan who sadly found the ultimate way to avoid reading my thesis! Lastly, but certainly not least, thanks to Tim for your constant love and understanding and for sharing your life with me.

ABSTRACT

The work described in this thesis addresses two classes of mixed biopolymer systems:
(a) starches and sodium caseinate
(b) gelling seaweed polysaccharides (κ-carrageenan - both with and without locust bean gum - agar and alginate) and proteins (gelatin and blood plasma proteins).

The viscosity and swelling volume of a 1% potato starch paste in distilled, deionised water is markedly reduced in the presence of caseinate. Similar effects were seen with simple electrolytes suggesting that this occurs as a consequence of a non-specific ionic strength effect. In contrast a 4% maize starch paste in distilled, deionized water undergoes a viscosity and swelling volume increase in the presence of caseinate. However, when pasted in a 0.1M, pH 7.0 phosphate buffer caseinate addition has little effect on the viscosity of the fresh paste and at high concentrations appears to prevent retrogradation on ageing. It is suggested that in buffer caseinate prevents the leaching of starch polysaccharides from the swollen granule and therefore maintains amylose in the granular phase. This is attributable to the high ionic strength of the solvent, allowing caseinate and the starch polysaccharides to phase separate. In water the unfavourable entropy, change due to the uneven distribution of the counter-ions, prevents phase separation and results in an interpenetrating network.

Studies on the large deformation stress relaxation behaviour and melting points of 2% carrageenan, 0.5% carrageenan/0.5% locust bean gum (LBG) and 2% agar gels in a variety of solvent media indicate that the inclusions of 0-20% gelatin and 0-5% bovine serum albumin (BSA) give different results depending on both protein and polysaccharide. The main points of this study show that agar/gelatin mixed gel undergoes a distinct phase inversion at 4-7% gelatin levels, which is not seen with the carrageenan gels containing gelatin. Even when 20% gelatin is incorporated into a 2% carrageenan gel the melting point of the gel is unaltered from that of carrageenan alone. In the presence of high levels of BSA the carrageenan/LBG gel undergoes a marked increase in melting point. Investigations using locust bean gums of variable protein content suggests a possible (LBG)protein-BSA interaction since the melting point increases with the LBG protein content. It is shown that carrageenan/LBG gels have clear regions when formed by autoclaving in the presence of blood plasma. This supports the idea of an association between the protein in the insoluble husk and the blood plasma proteins.

The interaction mixtures of BSA with sodium alginate at the interface and in bulk solution have been studied through the techniques of microelectrophoresis and ultracentrifugation respectively, to further elucidate the association between denatured proteins above their isoelectric point and anionic polysaccharides. Both techniques clearly show that the macromolecules can associate electrostatically at pH's above the pI of the protein.

CHAPTER 1. INTRODUCTION

1.1 Why Study the Functional Behaviour of Mixed Protein-Polysaccharide Systems?

Foods constitute multi-component systems. Many of these components confer properties other than nutritional ones to the product; these we term functional properties. Proteins and polysaccharides are two macromolecular components that provide the key structural functions in food systems, being largely responsible for the structural, mechanical and other physico-chemical properties (Tolstoguzov, 1991). In addition the functional properties can be greatly affected by any protein-polysaccharide interactions within the system (Tolstoguzov, 1991). Food formulations frequently contain more than one hydrocolloid in order to give the desired functional properties and in these cases the properties of the mixtures may be superior to those of the isolated components (Morris, 1990). Ideally the performance of these hydrocolloids can be tailored by the food chemist to meet the specific needs of any food manufacturing requirement (Hart, 1992). Many of the combinations employed in this study involved measuring the rheological properties of gels, as proteins and polysaccharides also constitute the two major classes of gel-forming polymers (Stading, 1993).

The objectives of this PhD study were to investigate the interactions between a number of mixed protein-polysaccharide systems in relation to the functional behaviour and to establish how the combined functional properties differed from those of the isolated components.

This PhD was carried out within a MAFF sponsored consortium of three Universities (the Universities of Nottingham, Cranfield and Surrey) and a number of Companies (Nestle, Pedigree Petfoods, St. Ivel, Amylum and Four Square). In addition to being of fundamental, academic interest the study of the functional behaviour of mixed protein-polysaccharide systems also has great industrial importance. Indeed the hydrocolloids market represents a growth area of the European food industry, with a projected value of nearly $2 500m estimated for the year 2000 (Frost & Sullivan, 1995). One of the three main directions, in terms of technological trends, singled out by this latter report was that of the creation of hydrocolloid blends. Thus the question posed above is then answered as follows. In order to reap the benefits of interaction phenomena (be it the optimisation of current systems or the identification and development of new systems) the underlying fundamental behaviour needs to be understood.

1.2 The Components of Food Systems

Before the understanding of the behaviour of any mixed system can be achieved it is essential to first understand the nature of the individual components. The proteins and polysaccharides employed in this investigation are now reviewed.

1.2.1 The Polysaccharides

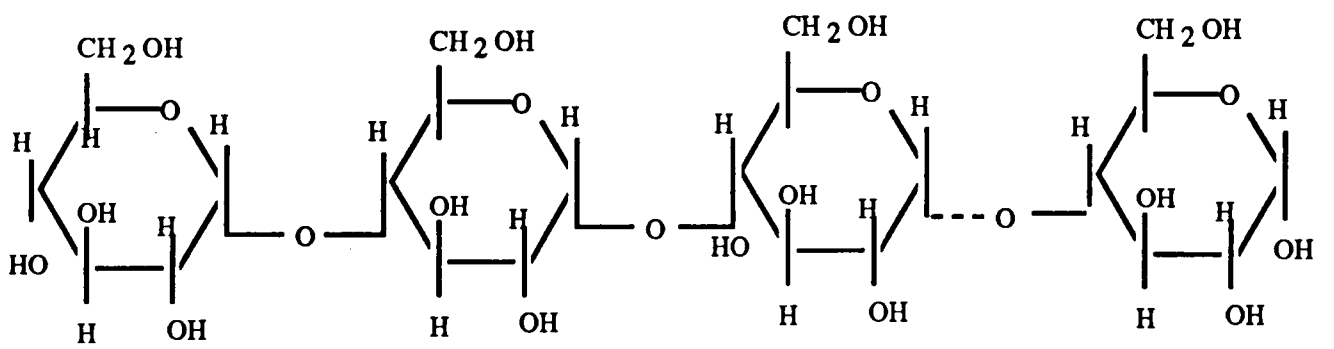
1.2.1.1 Starch

Starch, whilst serving Man as the dominant polysaccharide in the diet of the modern world (Ring, 1993), serves its primary function as the storage carbohydrate of plants, occurring in greatest abundance in tubers and the endosperms of seeds. The botanical sources from which starch is derived commercially are numerous and varied but the main sources of commercial starch are maize, potato, wheat and tapioca (also called cassava) (Swinkels, 1985). Starch is housed in the form of water-insoluble granules, the size and shape of which vary with botanical origin, spanning from 0.5μm-175μm (Zobel, 1988). In addition to species-dependence for granule size and shape the maturity of the plant is also a factor (Manners, 1989). The features of the granule are often characteristic of the source; diameters of up to 100μm are to be found in potato starch whilst wheat starch exhibits a bimodal size distribution (Swinkels, 1985).

The two major components of the starch granule are the polysaccharides amylose and amylopectin. The amylopectin component accounts for the greater proportion within the starch granule, usually 70-80% with amylose occupying the rest. The relative proportions of each component vary between starches of different origins. Generally the magnitude of amylose content increases in the order tuber<cereal<legume starches (Zobel, 1988; Ring, 1993; Howling 1980). Essentially single component systems can also be found in the form of the waxy starches (~100% amylopectin) and abnormally high amylose contents (~60%) can be found in amylomaize and wrinkled pea starches. Average molecular weights of amylose are of the order of 10^5-10^6 whilst for amylopectin weights of up to 10^8 have been reported (Coultate, 1990; Ring, 1993).

Whilst both amylose and amylopectin are based on the same α-(1-4) linked D-glucose residues the latter has a highly branched structure (Figure 1.2.1b) brought about through additional (1-6)-α-D-glycosidic linkages involving 20-25 of the linear residues and accounting for 4-5% of interchain linkages (Manners, 1989; Coultate,

1990). It is incorrect, yet frequently stated, that amylose is an entirely linear molecule. Instead interlinking of the much longer linear chains in the same way as in amylopectin occurs to produce a very lightly branched structure. Unlike amylopectin the degree of branching is not present to a sufficiently high extent to affect the physical properties (Manners, 1989). Amylose is often represented as a linear molecule to stress the essentially linear nature (Figure 1.2.1a). The structure of the amylose molecule results in the incorporation of a gradual twist giving rise to a helical structure overall (Zobel, 1988). The amylose molecule readily complexes with a number of chemical moieties of which the best known is the polyiodide-amylose helix forming the basis of the frequently used blue starch test.



Non-reducing end

Reducing end

Figure 1.2.1a. The α1-4 glycosidic bond of amylose

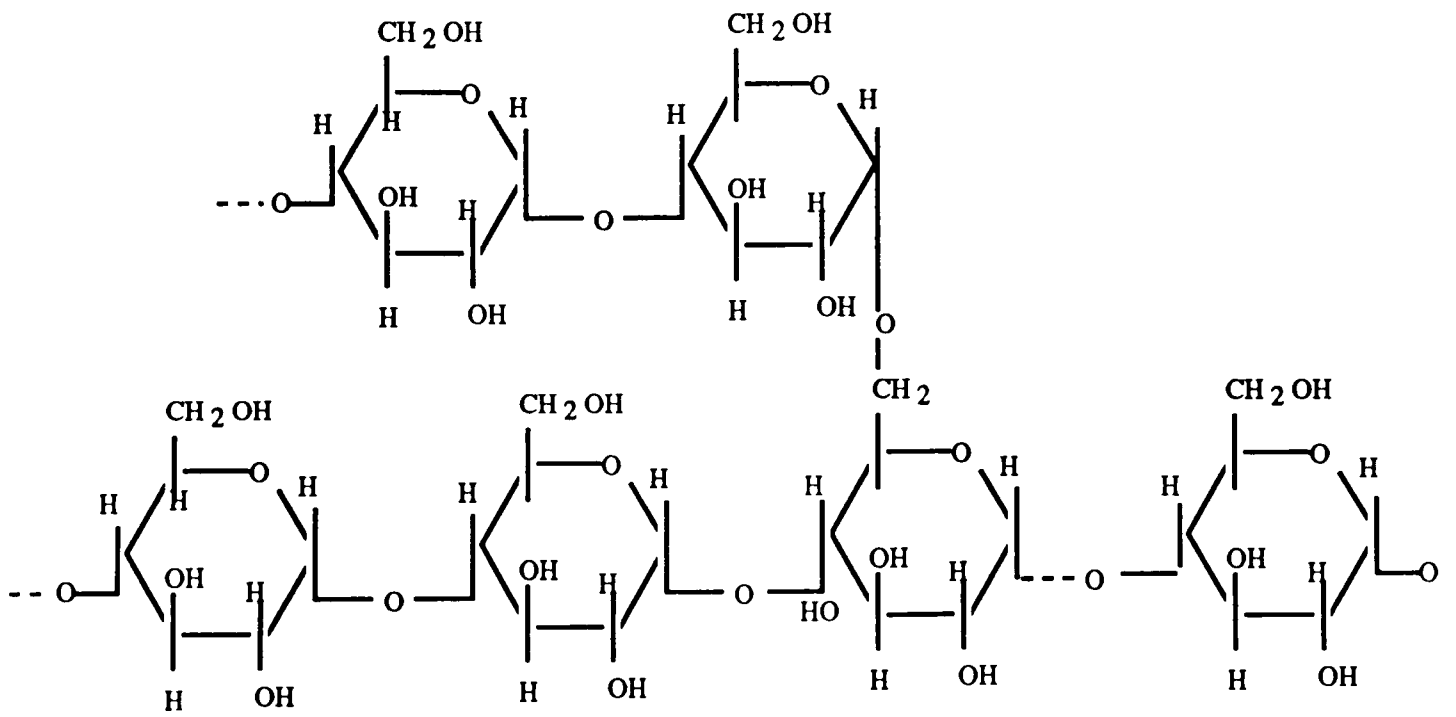


Figure 1.2.1b. The α1-6 branch of amylopectin

Much controversy has surrounded the structure of the amylopectin molecule with numerous models proposed in the past (see the excellent review by Manners, 1989 for details of these models). Today the accepted model is the "cluster" structure represented in Figure 1.2.2. Three different types of chain can be distinguished which are designated A, B, C. The single C chain carries the sole reducing group and a skeleton of branched B chains, each of around 25 glucose units long and connected by 1, 6 bonds. These B chains carry clusters of mostly unbranched A chains of ~15 glucose units bound at the 1, 6 position; additional A or B chains may be carried on the B chains at the primary hydroxyl groups (Zobel, 1988; Coultate, 1990; Manners, 1989). The non-reducing ends are then orientated towards the surface of the starch granule (Coultate, 1990). This non-random, clustered branching gives rise to interjacent linear segments, in turn forming thin (~5 nm) crystalline domains (Oostergetel & van Bruggen, 1993) which are visible under TEM with regular spacings of ~10 nm (Blanshard *et al.*, 1984).

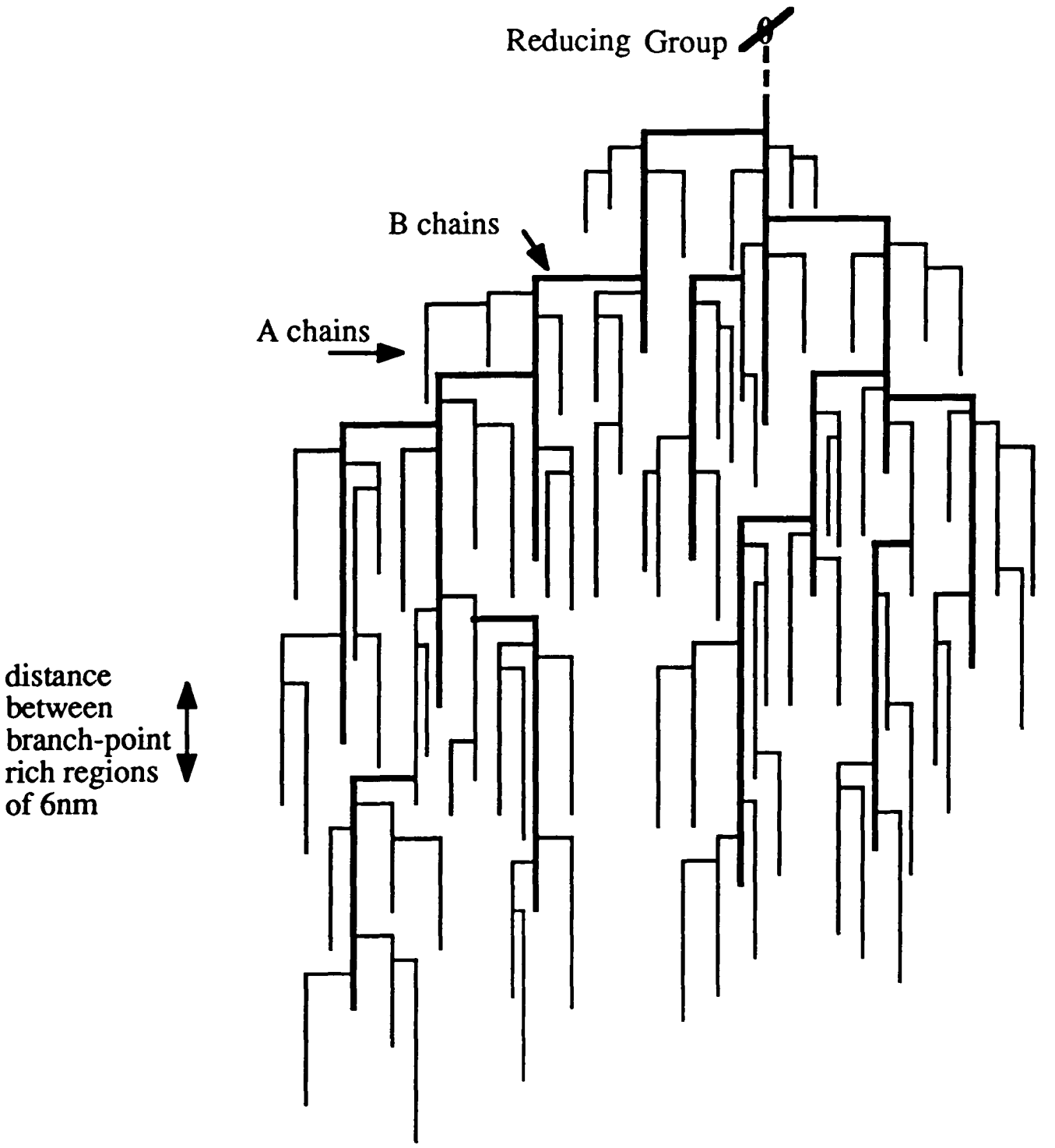


Figure 1.2.2. The cluster model of amylopectin (from Robin *et al.*, 1974)

How these two components (amylose and amylopectin) are arranged together within the granule is not well defined. That the granule contains an element of crystallinity is certain from the characteristic "maltese cross" patterns observed under the polarizing microscope. A recent study by Jenkins and co-workers (1993) suggested that the granular concentric rings observed microscopically by French in 1984 in fact represent a unifying feature of all starches, regardless of botanic origin. These "growth rings", of the order of 1200 Å - 4000Å in width, constitute bands of alternating semi-crystalline and amorphous nature (Yamaguchi *et al.*, 1979). Furthermore this semi-crystalline region is itself composed of stacks of alternating crystalline and amorphous lamellae (Yamaguchi *et al.*, 1979; Kassenbeck, 1978) due to the formation of double-helical short chain segments and branch-point dense regions of amylopectin respectively (Jenkins *et al.*, 1993). Evidence that the observed

crystallinity arises from the short chain clusters of amylopectin is provided by the similarity of the X-ray diffraction pattern produced by normal and amylose-deficient starches (Coultate, 1990). The small angle X-ray scattering studies of Jenkins and co-workers thus allows the determination of the average size of an amylopectin cluster to be made on the basis of the repeat distance. This study concluded that the structural periodicity of all the starches investigated was constant at ~9nm and thus independent of botanical source. Although no explanation for this occurrence could be given by these authors it was speculated that some aspect of the starch biosynthesis within the granule could be involved.

One method used in the attempt to elucidate the internal structure of a starch granule is that of chemical gelatinisation as used by Jane and Shen in 1993. From this study these workers showed that the concentration and molecular size of the amylose and amylopectin components within the potato starch granule varied with granular position, i.e. amylose at the core of the granule was both of a larger molecular size and less concentrated than at the periphery. This study also showed that the starch phosphate esters were more concentrated at the core.

Along with the groupings of starches based on their botanical origin classification into three groups designated A, B and C can be made on the basis of the molecular arrangement observed on X-ray powder diffraction (see Hizukuri, 1985). This method implies a relationship between the chain profiles of amylopectin and the crystalline structure of the starch granule. Whereas the A group starches (generally the cereal starches - see Zobel, 1988) share the common feature that amylose has little effect on amylopectin crystallinity, the B group starches (includes potato and amylomaize) display a crystallinity lowering on increasing amylose content and the C group exhibit no trend (Zobel, 1988). In the study by Hizukuri this third group also occupied the intermediate positioning in terms of amylopectin chain length between A (the longest) and B (the shortest), and this was rationalized to be due to the temperature sensitivity of group C to the surrounding environment which was not shown by either A or B groups. In work by Gidley, 1987, a close-packed double helical arrangement was assigned to type A starches with the group B starches having a more open arrangement with greater inter-helical water. Overall the nature and length of the amylopectin chain constitute an essential factor in determining the crystallinity of the starch granule (Hizukuri, 1985).

It should briefly be mentioned that in addition to the two major polysaccharides a third fraction intermediate between amylose and amylopectin also exists in some

starches of exceptionally high amylose content, but this becomes significant only when considering separation and fractionation procedures (Manners, 1989).

Along with the carbohydrate component the starch granule also contains a number of other components depending on the origin. A comparison of the composition of the major starches is given in Swinkels, 1985, and the major features of this highlighted for the starches of interest to this study - potato, maize and wheat. The cereal starches have a much higher lipid content (present as free fatty acids in maize, lysophospholipids in wheat and as an amylose-lipid inclusion complex in both), than that found in potato. The presence of such lipids can have effects on hydration, swelling and viscosity, produce cloudy pastes and contribute "off-flavours" as a result of lipid oxidation (Swinkels, 1985). Undesirable flavours can also be brought about through the protein content, much higher in maize and wheat than in potato starch. Indeed, potato proteins are considered to be amongst the most valuable vegetable proteins (Nuss & Hadziyev, 1980; Lindner *et al.*, 1981) and hence attempts to recover such proteins from the waste juice are made (Ahldén & Trägårdh, 1992). The non-carbohydrate component that is significant in potato starch is the phosphate content, responsible for many of the desirable properties that consequently arise (Bay-Schmidt *et al.*, 1994). The high content (up to 0.1%) and nature of its presence (covalently bound monoester groups linked to the amylopectin molecules at the rate of ~1 phosphate group per 300 glucose units) give rise to an extended conformation in solution through repulsion of the negative charges (Swinkels, 1985). The consequent increase in paste viscosity with increasing degree of phosphorylation has long been known (Veselovsky, 1940). Any phosphate in the cereal starches is present only as lysophospholipids and to a lower total phosphate content with no significant effect on granule properties (Swinkels, 1985). More specifically two sites of binding of the phosphate groups exist, namely the carbon 6 and carbon 3 of the glucosyl residue of the amylopectin chain with the former position (C-6) accounting for about two-thirds of the starch bound phosphate (Hizukuri *et al.*, 1970). In addition whereas the C-3 phosphate occurs at a constant rate the C-6 binding is variable and can in fact be correlated to total bound phosphate (Bay-Schmidt *et al.*, 1994). Similarly differences in content between potato varieties was only to be found with C-6 bound phosphate (Muhrbeck & Tellier, 1991). Studies by these latter workers showed that the actual distribution of phosphate within the potato tuber varied with position; the variable C-6 bound phosphate was found to increase from the cortex to the pith with a contrasting decrease in starch in the same direction. Furthermore, only ~33% of the phosphate is to be found at the innermost regions of the B chains of the amylopectin molecule with the rest accounted for at the outermost parts of the B chains and the A chains (Takeda & Hizukuri, 1982). Environmental growth conditions during starch

synthesis can also have an affect on phosphorous content (Muhrbeck *et al.*, 1991). The phosphate content of potato starches can be inversely related to the level of crystallinity, believed to be attributable to the C-6 phosphates, which orientate themselves out of the α-helices and thus disturb the crystallisation of the amylopectin during starch synthesis (Muhrbeck *et al.*, 1991). Any phosphate attached to the C-3 position - and thus facing the interior cavity of the double helix (Sarko & Wu, 1978) - will not be able to interfere with the crystal structure through their inability to interact with any other large molecules (Muhrbeck *et al.*, 1991).

An important feature of starches is the ability to undergo gelatinisation in the presence of the appropriate conditions to form starch pastes and this has been ascribed as the key to any starch use (Fannon and BeMiller, 1992). Undamaged starch granules are insoluble in cold water due to the overall strength afforded by the inter-chain hydrogen bonding (Oosten, 1982). Such granules will merely settle out of solution. On heating a starch suspension above a critical temperature, defined as the initial gelatinisation temperature, in an excess of water, the granule swells and carbohydrate is released (see for example, Mat Hashim *et al.*, 1992). As water is imbibed intermolecular hydrogen bonds are broken down causing the destruction of granule integrity and a concurrent loss of birefringence (Howling, 1980). For this reason starch gelatinisation has been termed "a phase transition from order to disorder" (Ghiasi *et al.*, 1982). Although gelatinisation most usually occurs in water it has been shown to also take place in other solvents with a high dielectric constant (Oosten, 1982). It is usually understood that shear forces are needed to assist in the gelatinisation process as well as maintain the damaged granules in suspension (Miles *et al.*, 1985a). It has been stated that potato starch, held at 121°C for one hour, in the absence of shear forces will still not yield a molecular disperse solution (Leach, 1965). The onset of gelatinisation is marked by a rapid increase in viscosity traditionally followed by the Brabender amylograph as swollen granules start to impinge on one another and amylose begins to leach out of solution and contribute itself to the viscosity of the paste (Coultate, 1990) through the formation of an inter-granular matrix from the exudate (Miller *et al.*, 1973). Normally amylose leaves the granule first with amylopectin not following until much higher temperatures have been reached, usually exceeding 100°C (Doublier, 1992) but this is not strictly true for all starches (Launay *et al.*, 1986).

With maintenance of heating the swelling granules eventually burst and collapse, producing what is termed a "ghost" (Hoseney *et al.*, 1977) with an accompanying rapid loss of viscosity (Fannon & BeMiller, 1992). These remnant ghosts constitute the only identifiable feature to indicate complete gelatinisation (Rockland *et al.*,

1977). The original contents of these flexible remnants are replaced by the same solution as that in the continuous phase (Bowler *et al.*, 1980). Within the paste model of a three-dimensional matrix of dispersed molecules surrounding the swollen, collapsed granule ghosts, the ghost itself contributes to the paste functional properties through its rigidity and volume along with any interactions it may undergo with the continuous phase molecules (Morris, V.J., 1990).

The continuous phase of the resultant paste contains the water soluble starch components whilst the particles of the dispersed phase arise as the residues of the original deformed starch granules containing the insoluble amylose and amylopectin fractions (Hoseney *et al.*, 1977; Bowler *et al.*, 1987). Within this inhomogenous continuous phase discrete microgel areas of varying mechanical strength occur which possibly contribute to the sudden breakdown of gel structure at low shear rates (Ketz *et al.*, 1988). Indeed starch gels (formed on cooling sufficiently high concentrations, of the order of 6%) may be regarded as composites containing gelatinised granules embedded in an amylose matrix (Miles *et al.*, 1985a).

Starches from different origins show different patterns of gelatinisation behaviour (Mat Hashim *et al.*, 1992). Cereal starches have been shown to undergo a two stage gelatinisation (Whistler & Paschall, 1956) indicative of the different forces present in the highly associated and amorphous regions of the granule (Howling, 1980). Multi-stage swelling in root and tuber starches has been quoted by some authors (Leach *et al.*, 1959; Howling, 1980) as not occurring and thus was thought to suggest more uniform association. However, the work of Eliasson (1986) on potato starch has shown that this is, in fact, not the case; potato starch too shows multi-stage swelling.

Under steady shear conditions starch pastes behave as shear thinning liquids with a yield stress evident at low shear rates (Lelièvre & Husbands, 1989). Along with the starch particle size distribution having a bearing on rheological properties (Wong & Lelièvre, 1982) the absolute granule size effects order of gelatinisation; larger granules swelling before their smaller counterparts (Badenhuisen, 1949).

On storage starch pastes can undergo the process of retrogradation which can be regarded as a return to the ordered state, indicated by the B-type X-ray diffraction pattern produced (see Miles *et al.*, 1985a). The leached amylose components tend to develop into double helices which by mutual side by side bounding partially coagulate and form a rough macromolecular gel network (Djakovic *et al.*, 1990). This process is characterised by an increase in paste viscosity (Coultate, 1990; Fannon & BeMiller, 1992). As the paste is cooled and within the first few hours after

preparation the rheological properties change as recrystallisation occurs and the number of double helices increase (Djakovic *et al.*, 1990). This is brought about by the formation of junction zones between the linearly aligned amylose molecules re-establishing the intermolecular hydrogen bonds (Howling, 1980). This behaviour is seen particularly in isolated amylose solutions (Wu & Sarko, 1978) evidenced visually by the free-standing opaque amylose gel that sets up within a few minutes whilst the equivalent concentration of amylopectin merely remains as a solution (Zobel, 1988). Incidentally it is believed that this opacity arises from a phase separation into polymer-rich and polymer-deficient regions on gelation (Miles *et al.*, 1984; 1985b). Retrograded pastes undergo such a loss of clarity (Howling, 1980; Swinkels, 1985). Since it is known that such gels tend to exhibit syneresis and are unstable to freeze-thaw cycles (Howling, 1980) the processor will often seek out new modified starches along with traditional waxy varieties to overcome this problem in the food product (Coultate, 1990) based on the fact that amylopectin has a very low rate of retrogradation due to its highly branched nature (Swinkels, 1985). Indeed amylopectin restricts the association of the amylose (Sievert & Würsch, 1993). However at concentrations exceeding c*, where c* is defined as the overlap concentration,. a very slow aggregation can also take place (Aberle *et al.*, 1994) and therefore the overall composition of the starch, amylose/amylopectin ratio, will to some extent determine the rate of retrogradation. This is because retrogradation is essentially a two-stage process: the short-term, irreversible crystallisation of the amylose fraction followed over longer times by the "reversible" (i.e. under the right conditions) retrogradation of the amylopectin fraction (Coultate, 1990; Miles *et al.*, 1985a). More recently (van Soest *et al.*, 1994) a three-stage process of retrogradation was observed for potato starch. The first stage involved the fast formation of crystalline amylose regions from amylose helices. The second stage was then attributed to the induction time for the amylopectin helix aggregation with the third stage designated as the helix-helix aggregation and crystallisation of amylopectin (van Soest *et al.*, 1994).

In addition to the modifications to retrogradation behaviour described above it may be necessary to modify other aspects of the native starch in order to impart the desired functional properties to the end-use application. Much of the corn starch, for example, used in the food industry for viscosity, texture or mouthfeel applications is chemically modified to enhance its rheological behaviour (Fannon & BeMiller, 1992). Increased stability of the granules to heat, shear and acid can be achieved through chemical crosslinking. Indeed, microscopic examination of the granule remnant "ghosts" of both native maize and chemically cross-linked modified maize starches reveals that as the degree of cross-linking increases in the modified starches the walls of the ghosts

are much thicker and undergo less damage or collapse (Fannon & BeMiller, 1992). Small particle starches such as amaranth and quinoa with granule diameters of 1-3μm offer the potential in industrial applications as fat substitutes imparting the necessary mouthfeel (Kettlitz, 1994) through the similar sizing to the fat micelle (Daniel & Whistler, 1990). However because of the high cost and problematic isolation of naturally occurring small particle starches (Jane *et al.*, 1992) the physical and chemical modification of larger size starches can be employed to produce such smaller granules (Jane *et al.*, 1992; Kettlitz, 1994). Along with the physical modification of starches the knowledge of starch biosynthesis has led to the development of new genetic varieties such as double mutant starches from maize offering differences in the size, structure and branching patterns of amylose and amylopectin (Kettlitz, 1994).

A summary of the main features of the starches investigated in this study now follows.

Table 1.2.1. Major characteristics of potato, corn and wheat starches

	Potato	Corn	Wheat
Amylose[a] % *1*	21	28	28
2	22	27	23
Granule shape *1*	oval, spherical	round, polygonal	round, lenticular
Dia. range (μm) *1*	5-100	2-30	1-45
No. of granules per g starch (x 10^6) *1*	60	1300	2600
Ave. no. of starch molecules in one granule (x 10^{12}) *1*	50	10	5
Paste viscosity *1,3*	very high	medium	medium-low
Rate of retrogradation *1*	medium-low	high	high
Paste clarity *1*	very clear	opaque	cloudy
3	clear	opaque	opaque
Gelatinisation temperature (C) *1*	60-65	75-80	80-85
2 b	67.3	71.3	63.5
Gel texture *3*	very cohesive	short	short
Resistance to shear *3*	poor	fair	fair
Amount of taste & odour substances *1*	low	relatively high	relatively high
Phosphorous (% on dry substance) *1*	0.08	0.02	0.06
Ash (% on d.s) *1*	0.4	0.1	0.2
Proteins (% on ds)*1*	0.06	0.35	0.4
Lipids (% on d.s.) *1*	0.05	0.7	0.8
World production (million t) 1985*1*	2	13	0.8
Main commercial uses *1*	food, paper, adhesives	sugars, paper, corrugtd. cardboard	Bakery, adhesives,sugars

References:

1. Swinkels, 1985
2. Zobel, 1988
3. Howling, 1980

Notes:

a. For corresponding amylopectin value amylose+amylopectin = 100

b. Represents mid-point in gelatinisation range

1.2.1.2 Carrageenan

The carrageenans represent a family of linear sulphated galactans extracted from *rhodophycaea,* or the red seaweeds (Painter, 1983). These soluble cell-wall polysaccharides are composed of alternating α(1-3) and ß(1-4) linked D-galactose residues (Singh & Jacobsson, 1994) with varying degrees of sulphation forming different fractions (Coultate, 1990). The general term carrabiose (Figure 1.2.3) is applied to the repeat disaccharide unit of carrageenan (Stanley, 1990) and substitution of the basic copolymer gives rise to a spectrum of fractions. The primary forms are denoted kappa (κ-), iota (ι–) and lambda (λ) but a total of seven limit forms are recognised (Guiseley *et al.*, 1980). This resulting continuous spectrum means that no one pure form exists naturally but rather particular species dominate within each fraction (see for example, Piculell & Rochas, 1990; Piculell *et al.*, 1992). The constitutional monosaccharides of the primary forms are comprehensively outlined in Coultate (1990).

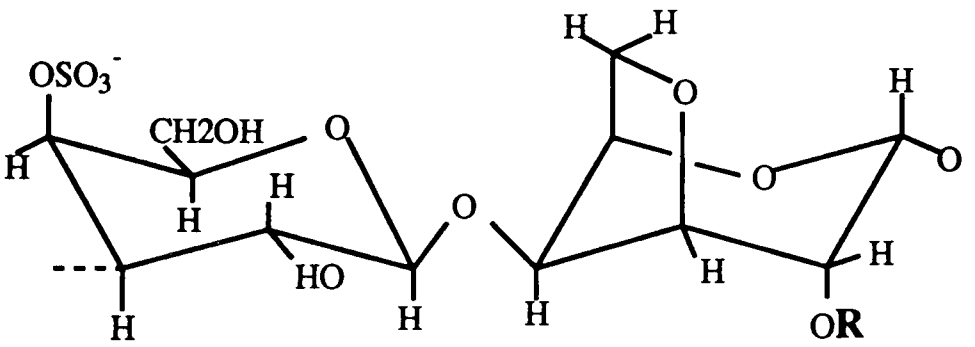


Figure 1.2.3 Repeat disaccharide unit of carrabiose: κ- (R=H), ι- (R= SO_3^-)

Only the kappa- and iota- fractions gel; the former type producing a much stronger, harder and highly brittle gel in contrast to the soft, weak and elastic gel of the latter (Parker *et al.*, 1993; Rochas *et al.*, 1989). Frequently the two forms are added together to provide optimum properties for a specific industrial application (Parker *et al.*,

1993; Coultate, 1990). The lambda form does not gel at all but merely forms viscous solutions and so has found applications in systems that require only mild thickening (Singh & Jacobsson, 1994).

Gelation of the gel-forming types is brought about by essentially a similar process (Rees *et al.*, 1982). The first step requires the formation of double helices through galactan chains winding around each other (Rees, 1977) induced by the presence of certain salts and a temperature lowering (Rees *et al.*, 1982). Other forms of ordered conformation have been proposed in the past - single helices (Smidsrød, 1980) and helical dimers (Rochas & Rinaudo, 1984; Rochas & Landry, 1987) - but the double helix model (Morris *et al.*, 1980a) is thought to be the most likely today (Fernandes *et al.*, 1993). This step in itself is insufficient for gelation; this then only occurs via some sort of network formation (Rees *et al.*, 1982).

An understanding of the mechanisms involved is fundamental in explaining why lambda carrageenan does not gel. With this fraction the first stage of helix formation is not permitted due to the intervention of residues lacking the 3, α-anhydro bridge (Coultate, 1990), a pre-requisite for inversion of the ring geometry from the normal $^{4}C_{1}$ chair form to the $^{1}C_{4}$ (Dagleish & Morris, 1988); failure to do so results in the random coils persisting under all states of environment within this type (Dagleish & Morris, 1988). The other structural feature inhibiting lambda gelation is the degree of sulphation. This is known to have an effect on rheological properties of all the carrageenan types - increasing this level reduces the gelling power (Coultate, 1990). In addition to the sulphation at the oxygen (4) of the 3-linked residue in the kappa and iota forms, iota, the weaker gel also has sulphation at the O(2) of the 4-linked anhydride residue, in turn imparting twice the charge density as that of kappa carrageenan (Dagleish & Morris, 1988). In the case of lambda carrageenan an even greater degree of sulphation is apparent, a disulphate being substituted at the 4-linked residue as well as the variable sulphation of the 3-linked residues (Dagleish & Morris, 1988).

Even though formation of kappa- and iota- carrageenan gels may be similar (Rees, 1982) the resultant conformations and networks have marked differences (Piculell *et al.*, 1992). It is now well established that the helices in kappa-carrageenan undergo extensive aggregation (Rees *et al.*, 1982; Hermansson *et al.*, 1991; Rochas & Rinaudo, 1984) with the formation of "super-junctions" (Coultate, 1990; Brigham *et al.*, 1994). This can be shown through the pronounced thermal hysteresis in the conformational transition (Piculell *et al.*, 1992). This effect is not observed for the iota fraction indicating the reduction or lack of inter-helical aggregation (Piculell *et*

al., 1992). In fact it has been suggested that iota-carrageenan helices are able to exist as stable entities without the imminent formation of a gel depending on the cationic environment (Morris *et al.*, 1980).

It is accepted that gelation is dependant on the surrounding ion environment (Clark & Ross-Murphy, 1987) but the debate on specific ion effects continues (Parker *et al.*, 1993). The presence of the negative charge requires an addition of neutralising cations to the system before gelation can be achieved (Coultate, 1990). Kappa-carrageenan helices have been shown to specifically bind certain monovalent cations - notably potassium, rubidium, caesium and ammonium (Grasdalen & Smidsrød, 1981; Norton *et al.*, 1983) - and induce helix formation at relatively low levels (Nilsson & Piculell, 1991). Involvement with other cations is believed to take the form of predominantly long-range electrostatic interactions (Nilsson & Piculell, 1989). Kappa-carrageenan in the coil form does not show any ion specificity (Nilsson & Piculell, 1992).

The traditional cation for iota-carrageenan gelation is usually calcium (Coultate, 1990) but recent studies suggest that ι-carrageenan shows no specific ion binding (Piculell *et al.*, 1992). The particular preference for divalent cations may be accounted for on the basis of the greater charge (Nilsson *et al.*, 1989). Due to the highly non-homogeneous nature of individual fractions (Parker *et al.*, 1993) the specific ion binding previously thought to be shown by iota-carragenan (Robinson *et al.*, 1980) can now in fact be attributed to the presence of up to a 5% kappa fraction impurity (Piculell & Rochas, 1990). In this latter study, use was made of a κ-carrageenase to isolate the two order-disorder transitions shown by a sample of "iota" carrageenan. Indeed this amount of contamination of one fraction by another is partly dependant on the algal source and 10% or more kappa content has been reported for the predominantly iota fraction (Bellion *et al.*, 1981).

Particular attention will now be paid to kappa-carragenan, the fraction of major interest to this thesis, and its specific ion effects. Much work has been done on this area in the past (Grasdalen & Smidsrød, 1981; Hermansson 1989; Hermansson *et al.*, 1991). The specificity for the alkali metal ions has already been mentioned. The mode of action is thought to be both during first stage transition and second stage aggregation (Hermansson *et al.*, 1991). Investigations into the influence of cations on the coil-helix transition suggest a concentration requirement in the order: $Rb^+ \leq Cs^+ = K^+ << Na^+ < Li^+$ (Rochas and Rinaudo, 1980). In another study the resultant magnitude of elastic and shear modulii were reported as $Cs^+ > K^+ >> Na^+ > Li^+$ (Watanase & Nishinari, 1981; Morris & Chilvers, 1983) which makes good sense

considering sodium and lithium show non-specific ion binding (Nilsson & Piculell, 1989; Nilsson *et al.*, 1989).

The most common counter-ions found in foods are sodium, potassium and calcium (Stading & Hermansson, 1993). As well as externally adding salts to the carrageenan system commercially available κ-carrageenan preparations vary intrinsically in their ion forms (Stading & Hermansson, 1993). Hermansson and co-workers (1991) suggest that work on calcium effects has yielded confusing and non-conclusive data. Considering divalent cationic environments in general, Rochas and Rinaudo (1980) reported that a small dependence exists with regard to transition temperature on melt-down of the helices. For these reasons potassium is usually the salt of choice in which κ–carrageenan is gelled.

The amount of potassium available is in itself a variable on the type of structure formed. At KCl concentrations of 0.1M the gel network consists of coarse, rigid superstrands only whereas at levels of 0.2M fine strands can be observed in addition (Hermansson 1989; Hermansson *et al.*, 1991). The rheological implications of this study indicated that the mixed network gave rise to the strongest gel with fine strands adding support and reducing brittleness. On lowering the KCl concentration even further these workers found that the superstrands are taken out of any gel network and merely left as a suspension in a fine network.

Very recent work by Brigham and co-workers (1994) using fast-freeze deep-etched rotary-shadowed replica microscopy confirm the presence of "superstrands" with junction zone formation over a distance of ~100nm. This was found to be similar for very low (0.01%) as well as high (2%) concentrations. It was also found that kappa-carrageenan in 0.1M KCl shows some strand thickness heterogeneity. Interestingly iota-carrageenan (in 0.05M $CaCl_2$) showed a greater density but of thinner strands suggesting that the dominant entity in ordered iota forms is a double helix with significantly less aggregation than for kappa (K^+) forms (Brigham *et al.*, 1994) confirming the afore mentioned differences in gelation behaviour between the two fractions.

Rochas and Rinaudo (1980) summarised the ionic effect on κ-carrageenan in the following way. They stated that the inverse of the melting temperature of the ordered conformation is linearly dependant on the logarithm of total ionic concentration.

As well as being used alone in its own right κ-carrageenan is often used in combination with other polymers. In milk based products the interaction with κ-

casein has been well documented (Dagleish & Morris, 1988) but one of the most important one in terms of food gels is the synergistic interaction with locust bean gum, or carob gum (see for example, Fernandes *et al.*, 1993).

1.2.1.3 Locust bean gum

Locust bean gum (LBG), extracted from the seeds of *ceratonia siliqua* , or the carob tree, belongs to a class of hydrocolloids known as the galatomannans. The main feature of chemical structure of this group of polysaccharides is the linear backbone of ß-1,4-D-mannopyranosyl (M) residues substituted to varying degrees with single unit side chains of α-D-galactopyranosyl (G) at the 6 position (Dey, 1978). The distinctive rheological aspect of the gum family as a whole is the ability to form highly viscous solutions, but the inability to gel alone under normal conditions (Coultate, 1990). In the absence of any strong interactions between macromolecular chains such solution properties are mainly determined by the topological entanglements they contain (Fernandes, 1994a). This in turn is primarily governed by molecular weight and concentration considerations (Launay *et al.*, 1986).

...→4)–D–Man–(β1→4) –D–Man–(β1→4)–D–Man–(β1→4)–D–Man–(β1→...
6
↑
D-Gal–α

Figure 1.2.4 Schematic structure of locust bean gum

The degree of substitution of locust bean gum, as expressed by the mannose to galactose ratio, varies according to botanical source and preparation (Stading & Hermansson, 1993). Typical values of M/G ratio range from about 3 to about 5 (Stading & Hermansson, 1993) with Fernandes and co-workers (1994a) stating a mid-point value of 4:1. A high M/G ratio causes a reduction in solubility (Stading & Hermansson, 1993) and this forms the basis of the hot- and cold-water soluble fractions commercially available. Evidence of solubility differences between "standard" and "quick-soluble" fractions is provided microscopically by the work of Brigham and co-workers (1994) who used fast-freeze deep-etched rotary-shadowed replica microscopy to show clearly convincing white patches in the standard grade, but absent from the higher-soluble LBG, suggestive of undissolved material.

If the galactose content of LBG is put at ~20% (Fernandes *et al.*, 1993) then in comparison, guar gum (extracted from *Cyamopsis tetragonoblus* (Fernandes *et al.*, 1994a), the other major commercially exploited galactomannan, has 33%. For completion, tara gum (a "new" galactomannan with potential future applications) has a galactose content of ~ 25% (Fernandes *et al.*, 1993).

Much work has been done in the past in determining the arrangement of G residues along the mannose backbone of LBG. A number of distributions have been proposed: the partial block theory of Courtois and Le Dizet of 1970; the three types of blocks- unsubstituted, alternatively substituted and total substitution - of Hoffmann and Svensson in 1978; the non-regularity with high proportions of substituted couplets, lesser amount of triplets and absence of blocks of substitution of McCleary *et al.* in 1985. Total randomness of distribution was ruled out by these last workers based on the fact that statistically there was a higher proportion of blocks of intermediate size. Similar uncertainties have surrounded the elucidation of structures in other galactomannans.

In the first paragraph it was stated that gums did not gel under *normal* conditions. It has been shown in the past (Dea *et al.*, 1977) and again more recently (Brigham *et al.*, 1994) that a set of conditions *do* exist under which LBG will gel, namely the subjecting of a dilute solution of LBG to a freeze-thaw cycle. This phenomena is not shared by guar gum and this has been attributed to its lower M/G ratio (Dea *et al.*, 1977). Specialist microscopic examination of LBG gels using the technique of fast-freeze deep-etched rotary-shadowed replica microscopy (Brigham *et al.*, 1994) reveals a fine network structure of numerous single chains. Gidley and co-workers have rationalised in the past (1991) that LBG gelation does not involve a significant conformational transition but rather that weak junction zones are due to low galactose substituted regions; this can be confirmed by the excellent afore mentioned micrographs.

1.2.1.4 The Locust Bean Gum /κ-Carrageenan Interaction

Synergistic polysaccharide-polysaccharide interactions have long been employed commercially (Glicksman, 1968) in the pursuit of cheaper gelling formulations and to generate new functionality's as well the manipulation of current rheologies (Cairns *et al.*, 1987). The most widely exploited of such systems is that of κ-carrageenan/LBG (Fernandes *et al.*, 1993).

The normally non-gelling LBG can interact with carrageenan (amongst other polysaccharides not of concern here) to produce gels of a lower total polymer concentration than that required for κ-carrageenan gelation alone (Dea & Morrison, 1975; Fernandes *et al.*, 1994b). This binary blend has a significantly enhanced gel strength though as would be expected this will depend on the mixing ratio. Fernandes *et al.* (1994a) cite the results of a number of workers who put the range of optimum ratios to lie anywhere between 10:1 (car/LBG) right down to 1:1 depending on KCl environment and total polymer concentration. The influence of salt concentration is demonstrated by Turquois and co-workers (1992) who show that in 0.1M KCl the best gel is observed at equal mixing ratios whereas reducing the KCl content to 0.05M changes this ratio to 3:2 (car/LBG). Fernandes and co-workers (1991), who set this maximum synergy themselves at 4:1, state that the synergistic maxima are related to the intrinsic viscosity and the M/G ratio, suggesting that the mechanism can be ascribed to both the "smooth" regions and the molecular weight.

The traditional view of the gelation mechanism (Dea & Morrison, 1975) involved the "smooth" (unsubstituted) regions of the mannan chain in LBG interacting specifically with the κ-carrageenan double helix. The "hairy" regions can then contribute to the gel strength by cross-linking the overall network (Morris, 1990). However, based on a lack of evidence for intermolecular binding requisite for this model, another idea has been postulated by Cairns and co-workers (1987) which suggests that the LBG is contained as a solution within a continuous carrageenan gel network. The possibilities arising from the mixing of two such components are summarised below (Cairns *et al.*, 1987):

(1) With no contribution from the LBG (denoted polysaccharide A) the simplest structure consists of a carrageenan (denoted polysaccharide B) network which merely contains A within it.

(2) When both components are able to contribute to the gel we can then have:

(i) an interpenetrating network - arising from the independent association of both A and B to produce interlaced separate networks

(ii) a phase separated network - brought about by demixing prior to gelation

(iii) a coupled network - the binding of A to B, or *vice versa*, results in specific junction zones.

It is apparent then that the traditionally held view fits the last model. Studies based on NMR and rheological techniques (Tvaroska *et al.*, 1992) uphold this theory of intermolecular binding between the two polymers via unsubstituted regions. The

proposal of Cairns and co-workers (1987), which satisfies the first model, does not denote a specific role to the galactomannan (Fernandes *et al.*, 1991). These latter workers believe that the galactomannan contributes a secondary network, through galactomannan-galactomannan aggregation at "smooth" regions of the mannan chain, producing an interpenetrating polymer network overall. Turquois *et al.* (1992) lend support towards the original idea of intermolecular binding but agree that the model of self-association of the galactomannan chains promoted by κ–carrageenan chains should not be rejected either.

Regardless of the mechanism the structure of LBG is obviously a crucial consideration in dictating the outcome of gelation, and this has been shown by other workers through studies on κ-carrageenan/galatomannan interactions. Unlike LBG guar gum does not exhibit such a synergy (Fernandes *et al.*, 1993) and this has been attributed to its lower M/G ratio. By using an enzyme modified guar gum however this interaction can occur (McCleary & Neukom, 1982). Further support for the importance of M/G ratio is given by the fact that tara gum with a galactose content of ~25% (Fernandes *et al.*, 1993) will also undergo a synergistic interaction with κ-carrageenan (Cairns *et al.*, 1987).

The total polymer concentration also plays a role in the type of gelation observed. Studies at very low concentrations of κ-carrageenan (Fernandes *et al.*, 1994a) reveal that LBG and guar gum mixtures produce similar viscoelastic properties. At low levels (0.01%) of carrageenan, network formation does not occur but rather a continuous dispersion of aggregates of "micro-gels" exists over the whole volume (Fernandes *et al.*, 1994a). The other phase, the continuous phase, is then provided by the galactomannan which in turn determines the overall rheological properties. On increasing the carrageenan to 0.05% (in adequate levels of KCl) a carragenan network forms, entrapping the galactomannan solution and consequently producing a gel. In this situation the rheological properties are dictated by the carrageenan (Fernandes *et al.*, 1994a). However work at low concentrations by Goycoolea and co-workers (1993) show enthalpic and mechanical interactions which are best interpreted by inter-molecular coupling.

Microscopic imaging of κ-carrageenan/LBG gels (Brigham *et al.*, 1994) clearly shows recognisable features corresponding to each component. These workers suggest that an intimate mix of a carrageenan network, with the same strand and pore features as for the carrageenan control, and an LBG network exists. However, the debate over gel mechanism is not over; although this work eliminates the possibility of bulk phase separation these workers argue that the inter-network connections

observed could be an artefact of the technique. Hopefully the combined research over the next few years should establish once and for all the underlying mechanisms in κ–carrageenan/LBG gelation.

1.2.1.5 Agar

Extracted from the family of red seaweed called the *Rhodophyceae*, agar is a complex sulphated galactan. The main structural feature is repeating units of 1, 3-linked ß-D-galactose and 1, 4-linked 3, 6-anhydro-α-L-galactose. This disaccharide is known as agarobiose (see for example Matsuhashi, 1990).

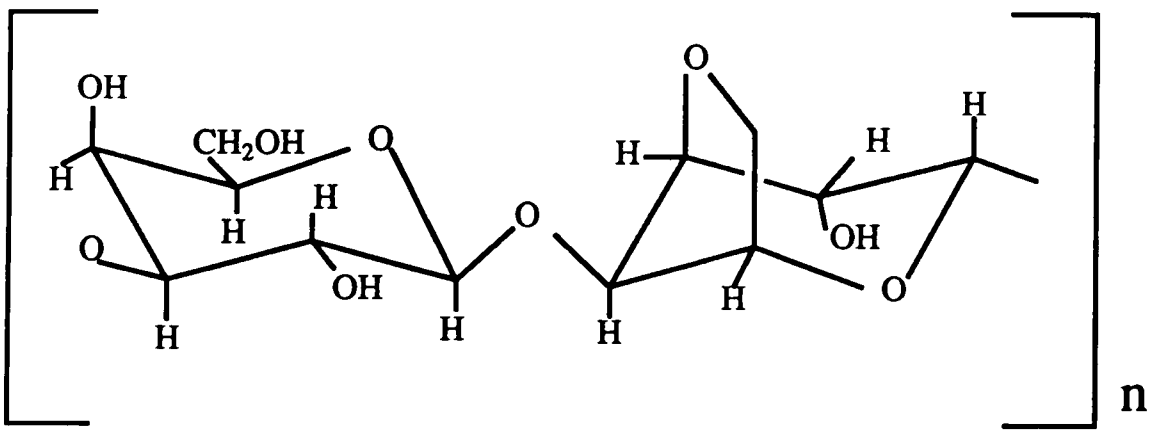


Figure 1.2.5 Idealised structure of agarose

With a similar basic structure to the other red seaweed derivatives - the carrageenans - but with the lowest ester sulphate content of the group, agar is known for its ability to form strong gels (Coultate, 1990). Indeed agar is one of the most potent gel formers known; gelation threshold concentrations being quoted down as low as 0.04% (Selby & Whistler, 1993). Along with the sulphate content (and positioning of the groups) the rheological properties of agar have been shown to be also dependant on the molecular weight and repeating pattern of galactose residues (Watase & Nishinari, 1983).

Within agar itself there are two different groups of polysaccharides - a neutral fraction called agarose and the more complicated, charged fraction of agaropectin (see for example, Matsuhashi, 1990). Both groups have a common feature of an agarobiose skeleton but the latter contains D-guluronic and pyruvic acids plus other trace sugars (Matsuhashi, 1990). Araki (1956a,b), whilst elucidating the primary structure of agar, isolated and named the component with the highest gelling power, agarose

(Arnott *et al.*, 1974). In accordance with the gel strength series to be found in the carrageenans, the other red seaweeds which share a similar structure to agar, the low sulphate content and neutral charge can explain this observation. In common with other polysaccharides it is more usual to have a distribution of fractions within a sample rather than well-defined separate entities (see Arnott *et al.*, 1974).

Agarose is believed to form gels as a result of association through a three-fold left-handed parallel double helix with a pitch of 1.90 nm and a central cavity along the helical axis (Arnott *et al.*, 1974). This cavity, which is relatively small with a minimum diameter of 0.45nm (Arnott *et al.*, 1974), can accommodate water molecules without too much steric hindrance (Matsuhashi, 1990). This is achieved through the hydroxyl groups lining the cavity's interior participating in hydrogen bonding with water (Morris & Norton, 1983). NMR experimental studies by Ablett and co-workers (1978) have indicated the presence of bound water within the helix. This helical-water binding seems to be a unique feature of agar (Arnott *et al.*, 1974).

Agar must be boiled at neutral pH to produce a "true" gel (Matsuhashi, 1990). Although the sol sets to a gel at ~30°C-40°C remelting of the gel requires 85°C-95°C (Glicksman, 1968; Matsuhashi, 1990). Thus gels of agar show thermal hysteresis. Although different authors quote different absolute temperatures this is reinforced by more recent literature; the firm gels formed on heating the polymer solution to above 90°C then cooling to ~30°C require remelting temperatures of ~80°C (Ross-Murphy & Shatwell, 1993). These authors describe its gelation mechanism as chain disordering (and consequently double helix melting) at temperatures above its coil-helix transition temperature. On re-cooling, these individual chains partly revert to the native double helix form and physical cross-links are brought about by the sharing of helices between different polymer chains (Ross-Murphy & Shatwell, 1993). The turbidity of agar gels, unlike most polysaccharide gels, is evidence for the association of double helices and this in turn accounts for the characteristic stiffness (Arnott *et al.*, 1974). Extensive helical aggregation would give rise to a network of side-by-side assembly around a large void, producing internal channels, and it is this special property that renders the gel an excellent separation medium for biochemical techniques (Arnott *et al.*, 1974).

The technique of fast-freeze deep-etch rotary-shadowed microscopy was used by Brigham and co-workers (1994) to reveal the microstructure of agar gels to consist of a broader strand distribution and more heterogeneous pore size than for carrageenan gels.

As well as having applications in biochemistry agar has also found invaluable applications in microbiology and medicine (Selby & Whistler, 1993). Applications to the food industry have been slow, but their use as gelling agents in dairy products and as stabilizers in other products is increasing (Coultate, 1990). The uncharacteristic high melting temperature of agar gels (coupled with the good emulsifying, stabilizing and gelling properties) make it extremely useful in a variety of food products including confectionery and baked products and also vegetarian products through the replacement of gelatin by agar (Selby & Whistler, 1993).

1.2.1.6 Alginate

Whereas the agars and carrageenans collectively constitute the red seaweeds, the alginates are the salts of alginic acid, the primary structural polysaccharide of the brown algae, or the *Pheophyceae* (Coultate, 1990). Alginate consists of the two monosaccharide units of ß-D-mannuronic acid (M) and α-L-guluronic acid (G) (Figure 1.2.6a, 1.2.6b) arranged as a linear co-polymer. In addition alginates can also be excreted as extracellular mucilages by some species of bacteria (Percival & McDowell, 1967; Sandford, 1979). From bacterial sources various levels of acetylation at C-2 and/or C-3 are observed (Yalpani, 1988).

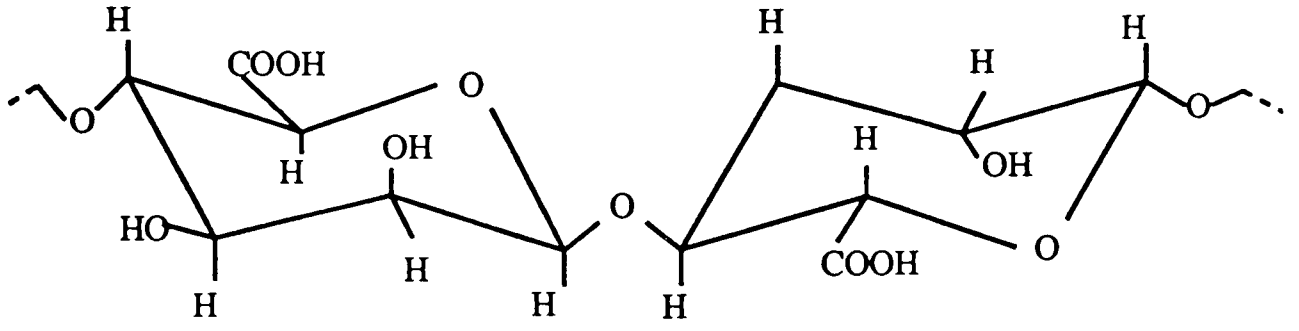


Figure 1.2.6a Mannuronic acid

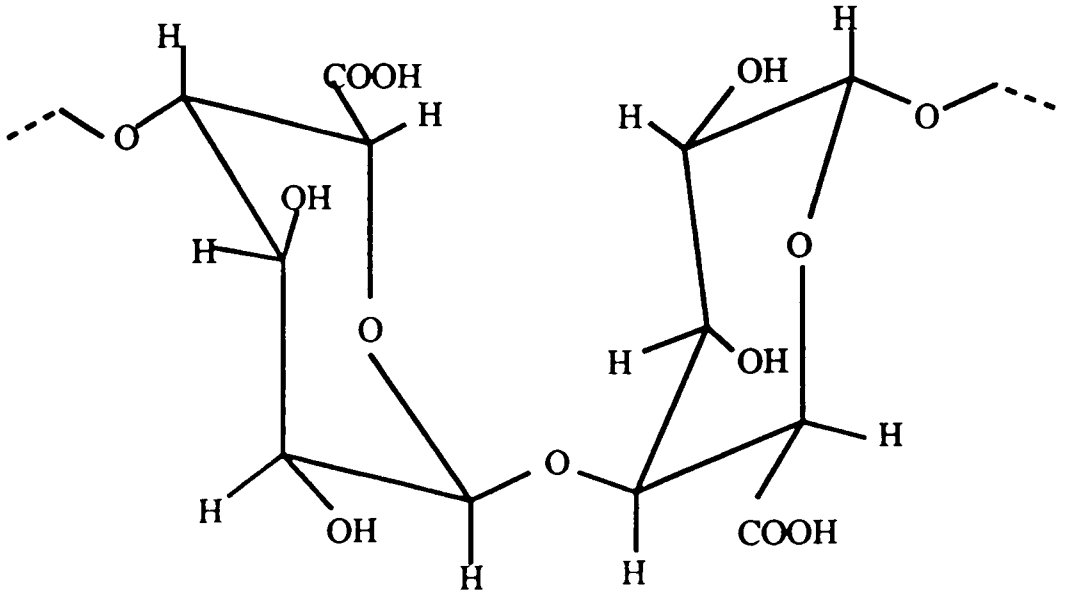


Figure 1.2.6b Guluronic acid

These residues are C-5 epimers of each other and are arranged in homopolymeric (GG and MM) and heteropolymeric (MG) sequences in varying proportions and distribution patterns (Painter, 1983). In each case this is achieved by 1-4 linkages (Haug *et al.*, 1966, 1967; Larsen *et al.*, 1969). The G blocks have been shown by ion binding studies (Kohn, 1975) and gelation studies (Morris *et al.*, 1973; Kohn & Sticzay, 1977) to vary in length but to always be greater than 20 residues long. In contrast the M blocks are constantly of a uniform length (~24 residues) , as shown through enzymatic studies (Boyd & Turvey, 1978). This study also revealed that the MG blocks deviate significantly from regular alternation. As a consequence of the shape and linkages of these residues the geometries of the G block regions, M block regions and alternating regions are very different; the G blocks are buckled whilst the M blocks adopt an extended ribbon conformation (Sime, 1983).

The molecular weights of commercial alginates usually exceed 150 000 (Ball, 1989) but molecular weight distributions are influenced by the method of extraction and algal source. This in turn affects the quality of the gel formed; lower molecular weights produce brittle gels whereas the gel of a higher molecular weight alginate will be more elastic (Sime, 1983).

Whereas alginic acid is generally insoluble in water (the only exception being the fractions with a high content of alternating block sequences) sodium alginate along with most other alginates of monovalent metals, are soluble in water and give solutions of high viscosity (Smidsrød, 1970). For alginate gelation to occur it is necessary to include divalent ions in the system (Mitchell & Blanshard, 1976). In the presence of calcium ions, strong gels readily form from a well characterised arrangement known as the "egg-box" model (Sime, 1983; Coultate, 1990). The alignment of two buckled shape guluronate chains yields an intermediary cavity which exactly accommodates a calcium ion (Sime, 1983) and for this reason junction zones only arise at guluronate repeating sequences (Coultate, 1990) and more particularly, only with chains exceeding ~20 residues (Kohn, 1975). In the process of gelation, chain dimerisation constitutes the main feature (Morris *et al.*, 1977) with secondary aggregation of the chains then also possibly following (Sime, 1983). The ability of calcium alginate gels to withstand very high temperatures (they do not melt below 100°C) enables their practical application in a number of food uses, such as synthetic fruit pieces (Coultate, 1990).

However the interest of alginate in this thesis is the surface adsorption potential in the presence of proteins at their isoelectric point (see Chapter 6). Under acidic conditions anionic polymers such as sodium alginate will interact with proteins, in this case

bovine serum albumin (BSA) in what is described as an electrostatic interaction (Imeson, 1983).

1.2.2 The Proteins

1.2.2.1 Gelatin

Gelatins are polydisperse water-soluble products derived by denaturation and partial degradation from the insoluble, fibrous protein collagen - the principle protein component of skin, bone and other connective tissue (Ross-Murphy, 1992). Gelatin is unique amongst the proteins in that it exhibits thermo-reversible gelation, a property normally only seen in polysaccharide gels, and perhaps providing the most common example of a melting gel (Clark *et al.*, 1994). Indeed the term "gel" is derived from this protein (see Ross-Murphy, 1992). Along with its many food-related uses gelatin has also found numerous applications in the pharmaceutical and photographic industries (Gautam & Schott, 1994; Fruhner & Kretzschmar, 1992).

As already stated gelatin is produced from collagen and a brief description of the latter will now be given. Collagen is a complex arrangement of fibres; each level of arrangement consisting itself of a fibrilar arrangement. At its highest level a collagen fibre contains bundles of smaller fibrils, strengthened by covalent intra- and intermolecular crosslinks rendering the animal tissue insoluble, with each fibril itself consisting of 5 micro-fibrils (Djabourov *et al.*, 1993). Each microfibril consists of a number of crosslinked, longitudinally arranged tropocollagen molecules of 280-300nm in length, with a pattern of transverse banding across the fibre of repeat distance 64nm (Coultate, 1990; Djabourov *et al.*, 1993). Tropocollagen molecules are actually a right-handed triple helix of polypeptide chains with each polypeptide chain itself a left-handed helix. The triple helix is stabilized by intramolecular hydrogen bonds (Coultate, 1990; Djabourov *et al.*, 1993). When thermal or chemical treatments are applied to collagen the hydrogen and covalent bonds are broken resulting in the disordered, soluble gelatin state containing random, flexible coils of ~ 300nm radius of gyration (Djabourov *et al.*, 1993).

The amino acid chain of gelatin is characterized by the high occurrence of glycine, proline and hydroxyproline with trace amounts of tryptophan and cysteine (Janssens & Muyldermans, 1994). Such an occurrence of amino acids does not arise by chance. At the tropocollagen level the glycine, accounting for approximately one third of the amino acids and occupying every third residue along the chain, has been shown through structural studies, to exist at the points in the helical chain where the three

strands approach closely - i.e. where only the single hydrogen atom of glycine would be accommodated (Coultate, 1990). Equally fascinating is the significance of the two imino acids (accounting for 20-25%) whose particular form of the peptide bond gives the ideal bond angles of the tropocollagen helix (Coultate, 1990). Of these two the proline content is particularly important, as it tends to promote formation of the polyproline II helix, which ultimately determines the form of the tropocollagen trimer (Ross-Murphy, 1992).

Two distinct types of gelatin, with distinct differences in isoelectric point and viscosity, occur depending on the nature of the hydrolysis (Janssens & Muyldermans, 1994). Hydrolysis in dilute inorganic acid results in type A with a pI value always higher than 6.5. Alkaline hydrolysis gives rise to type B with a pI of ~5 and the greater viscosity (Janssens & Muyldermans, 1994). The type is often dictated by the source; pigskin gelatins are always type A, hide (bovine) gelatins are always type B whilst bone gelatins can be either type (Janssens & Muyldermans, 1994).

Gelation of gelatin occurs readily on cooling a hot ($\geq$ 40°C) solution in conditions where a critical concentration, typically 0.4%-1.0%, has been exceeded (Ross-Murphy, 1992). The willingness of gelatin to form gels is demonstrated by the fact that the solution only has to be cooled below 30°C before the onset of gelation (Djabourov *et al.*, 1993; Clark *et al.*, 1983). The mechanism underlying this process is thought to involve the partial reversion of the random coils to a helical state similar to tropocollagen (Djabourov *et al.*, 1993), likened to a "renaturation" by some authors Djakovic *et al.*, 1990) whereby ordered quasi-crystalline sequences form junction zones separated along the chain by regions of disorder (Clark *et al.*, 1983; Ross-Murphy, 1992). Unlike other gelling systems, when a coil-helix transition occurs rapidly and in the vicinity of the transition temperature, in the formation of gelatin gels an initial, helix nucleation phase must first be achieved after ~hours and is then followed by an even slower, propagation process (Ross-Murphy, 1992). In the same excellent review by Ross-Murphy an alternative theory to the traditional view of triple helix formation - separately wound "tropocollagen" peptide chains each involved in several junction zones - is highlighted. Here work by Busnel and co-workers (1988;1989) suggest that in the first instance helix nucleation is a bimolecular process involving two gelatin macromolecules, one with an intramolecular ß-turn. Only then when a third molecule comes into contact with a correctly orientated kink will helix formation be initiated. This alternative approach also has significance for the chemical sequencing; the "hair-pin" bend of the intramolecular ß-turn requires a triple peptide with only one imino acid (Stainsby,

1991) in opposition of the traditional belief that adjacent proline rich regions were necessary (Ross-Murphy, 1992).

Melt-down of gelatin gels, since they exhibit thermo-reversibility, has been related to concentration and molecular weight and described in the Flory-Stockmayer condition as a "shift in a reversible cross-linking equilibrium to the point where the degree of cross-linking falls below that critical threshold needed for gelation" (see Clark *et al.*, 1994).

The measure of gel strength is given by the bloom value and exists under very well-defined conditions (see Janssens & Muyldermans, 1994, for conditions). Three categories of bloom value exist:

Low Bloom	<150
Medium Bloom	150-220
High Bloom	>220

Commercial gelatins are usually of the range 60-280 (Janssens & Muyldermans, 1994). Functionally, gelatin is used as a thickener, emulsifier, foaming agent, water binder, whipping agent and coacervation agent along with its ability to form a melt-in-the-mouth gel. It currently finds new applications as fat replacers in many low-fat spreads. Indeed the basis for one of these, commercially known as Slimgel, involves a gelatin-polysaccharide interaction. The thermodynamic incompatibility between gelatin and guar or locust bean gum results in phase separation, which on dissolution and setting, produces a dispersion of discrete gelatin beads in a continuous viscous galactomannan matrix as a water/water emulsion (Janssens & Muyldermans, 1994).

A number of other gelatin-polysaccharide interactions have been studied: gelatin-gum arabic coacervate films were formed by Peker-Basara and co-workers (1993); starch-gelatin gels were investigated by Djakovic and co-workers (1990); the effect of chitosan on gelatin gels was the subject of Ichikawa and co-workers (1992). Gelatin-whey mixtures have also been investigated by Walkenström and Hermansson (1994). A particularly relevant investigation was made by Clark and co-workers in 1983. This study of agar-gelatin gels revealed an antagonistic effect of each component resulting in phase-separation (an unevenly dispersed microgranular agar gel in a gelatin gel medium) and ultimately phase inversion at specific concentrations. In another study this phase inversion was stated to occur at levels of 0.5% agar/0.5% gelatin (Moritaka *et al.*, 1980).

1.2.2.2 Sodium Caseinate

Milk from the domestic cow is a significant contributor to the protein intake of the human diet. In addition to its impact as a food in its own right, bovine milk is the only milk out of the 5 or 6 mammalian milks used in total by Man that is also utilized as a source of functional proteins (Fox, 1989). Its long inclusion in the diet combined with the relative ease of isolation of the fractions has meant that milk proteins are the best characterized of all food proteins (Swaisgood, 1982; Fox & Flynn, 1992) with the primary structure now known for all the major components (Swaisgood, 1982).

Bovine milk contains ~ 13% solids made up of fat, lactose, protein and organic and inorganic salts (Mulvihill, 1989). Of the 3.5% (w/v) protein content of normal milk two distinct groupings can be made based on solubility at pH 4.6 and from this test the definitions of casein and whey proteins arise: 80% of the total nitrogen precipitates - this represents the casein fraction - with the remaining 20% is soluble in the whey or serum (Mulvihill, 1989). It is sufficient to say that the whey proteins account for the largest part of this soluble fraction (15%/20%) whilst the remainder is made up of non-protein nitrogenous material (Mulvihill, 1989). Within the casein fraction are four primary proteins termed α- (subdivided into α_{s1}- and α_{s2}-casein on the basis of its sensitivity to calcium ions), ß- and κ-casein of which the molecular weights are known to be 23 500, 24 000 and 19 000 respectively (Mulvihill, 1989; Coultate, 1990). Several minor proteins have also been quoted, one being γ-casein, which can account for as much as 4% of the casein proteins (Coultate, 1990), but which is now thought to be merely a breakdown product of β-casein brought about during the separation process (Swaisgood, 1973) by the action of proteolytic enzymes in milk (Coultate, 1990). Indeed milk contains up to 60 indigenous enzymes (Fox & Flynn, 1992) but these will not be discussed here. There are also known to be a number of different versions of each casein, differing in their amino acid sequences, but this discussion will be limited to the four primary proteins. Within the micelle the α_{s1}-, α_{s2}- , β- and κ-casein exist in the ratios of 3: 0.8: 3: 1 (Aoki, 1991) .

All the caseins are phospho-proteins; bovine α_{s1}-, α_{s2}-, β- and κ-caseins contain 8-9, 10-13, 4-5 and 1-2 P per mole of protein respectively (Fox & Flynn, 1992). These phosphate residues, present as monoesters of serine, can strongly bind metal ions, with the formation of the casein micelle being attributable to the high affinity of the α- and β-caseins to bind calcium (Fox & Flynn, 1992).

Whilst the knowledge of the individual caseins is well established - a detailed chemistry of the caseins can be found in Rollema, 1992 - the arrangement of these within the colloidal particle known as the micelle is less clear. What *is* known is that the casein micelle is a large, spherical particle with diameters in the range 50-300nm and amounting to ~10^{15} per dm^3 of milk (Coultate, 1990). For this reason milk can be regarded as essentially a two-phase system consisting of a micellar and serum phase (Rollema, 1992). Rotary shadowing electron microscopy clearly indicates the spherical shape and large size of the micelle (Kalab *et al.*, 1982). Approximately half the inorganic phosphate and two-thirds of the calcium are present in bovine milk in the colloidal form, with the remainder being soluble (Pyne, 1962).

Many models for the structure of the casein micelle have evolved over the decades (see Rollema, 1992) based on either core-coat, internal structure or sub-unit models but only the three models most usually accepted will be discussed here. All three models represent examples of sub-unit models but can be placed into two types: that of Slattery and Evard (1973) and the models of Schmidt (1982) and Walstra (1990).

The casein micelle is often depicted as the Slattery and Evard (1973) model, (Figure 1.2.7) whereby the micelle is an aggregate of submicelles, each consisting of 25-30 molecules of casein fractions in similar proportions to that found in milk. Submicelle formation is brought about by the association of the different casein molecules, each in an ellipsoidal shape due to the folding of the polypeptide chain, by the hydrophobic ends. The sub-micelles are then linked together through the reaction of calcium ions with the phosphoserine residues at the polar ends of the α- and β-caseins. The κ-casein fraction however is deficient of phosphate, carrying instead a trisaccharide unit on its threonine residues, and this serves to yield an area where no further sub-micelle binding can occur preventing an indefinite increase in micelle size (Coultate, 1990).

(A)

(B)

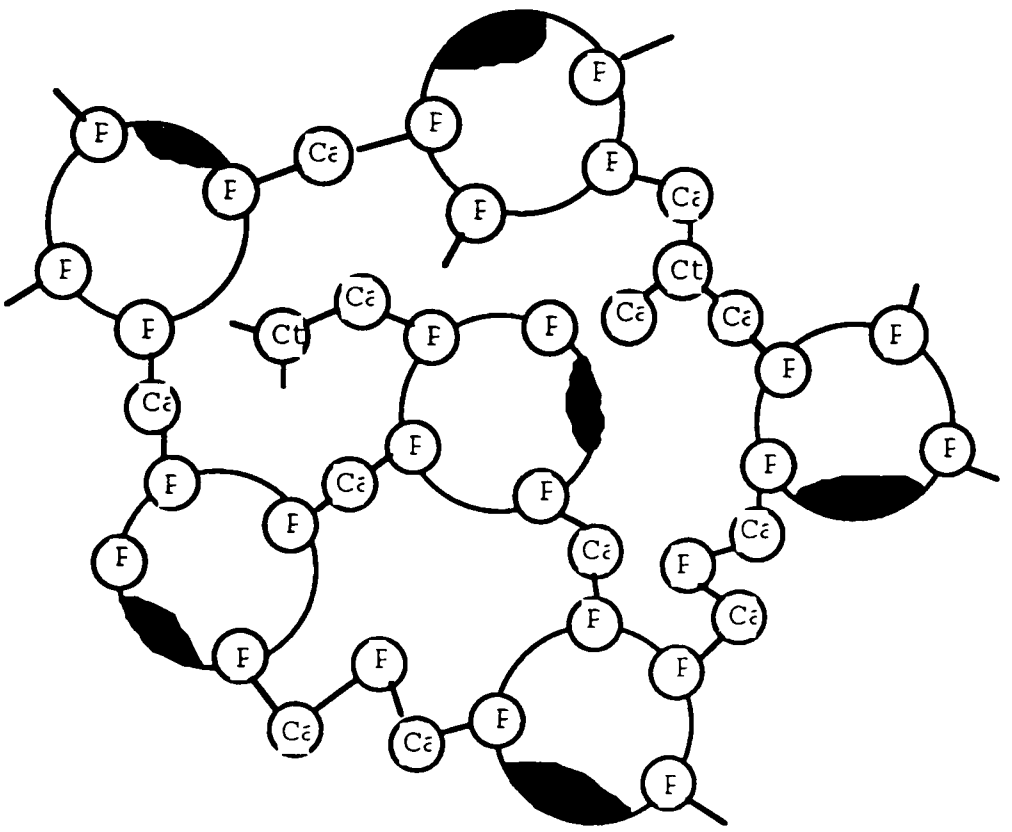
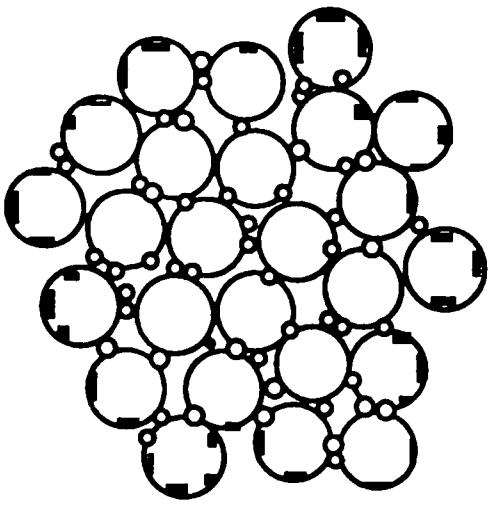


Figure 1.2.7 The casein micelle (model of Slattery & Evard, 1973); (A) The micelle (B) Cross-link formation between sub-micelles : P = phosphate, Ca = calcium, Ct = citrate, dark areas = non-linking

Adaptations of this model exist in the form of the Schmidt (1982) and the Walstra (1990) models. In the Schmidt model however the sole linking mechanism of the submicelles is by calcium phosphate, since there are no inter-subunit protein-protein interactions, with the assumption that a calcium ion is attached to virtually all of the ester phosphate groups and some groups are linked in pairs by $Ca_9(PO_4)_6$ cluster.



o = $Ca_9(PO_4)_6$ cluster linking sub-micelles

dark regions on peripheral micelles represent non-bonding κ-casein molecule.

Figure 1.2.8 Micelle model proposed by Schmidt (1984)

In the later model of Walstra (1990) the concept of steric stabilization of the micelle by κ-casein was added to the model of Schmidt. With the Walstra model the C-terminal parts of the κ-casein protrude from the micelle surface into the surrounding solvent.

It has been concluded that the combination of all three models best describes the experimental evidence to date with the overall implication that hydrophobic interactions and a calcium phosphate phase ensure the integrity of the micelle with the additional stabilizing action of κ-casein afforded by the Walstra model (Rollema, 1992). The role of colloidal calcium phosphate (CCP) in micelle structure is detailed by Aoki, 1991.

Casein is isolated from milk by principally one of two ways: (1) isoelectric precipitation, through either mineral acids, ion exchange resins or a lactic starter or (2) proteolytic coagulation using calf rennet or a substitute. A number of refining states are then employed including cooking and dewheying before final form casein is obtained. An expansion of the stages involved can be found in Mulvihill, 1992. A variety of plant designs for producing casein are given in Muller, 1982. In addition to the solubility differences between casein and whey, exploited as the basis of isoelectric precipitation, another physical difference between the fractions is that caseins are extremely heat stable whereas whey proteins are fairly easily denatured. This reflects the essentially random coil nature of the casein components.

Caseinate constitutes one of the four primary protein products from skim-milk, the others being acid casein, rennet casein and milk-protein co-precipitates as shown

below (Kirkpatrick & Walker, 1985). Secondary products include lactalbumin and whey protein concentrate, derived from the whey by-product of casein manufacture.

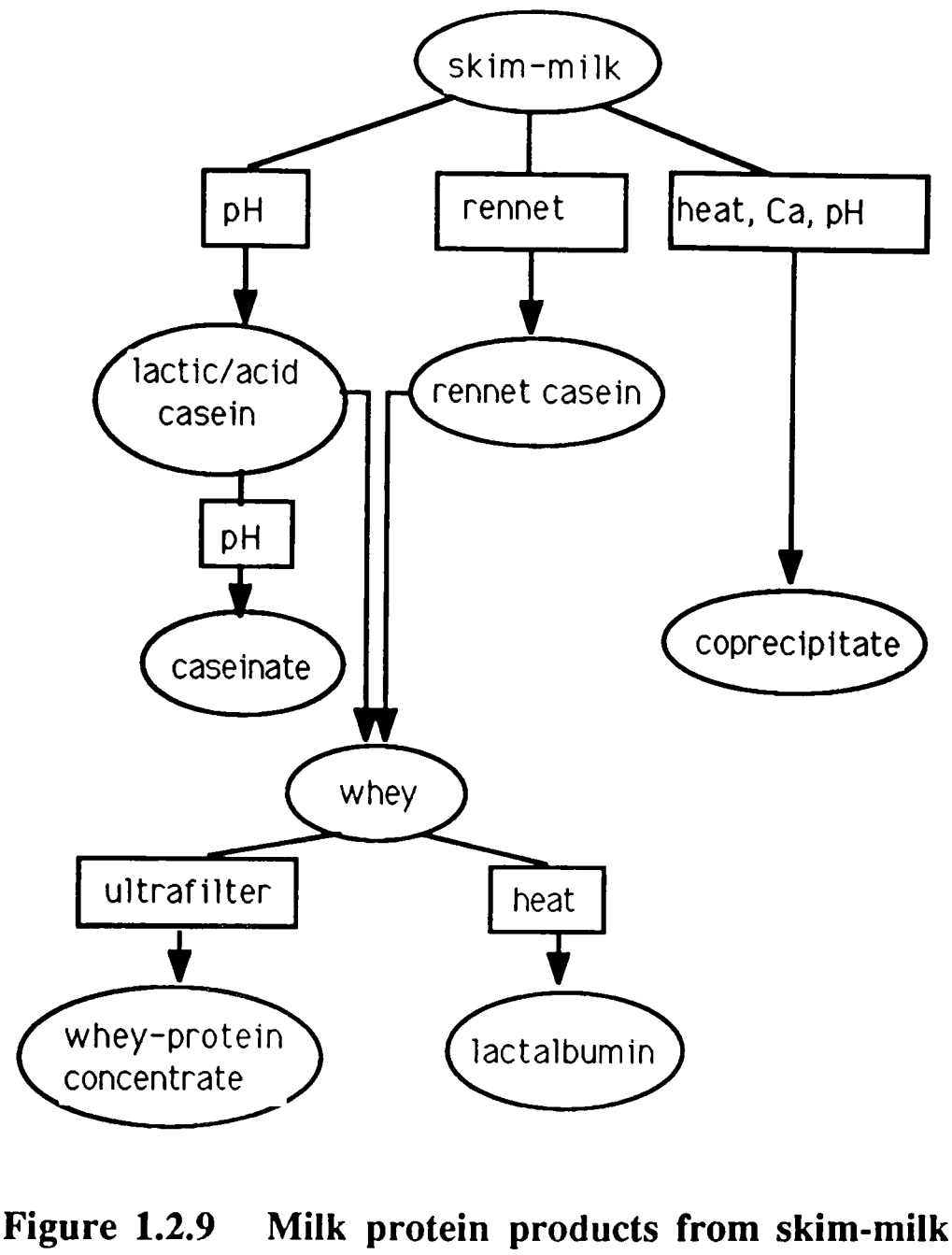


Figure 1.2.9 Milk protein products from skim-milk (from Kirkpatrick & Walker, 1984)

Sodium caseinate, the water soluble caseinate most commonly used in foods, can be prepared by a number of methods. A detailed description of these methods can be found in Mulvihill, 1992. Traditionally caseinate is derived from the starting material of curd or acid casein but alternative approaches such as skim milk powder extrusion are also reported in the literature (Barraquio & van de Voort, 1991) and such methods are discussed by Mann in his 1991 review. To obtain a bland flavoured caseinate it is better to use a fresh, undried acid casein curd rather than dried casein (Bergman, 1972; Southward, 1985).

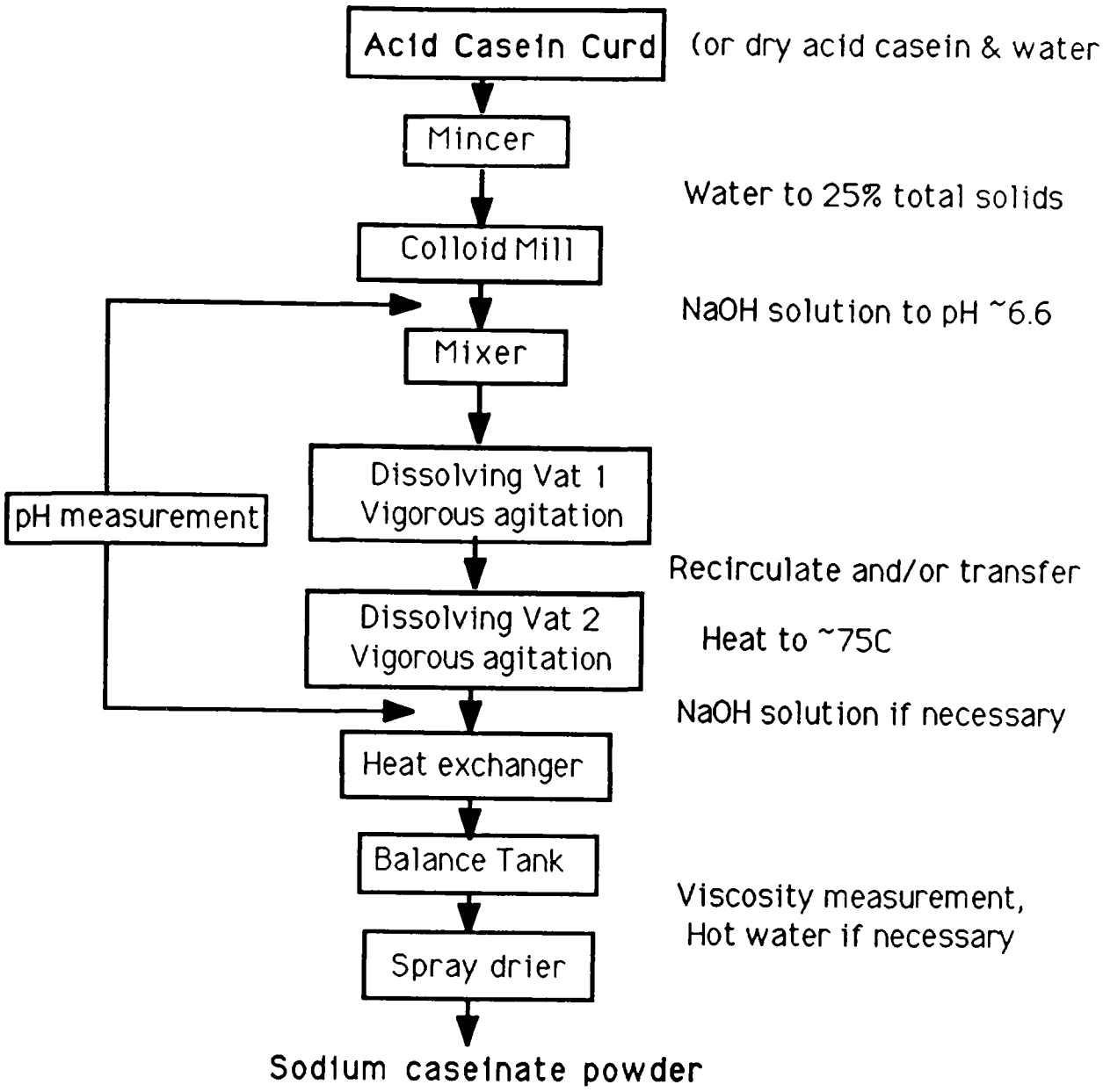


Figure 1.2.10 Production of sodium caseinate (from Mulvihill, 1992)

The molecular weight of sodium caseinate is 250 000 (Visser, 1988). Whereas the micelle is an aggregate of α- and β- casein held together by amorphous calcium phosphate, with the whole micelle stabilized against flocculation by a coat of κ-casein, disruption of the micelle using alkali yields the sub-micelle structure in isolation (Visser, 1988). Caseinates represent the functional milk proteins in the most concentrated (low lactose) and most functional (low calcium) form (Visser, 1984).

Sodium caseinate, which forms a pseudo-plastic solution (Towler, 1974), has found numerous uses in foods. In the meat industry caseinates are utilized, amongst other things, for their excellent emulsifying capacity (Visser, 1984). Applications specifically related to sodium caseinate include: yoghurts (reduction of syneresis, increase in gel firmness); milk shakes (emulsifier and foaming agent); powdered coffee creamers (emulsifier/fat encapsulator, adds body, prevents coagulation and whitens all together produce a high convenience - long shelf life/no refrigeration -

alternative) and imitation cheeses (optimized melting behaviour and stringiness) (Mulvihill, 1992). In addition to food uses caseinates also find applications in pharmaceutical and medical fields.

1.2.2.3 Bovine Serum Albumin

Albumins are secreted proteins, made in the liver, and present in high concentrations - 60% of the total plasma protein (Peters, 1985). In human plasma the albumin level is stated as 42g/L and is thus the major circulatory support protein but unlike other secreted proteins albumin lacks any carbohydrate (Peters, 1985). The molecular weight of bovine serum albumin (BSA) is 66 267 (Ziegler & Foegeding, 1990).

The sequence of BSA is now well defined. A highly detailed description of this sequence, along with the molecular genetics and physiological implications, can be found in the review by Peters (1985) but will not be given in such lengths here. It is sufficient to say that the molecule is constructed through the folding of a single peptide chain into a series of nine loops stabilized by 17 disulphide bridges with one free sulphydryl group. These loops are further organized into three homologous domains of three loops each, all but the first being double-loop arrangements based upon a Cys-Cys repeat sequence. Each double loop has a long and short "arm" and the former have been suggested to be of a helical nature (Figure 1.2.11).

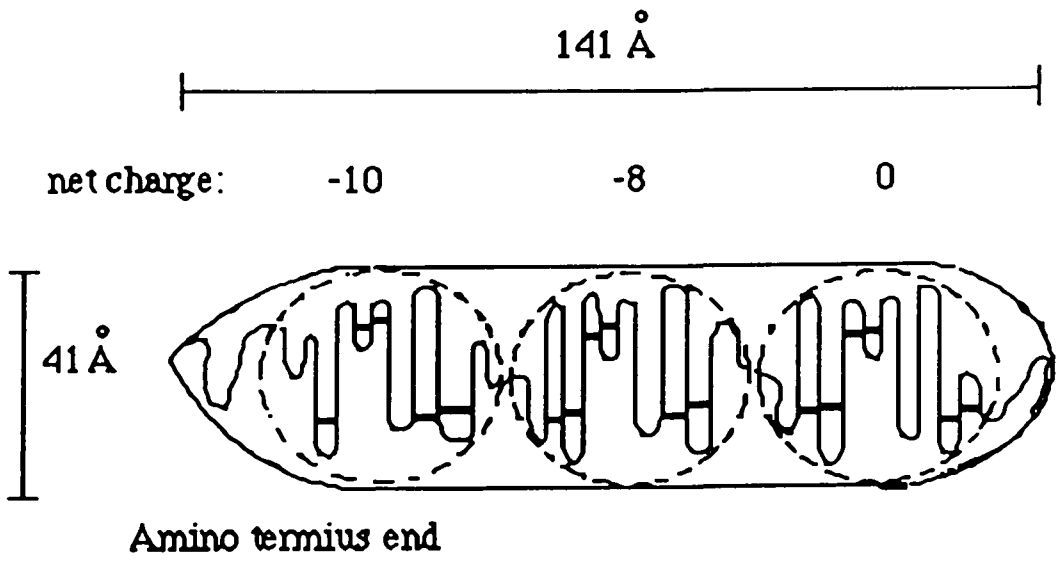


Figure 1.2.11 Domain structure of BSA (from Peters, 1985)

These domains are capable of folding to form hydrophobic pockets accommodating large insoluble anions (Peters, 1985). This backbone structure gives the molecule an ellipsoidal shape with major and minor axes of 140.9Å and 41.6Å respectively (Wright & Thompson, 1975). The molecule is highly polar with a potential for 100

negative and 82 positive charges. The electrical asymmetry of the molecule is attributed to the greater negative charge at the N-terminal domain than at the C-terminal end. The surrounding volume of the ellipsoid is filled with a "hydration shell" of water molecules (estimated at ~20) ultimately affected by the immediate closeness of the protein (Kuntz & Kauzmann, 1974). Held even closer to the protein molecule by hydrogen bonding is a zone of unfreezable water estimated at 1-2 water molecules per amino acid residue (Peters, 1985).

BSA denaturation, brought about by prolonged heating or exposure to alkali, proceeds through a series of intermediates possibly indicating the involvement of the loop domains mentioned above (Aoki *et al.*, 1973). The disulphide bridges stabilizing these domains may undergo an irreversible destruction in the presence of high pH through oxidation of the S^- form of cysteine by oxygen (Noel & Hunter, 1972; Wallevik, 1976). As aggregation follows denaturation - BSA has a denaturation temperature, T_D, of 58°C (Tolstoguzov, 1990) - the proportion of ß-structure increases (Peters, 1985). At thermal denaturation temperatures of ~60°C at pH 7 aggregation and precipitation of most of the albumin occurs. In the native state bovine albumins are estimated to contain ~55% α-helix and ~16% β-pleated sheet (Peters, 1985). A fraction remains soluble however and this is stable to temperatures of 78°C or higher. This effect is partly due to the influence of fatty acids present; in the precipitated fraction there is an absence of any fatty acids and all of the bound fatty acids appear in the soluble form (Aoki *et al.*, 1973; Brandt & Andersson, 1976; Shrake *et al.*, 1984).

Although BSA is usually described as an irreversibly denaturing protein under certain conditions, it can "temporarily" denature, i.e. the unfolding process is then reversible. Having unfolded through the deliberate reduction of the disulphide bonds all 17 bridges can spontaneously reform on re-oxidation (Peters, 1985). Such an ability to unfold-refold demonstrates the flexibility of the albumin molecule, brought about because of the loop-link-loop structure of the peptide chain with no long distance linking by disulphide bonds (Peters, 1985).

The increase in β-sheet conformation is associated with the initial onset of gelation (Lin & Koenig, 1976; Clark *et al.*, 1981). However the knowledge that the amount of β-sheets within BSA gels of very differing rheological properties is fairly constant sheds uncertainty on the actual role β-sheets play (Clark & Lee-Tuffnell, 1986). It is most probably part of the aggregated structure since the amount of β-structure is decreased on cooling when refolding is favoured (Clark & Lee-Tuffnell, 1986). Depending on gelling conditions and how gelation is evaluated the minimum gelling

concentration of BSA can span a whole decade, from 0.6% to 6% (see Ziegler & Foegeding, 1990). In addition to protein concentration any variations in pH and ionic strength, which in turn determine electrostatic forces, will produce significant effects in BSA gels, observed visually as changes in gel clarity (Richardson & Ross-Murphy, 1981).

Due to its well-characterized structure and potential for extremely high levels of purification BSA has found varied applications in numerous research fields.

1.2.2.4 Dried Blood Plasma

BSA is an expensive, highly characterized protein with far-reaching applications in biochemistry and molecular chemistry. This cost factor makes it unsuitable for experimental use involving large quantities and indeed practical end-use food applications.

To satisfy the requirements for a BSA replacer, dried blood plasma protein (abbreviated to DBP in this thesis) was selected. This material is a serum protein of bovine or porcine origin produced through the fractionation and atomisation of the blood. In fact a number of proteins that are used in food processing are derived this way, as a by-product at slaughter (Wismer-Pederson, 1979). The whole blood is separated into plasma and red blood cells. The latter group are then further processed into haemoglobin or globin. The suitability of DBP as a replacer for BSA is shown in the fact that, of the wide variety of proteins contained within the plasma fraction serum albumin is the most abundant (Ziegler & Foegeding, 1990). Due to the nature of the separation procedure and the diversity of the starting material different dried plasma proteins will contain a variety of proteins, salts and anti-coagulants and thus gelation properties will be directly affected by this. For the dried blood plasma used in this study (Vepro® 75) the total protein content is 75% minimum, with maximum limits of 15% total mineral matter, 2% lipids and 8.5% salt (sodium citrate). The nutritional aspects aside it is also stated by the manufacturers to have a high emulsifying and gelling capacity to stabilize in water and lipids and ensure cohesion in meat mixes.

Studies on plasma proteins in the past have indicated that gelation occurs as a product of both concentration and temperature; plasmas from bovine and porcine sources formed gels at a concentration of 3.5% when heated to 82°C but at protein concentrations of 5% a heating temperature of 72°C will bring about gelation (Hermansson and Lucisano, 1982).

1.2.3 Protein-Polysaccharide Interactions

Interest in the interactions between these two macromolecules has been apparent for at least the last two decades (see for example, Imeson *et al.*, 1977; Ledward, 1979). More recently interest in protein-polysaccharide interactions has been shown by this laboratory (Harding *et al.*, 1993; Kelly *et al.*, 1994). Since most foods are multi-component systems the knowledge of the combined behaviour becomes as important as that defined by the isolated polymers.

Tolstoguzov (1986) reviewed the possible outcome of mixing solutions of proteins and polysaccharides. Here he stated that three situations could arise namely:
(i) both macromolecules undergoing no interaction or collectively existing as soluble complexes resulting in a homogeneous system

(ii) both species existing in *different* phases due to their thermodynamic incompatibility resulting in a water-in-water emulsion

(iii) both components existing in the *same* phase as part of a two-phase system resulting in complex coacervation and the formation of insoluble electrostatic complexes.

If the concentration of either component is increased sufficiently or specific salts are included in the mix then the system may turn into a gel. Combination of two (or more) biopolymers in the presence of gel- promoting salts may produce a gelling system at concentrations below those required for gelation of the individual polymers or even the gelation of non-gelling biopolymers; the classic example of such a synergism is the κ-carrageenan/locust bean gum gel interaction described earlier. Food gels prepared from protein-polysaccharide mixtures are the focus of attention of this thesis.

Firstly what is a gel? Difficult in itself to define, a gel is a mid-state system, whose properties are neither purely solid nor liquid. Although consisting very often of almost 100% water, a gel has solid-like properties; it is usually self-supportive and exhibits a yield stress. For this reason gels are best described as visco-elastic materials however the search for the definitive definition is not an easy one! Indeed it is perhaps best used to encompass a whole class of materials with similar recognisable rheological properties (Ross-Murphy & Shatwell, 1993). Even though our technological evaluation of gels may have advanced significantly our ability to define it has not: in 1926 Jordan-Lloyd (see Ross-Murphy & Shatwell, 1993) came to the

conclusion that "the colloidal condition, the gel, is one which is easier to recognise than to define"!

Mixed polymer gels can form essentially through the following mechanisms (Morris, 1990):

(i) Covalent interactions between polymer chains
(ii) electrostatic interactions most commonly seen between a polyanion and a protein below its isoelectric point
(iii) phase separation and polymer exclusion - increasing the effective concentration of each component
(iv) co-operative association of the two polymers forming mixed "junction zones"

Few biopolymer systems contain covalent bonds (Morris, 1990; Ross-Murphy & Shatwell, 1993). One such example that does however is a denatured protein, which allows the formation of new intermolecular disulphide crosslinks (Ross-Murphy & Shatwell, 1993). Most food gels are of a physical nature, that is to say they possess physical cross-links which are not as strong or as permanent as the former mentioned covalent links (Ross-Murphy & Shatwell, 1993). A gel can vary from a strong, high integrity gel such as employed in meat and fruit chunk analogues and dessert jellies to a weak, thickening "gel" of the sort found in gravies and sauces. In this thesis gels of very different strengths can be seen in the starch pastes of Chapter 3 and the agar/carrageenan/gelatin gels of Chapter 4.

Recent studies into the development of a suitable model for food gels (Foegeding *et al.*, 1994) have indicated that many gels can be regarded as homogeneous and isotropic. This investigation showed that food gels do not generally show entropic elastic behaviour. This is attributed, by these workers, to a combination of bonds and interactions present in most food gels as opposed to the strictly covalent bonding found in most entropic elastic systems. Such combination of interactions to be found stabilizing food gels include covalent bonds, hydrogen bonds, electrostatic interactions and hydrophobic effects (Foegeding *et al.*, 1994).

The gelling ability of proteins and polysaccharides is dependant on a number of factors: the biopolymer itself (e.g., gelatin and agar represent two of the best gelling proteins and polysaccharides respectively); the presence of any gel-promoting salts (such as Ca^{++} ions in alginate gels, K^{+} ions in κ-carrageenan); temperature and time (e.g. the long times needed for development of gelatin gels in contrast to rapid setting gels such as pectins). The two different classes of macromolecules can usually be

regarded as forming gels of different type. However the low temperature gelation behaviour of gelatin gels are often said to be closer to that of a polysaccharide (see for example, Ross-Murphy & Shatwell 1993) whilst agar gels share the requirement of high heating temperatures with the thermo-gelling proteins. Polysaccharide gels are characterized by their fine texture and transparency, achieved through the low polymer concentrations required (Harris, 1990). Proteins gels, on the other hand, are characterized by a higher polymer concentration and almost always formed through heating (Harris, 1990).

1.3 Rheology

1.3.1 The Origins of Rheology

The concepts described in this section are well understood in the world of rheology and therefore individual sources are not outlined at every point. For further information the reader is referred to Ferguson & Kemblowski, 1991 and Barnes *et al.*, 1989 from which this section is adapted.

Firstly, what is rheology? In its simplest terms rheology concerns itself with the deformation of real bodies under the influence of external stresses. It is a relatively "new" science (indeed the term rheology was actually invented by Professor Bingham of Lafayette College as recently as 1926 from the literal meaning of "the study of flow" in Greek) representing a broad inter-disciplinary subject.

Although rheology is relatively new an understanding of the extreme states of materials - solids and liquids - was being developed in the 17th Century. The "True Theory of Elasticity" was presented by Robert Hooke in 1678 who suggested that "the power of any spring is in the same proportion with the tension thereof"; in simple terms this means doubling the force doubles the extension. A few years later in 1687 Isaac Newton stated that "the resistance which arises from lack of slipperiness of the parts of the liquid, other things being equal, is proportional to the velocity with which parts of the liquid are separated from one another". What Newton termed "lack of slipperiness" we now term viscosity and forms an important rheological parameter.

1.3.2 Viscoelasticity

The idealised states of solid and liquid described by Hooke and Newton can be represented by the spring model (a system whereby force is proportional to extension) and the dashpot model (a system whereby force is proportional to the rate of extension) respectively and these are shown below in Figure 1.3.1. Between these extremes lie materials which possess both viscous and elastic responses. Food gels exhibit such properties and are thus termed viscoelastic. Description of such materials is enabled by combination of the spring and dashpot models and the two simplest linear viscoelastic models are those of Maxwell and Kelvin. The Maxwell model has the spring and dashpot in series making the strains or strain-rates additive whereas the Kelvin model has the spring and dashpot in a parallel arrangement, which indicates that the strain or extension in the spring is equal at all times to that in the dashpot.

Reference to Barnes *et al.*, 1992 will detail the more complex models of Jeffreys and Burgers but since these can ultimately be reduced to the Maxwell and Kelvin models discussion of them here is not needed.

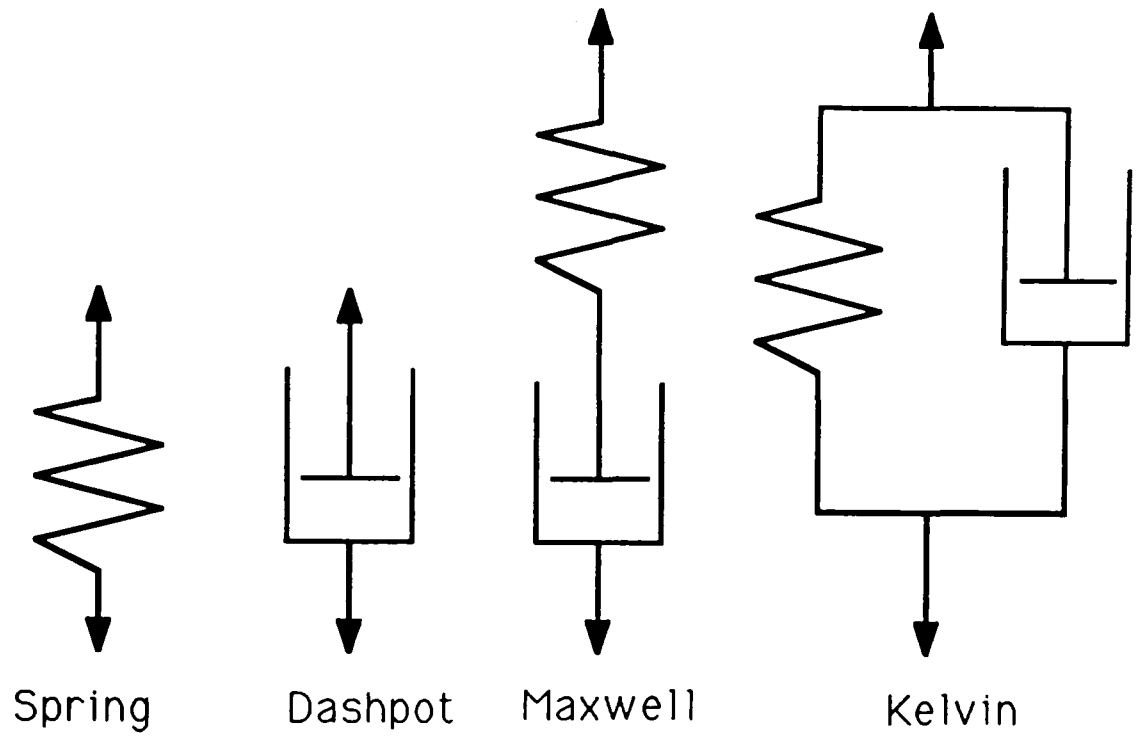


Figure 1.3.1 Models of solid, liquid and viscoelastic properties

Alongside viscoelastic materials, classically solid materials can display liquid-like properties and the converse is also true for liquids. To further add to this a material can behave as both a solid and liquid depending on the time scale of the deformation. The classification of such a time scale in rheology is made through the use of a dimensionless term called the "Deborah number", defined by Reiner (1945). This term came from the prophet Deborah's statement in the Bible, that "even the mountains flowed before God"! The Deborah number, D_e, is defined as:

$$D_e = \frac{\tau}{T}$$

where T is the characteristic time of the observed deformation process and τ is a characteristic time of the material; for a Hookean elastic solid this is infinite and for a Newtonian viscous liquid τ is zero. High values of D_e correspond to solid-like behaviour and, likewise, low numbers to liquid-like behaviour. This means that a material's "status" may change depending on the time scale of measurement.

1.3.3 Viscosity

The rheological parameter most often used to describe a material's properties is the viscosity. In its simplest terms the viscosity is a measure of a resistance to flow.

When a shear stress, σ, is applied to a material a deformation results, which in the case of Newtonian liquids will occur for the duration of the stress application, but for a Hookean solid, will result in an instantaneous deformation to the deformed state with no further movement and persisting whilst the stress is maintained. The amount of strain for a given stress is governed by the "rigidity modulus", G. The shear stress can thus be regarded as the force required per unit area to produce this deformation and is proportional to the velocity gradient or "shear rate" denoted as $\dot{\gamma}$

From this the simplest equation for viscosity, η, is obtained:

$$\eta = \frac{\sigma}{\dot{\gamma}}$$

In defining the viscosity of a material the shear rate of measurement should also be stated since viscosity is essentially "a plot not a dot"! This in turn can be referred to as the "shear viscosity", "apparent viscosity" or "shear-dependant viscosity". The S.I. unit of viscosity is the Pascal second (Pa.s). The viscosity of water at 20°C is 1mPas at all shear rates.

The ideas of Hooke and Newton both show linear relationships between the imposed stress and strain. However few materials show this or if they do, for only a limited range of stress. With linear viscoelasticity the strain response at any time is proportional to the magnitude of the initiating stress signal .

1.3.4 Newtonian Behaviour

This type of behaviour is evident when:
(i) the shear viscosity does not change with shear rate
(ii) the viscosity is constant over the time of shearing with the stress in the liquid falling immediately to zero once the shear is removed *and* if reapplied the viscosity should always assume the previous value irrespective of how long the sample was rested for.
(iii) the shear stress, σ, is the only stress generated in simple shear flow and the two normal stress differences are zero
(iv) the viscosities measured in different types of deformation are always in simple proportion to one another.

1.3.5 Non-Newtonian Behaviour

Thus if a material is not Newtonian it must deviate from these regimes in one of two principal ways - it can be known as (i) shear-thinning and undergo a viscosity reduction as shear rate increases or it can be termed (ii) shear-thickening and have an increase in its viscosity as the applied shear rate is increased. The former case is the more commonly seen and will be considered first.

If the viscosity of the material decreases as the shear rate increases then we say that material is "shear-thinning". Many food products (yoghurts, milk shakes, etc.) show this type of behaviour. Shear-thinning behaviour is also exhibited by such materials as polymer solutions and melts, colloidal suspensions and asymmetric particles (e.g. fibre suspensions). Since viscosity is a measure of a material's resistance to flow a reduction in viscosity essentially requires a reduction in the internal friction of the system.

For polymer solutions there are two major mechanisms for shear-thinning behaviour. These are: (i) the reduction in entanglements with increasing shear rate and (ii) the alignment of asymetric particles in the flow field. Polymers will disentangle as a shear force is applied and continue to do so as the shear rate increases. The reduction in interactions brings about a decrease in the internal friction of the system. This mechanism will be important at concentrations where the coils start to overlap and thus entangle. This critical concentration is termed the c^* concentration.

It therefore follows that polysaccharide solutions are highly shear-thinning above the c^* concentration but often show near Newtonian behaviour below c^*. Assymetric molecules (e.g. rods) will tend to align parallel to the streamlines in a flow field. This reduces the internal friction within the system even in very dilute solutions because the aligned "rods" perturb the velocity field less than randomly orientated rods. This mechanism explains the non-Newtonian behaviour found for dilute solutions of polysaccharides (below c^*) that have extended conformations, e.g. xanthan gum.

With shear-thinning materials the flow curve normally exhibits a lower and upper shear rate region of near-Newtonian behaviour but connected by a noticeable reduction in the viscosity. This can be explained through a grasp of these underlying molecular mechanisms. At the very low shear rates the orientating effects of shearing on asymmetric particles and the system structure are still completely representative of the disorientated state, whereas at high shear rates complete orientation has been achieved and internal friction is constant at its minimum. In the case of entangled

systems the low shear rate Newtonian "plateau" is the region where the entanglement density is a maximum. At very high shear rates the entanglement density approaches zero as molecules are not in contact long enough for these interactions to form.

The opposite of shear-thinning is shear-thickening but this type of behaviour is rarely seen and when it is, usually only occurs over a decade of shear rate with a shear-thinning pattern still observed at low shear rates. The underlying molecular mechanisms of this type of behaviour are more complicated but can generally be explained by the break-up in ordered layers of particles at high shear rates. The resulting decrease in particle-particle distance increases the level of inter-particle interactions and hence the viscosity. The most common occurrence of shear-thickening behaviour in food systems is in suspensions of ungelatinised starch granules.

1.3.6 Rheological Models

Shear rate dependence is the most rheologically influential factor for non-Newtonian materials. To predict this type of flow curve empirical curve-fitting equations are employed. In this study the "power law" equation was used which is given by:

$$\eta = K_2 \dot{\gamma}^{n-1}$$

where n is termed as the "power-law index" and K_2 is the "consistency". This equation fits over the central region of the flow curve over two or three decades and is therefore quite useful to a number of situations. However this model fails at high shear rates as viscosity approaches a constant value. The "power law" model is in fact derived from another equation, the Cross equation (when $\eta \gg \eta_\infty$) below:

$$\frac{\eta_0 - \eta}{\eta - \eta_\infty} = (K\dot{\gamma})^m$$

where η_0 and η_∞ are the asymptotic viscosity values at very low and very high shear rates respectively, K is a constant parameter with the dimension of time and m is a dimensionless constant. In order to fit equations to specific parts of the flow curve other viscosity models must be derived from the Cross equation by making a number of approximations. In the case of the power law equation this occurred when $\eta \gg \eta_\infty$. When $\eta \ll \eta_0$ the "Sisko" model is used:

$$\eta = \eta_\infty + K_2 \dot{\gamma}^{n-1}$$

The Sisko model equation fits in the mid-high shear rate range. Further more if, in the above equation, n is zero then we have:

$$\eta = \eta_\infty + (K_2/\dot{\gamma})$$

which through redefinition of parameters becomes the Bingham model equation:

$$\sigma = \sigma_y + \eta_p \dot{\gamma}$$

where σ_y and η_p are constants for the yield stress and the plastic viscosity respectively. In yield stress systems one or more phases are dispersed as particles or bubbles in the continuous liquid phase of a multi-phase system. At a high enough

concentration of dispersion particles an interaction may occur leading to a three-dimensional structure with enough strength and integrity to resist high stresses and thus under such conditions behaving as an elastic solid. On exceeding the critical yield stress the material breaks down instantaneously and completely and allows the material to assume viscous fluid properties, which often, can be equated to that of the continuous phase and thus can then show the types of flow seen with non-yield stress materials. Indeed on a stress-strain curve a Bingham fluid shows a parallel trend to that of the Newtonian liquid but is displaced upwards to a critical value on the stress axis with deviations of thinning and thickening behaviour common to both types of "Newtonian" behaviour.

This model is only applicable over a short range (one decade or so) and at low shear rates. Flow curves essentially similar to Bingham fluids but showing non-linear behaviour (i.e. shear-thinning or -thickening) can be most simply applied to the rheological model of Herschel-Buckley:

$$\sigma = \sigma_y + k\,\dot{\gamma}^n$$

1.3.7 Factors Affecting Viscosity Other than Shear Rate

As well as being dependant on shear-rate viscosity also depends to different extents on a number of other factors including temperature, pressure and time of shearing. Temperature is a major influence on viscosity; indeed the temperature sensitivity of water is 3% per ^{o}C at room temperature. However, the range of temperatures to be considered will depend on the application. For Newtonian liquids the temperature variation can be described by the Arrhenius equation:

$$\eta = Ae^{B/T}$$

where T is the absolute temperature and A and B are constants of the liquid. As a generalisation the higher the viscosity of the Newtonian liquid the greater is the temperature dependence. As well as considering the external heat on the system the heat generated by the material as it undergoes the shearing process must also be considered and ideally adequately dissipated if excessive internal heat build-up is to be avoided. Temperature effects can thus also depend on the instrument. In capillary rheometers the rate of heat extraction too can be important. In cone-and-plate and concentric cylinder type viscometers the only significant heat-transfer process is to the surface.

Within a two-phase system temperature effects can have significant rheological consequences. Normally the continuous phase governs the temperature sensitivity but in more complex situations, such as dispersions, the discontinuous (suspended) phase may contribute an effect of its own and perhaps have a discrete melting point which is seen as a sudden decrease in viscosity on heating. (Interestingly, the opposite effect is utilized in multi-grade oils which rely on the opening up of a polymer coil structure on heating to increase viscosity and thus offset the reduction seen in the continuous phase).

The influence of pressure effects on viscosity in most laboratory situations are so small as to be unimportant but in certain industries, such as oil-drilling, liquids are used under high pressures. The viscosity of most liquids increases exponentially with isotropic pressure; this is not so for water however where its viscosity first decreases before increasing exponentially.

Rheological phenomena such as the "climbing rod" (or Weissenberg effect) and die-swell effect can be explained in terms of Normal forces. It is adequate to say here that, in addition to those stresses already mentioned, such as shear stress, normal

stresses are simply the terms used to define the stresses of any flow that occur. Both effects can be explained in terms of extra tension along the streamlines, in the first case forcing the fluid inwards and upwards against the centrifugal and gravitational forces respectively and in the second case causing an axial contraction of the jet with simultaneous expansion in the radial direction.

1.3.8 Oscillation

If the stress is applied as a periodic oscillatory motion and changes in strain are seen with this varying stress then the material is under oscillation. This can be defined as:

$$\sigma = \sigma_0 \cos(\omega t)$$

where σ_0 is the amplitude of stress and ω is the frequency of oscillation. For ideal elastic solids ($\sigma = G\gamma$) where G is the elastic modulus of the material:

$$\gamma = (\sigma/G) = \sigma_0/G(\cos(\omega t)) = \gamma_0 \cos(\omega t)$$

where the first part of the equation represents the amplitude of strain of the elastic material and the strain response of the material is completely in phase with the stress. (Having said this the response is as close to instantaneous as possible since, theoretically, there is a diminutive delay between the two caused by the fact that wave propagation only travels at the speed of sound, but this is ignorable for all intents and purposes). In the case of an ideal viscous fluid ($\sigma = \eta \dot{\gamma}$) where η is viscosity:

$$\dot{\gamma} = (\sigma/\eta) = \sigma o/\eta(\cos(\omega t))$$

which through integration with time becomes: $\gamma = \gamma o \cos(\omega t - (\pi/2))$ and shows that, unlike the elastic solid above, the strain response will be 90^o out of phase with the stress. These two systems represent the ideal state. For a viscoelastic material a situation in between the two extremes must exist, i.e. the strain response will be out of phase to the applied sinusoidal response by some value δ related by:

$$\gamma = \gamma_0 \cos(\omega t - \delta)$$

where the phase lag is greater than zero but less than $\pi/2$. In oscillatory measurements complex numbers are employed to resolve the elasticity modulus, G, and the viscosity, η, into the elastic, in-phase component and the viscous, out-of-phase component and details of these can be found in the texts recommended. From this the

values of the complex modulus of elasticity, G^*, can then be defined as a ratio of the complex stress and strain. The much used rheological parameters of G' and G'' - the elastic and loss modulus respectively, [$G' = (\sigma_o/\gamma_o) \cos \sigma$ and $G'' = (\sigma_o/\gamma_o) \sin \sigma$] - are then derived from this, representing the elastic and viscous components of the complex modulus. The complex viscosity, η^*, can be defined as the complex stress over complex rate of strain. Two more rheological parameters are also used in the oscillation mode of testing to characterize a viscoelastic material. The phase lag, δ, mentioned in the equations above is often used as a parameter in its own right to describe the degree of solid- or liquid-like character, i.e. the magnitude of out-of-phase behaviour; thus approaches to 0^o indicate elastic or solid-like behaviour and conversely a lag approaching 90^o shows viscous or liquid-like behaviour. This is derived, from the definitions of G' and G'' earlier, as: $\tan \delta = G'' / G'$ or as: $\tan \delta = 1/(\tau\omega)$ where τ is the experimentally determined relaxation time.

1.3.9 Rheometry

The measurement of rheological parameters is termed rheometry. A number of rheometers (a rheometer is distinct from a viscometer in that the latter is only capable of measuring viscosity whereas a rheometer can measure the parameters described above and more) are available today based on a variety of different measuring systems, at different costs and with different levels of sophistication. Each has its own advantages and disadvantages and these, together with a brief outline of the ideas behind each design, are shown in Table 1.2 on the following page.

Table 1.3.1 Common rheometers

Rheometer	Controlled σ and / or $\dot{\gamma}$? *	Advantages	Disadvantages	Special Applications
Capillary	Both types, both good but constant σ is better for unstable fluids	cheap; easy to use; ^{o}C control easy; mimics Industrial use	Entrance and slip effects a problem. Not for suspension studies.	Die swell; melt fracture (the only rheometer capable of this)
Coaxial (Concentric Cylinder)	Both	Low viscosities possible; no time limit for shearing (unlike capillary types)	At high η's entrapment of air bubbles & loading problems; $\dot{\gamma}$ upper limits	Volatile fluids- a special guard ring prevents skinning
Cone-and-Plate	Both - stress model is newer.	Uniform shear; small sample volumes; gap easily adjusted; many values measurable	Gap setting must be accurate; max. shear rate may be limited	Many. Include oscillatory measurements - applicable also to high viscosities
Parallel Plate	Both (as cone-and-plate)	Gap setting not as crucial; precise oscillatory measurements	Difficult to monitor yield stress fluids	Far reaching. Oscillation.
Sliding Cylinder	Both	Sample loading easy; gap setting problem eliminated; no edge effects; smooth data	Loss of sample at low shear rates possible; cannot measure normal forces	Used with compressional equipment to obtain extra data; oscillation

** Both types of machine can produce identical data for fluids without internal structure but the use of a controlled stress machine over shear rates allows the measurement of fluids with internal structure, which under an excessive stress would be broken down. Instead controlled, small stresses can be used to merely deform the structure and can be applied to many industrial processes (e.g. paint sagging). The use of very low shear stresses also allows the determination of a yield stress.*

1.3.10 Large Deformation Studies

Another rheological method of assessing the properties of gels is that of large deformation stress relaxation, a type of test which can give information regarding the specimen's elasticity (Nussinovitch *et al.*, 1990). How is this achieved?

Considering the simpler case of a compression-decompression test first the ratio between recoverable deformation to total deformation can be used to assess the "degree of elasticity" (Mohsenin 1970) or alternatively, we can use the ratio between recoverable work to total work (Olkku & Sherman, 1979; Kaletunc *et al.*, 1991). The use of the second method is the more reliable since the need to accurately identify the point of maximum recovery is less significant, whereas the determination of the exact size of the recoverable deformation can be problematic (Nussinovitch *et al.*, 1990) but either way both forms rely on the dependence of deformation or strain level. In such a test the recoverable and irrecoverable work represent the "elastic" and "viscous" components respectively since in the case of an ideal viscoelastic body under a deformative stress energy is both stored (related to the strain) and dissipated (related to the rate) (Nussinovitch *et al.*, 1990). However since most gels under large deformations show non-linear viscoelastic behaviour accompanied by possible structural changes, a direct prediction of the stress-relaxation behaviour from the compression-decompression curve is not possible in theory. However on replacing the test with a stress-relaxation test the elastic component is then represented by the asymptotic modulus, E_a, to be described further on and this similarity allows the recoverable work and modulus to be related, e.g. a material with a high value for the percentage of recoverable work will have a correspondingly high value of E_a, comparisons always made at the same strain and under the same deformation history (Nussinovitch *et al.*, 1990).

Generally the stress relaxation phenomenon of solids can be described as (Peleg & Pollak, 1982):

$$E(t) = E_0 + f_1(t)$$

and for liquids as:

$$E(t) = f_2(t)$$

where E is the decaying modulus after time, t, E_0 is the modulus at equilibrium (in other words, at infinite time E(t) approaches E_0) and f_1 and f_2 are decay functions of time upholding that at infinite time both functions are zero (Peleg & Pollak, 1982).

However as already established gels represent mid-state systems between solids and liquids, namely viscoelastic materials. For such a property another treatment must be made - the Maxwell models as quoted by Peleg & Pollak:

$$E(t) = E_0 + \sum_{i=1}^{n} a_i \exp(-b_i t)$$

or:

$$E(t) = \sum_{i=1}^{n} a_i \exp(-b_i t)$$

where a and b are characteristic constants of the Maxwell elements. However this model represents problems when fitting to foods, for example a good fit of the equation would suggest liquidity of the food (see Peleg & Pollak, 1982) and thus normalization and linearization of the curves is a better procedure to the method described by Peleg (1980):

$$\frac{F_{(o)}t}{F_{(o)}-F(t)} = k_1 + k_2 t$$

where $F_{(o)}$ is the force at time zero, F(t) is the force after time t and k_1 and k_2 are constants. This constant k_2 can then be used to arrive at the asymptotic residual modulus, E_a, described earlier by:

$$E_a = \frac{F_o}{A(\varepsilon)\,\varepsilon} \cdot [\,1 - (1/k_2)\,]$$

where e is the imposed strain and A(e) is the corresponding cross-sectional area of the relaxing specimen, i.e. (from Peleg, 1980):

$$A(\varepsilon) = \frac{A_o L_o}{L_o - \Delta L}$$

What do the parameters described above in the various equations actually tell us about the gel? The simplest parameter to understand is perhaps F_o which indicates the gel's resistance to the deforming stress initially applied; higher F_o values would suggest a "stronger" or more rigid gel. The k_1 and k_2 data give information concerning the short and long time behaviours of the gel respectively. These are perhaps best understood through the equations used to describe them and the mathematical treatment of this can be found in the discussion of chapter 4. The consequences of E_a mean that "real numbers" can be assigned to the otherwise arbitrary functions. Details of the internal structure of the gel can be gained through the manipulation of the E_a value (Peleg, 1980). A knowledge of such allowed Nussinovitch and co-workers (1990) to suggest that carrageenan and agar are yielding

materials with a weakening of structure occurring on deformation with these gels, postulated from the decrease in E_a with increasing strain.

Since gels are vulnerable to dehydration the time period of the stress relaxation experiment must be kept short enough to prevent the gel specimen undergoing structural changes but also important is the meaningful representation of a time-scale. Peleg & Pollak (1982) showed that for the wide variety of foods tested the rheological information associated with the E_a value is already established after a fairly short time in relaxation. In fact these workers state that as long as the normalisation equation (Peleg, 1980) described above holds true the calculated value of E_a is independent of the test duration and can be calculated after ~minutes.

Another time-dependant factor worth considering is gel syneresis, apparent in a free-standing gel but also accelerated by the imposition of the strain, promoting liquid expulsion from the gel network, usually detectable on the time scale of the stress relaxation. In addition to the stress relaxation test defined here important information can also be gained from a simple failure test, where the gel is compressed under defined conditions to the point of rupture or failure. This test in itself can be used as an indicator of the gel strength (Nussinovitch *et al.*, 1989).

CHAPTER 2. MATERIALS AND METHODS

2.1 Starch-caseinate system

2.1.1 Materials

Native potato starch was supplied by Amylum, Belgium, native maize starch was supplied by Sigma Chemical Company, U.K. and the native wheat starch was provided by Pedigree Petfoods, U.K. The modified potato starches, namely an amylopectin potato sample and a low phosphate potato sample, were supplied by Avebe U.K. The modified waxy maize starch (an acetylated distarch adipate) was provided by National Starch, U.K. The sodium caseinate was provided by St. Ivel, U.K.. For the applications work a range of wheat flours were provided by Pedigree Petfoods, U.K. All samples were used as supplied and without further purification. The starches and flours were stored at ambient in dry, sealed containers and the caseinate was stored in hermetically sealed cans in a cold store. The moisture contents (mean of triplicate dryings ± 0.1%) of all the samples were found to be as follows:

	% H_2O
native corn starch	9.92
native potato starch	15.39
native wheat starch	11.79
amylopectin potato starch	14.77
low phosphate potato starch	16.92
modified waxy maize starch	12.17
sodium caseinate	5.79
<u>Wheat Flours</u>:	
Mercury standard	12.96
Mercury A	13.54
Mercury B	13.43
Mercury C	13.43
Emblem	14.28
Chlorinated	12.45

The low phosphate potato starch was stated by the manufacturer to have a phosphate content of 0.66mg/g as opposed to ~1.0 mg/g expected for standard potato starches (see Zobel, 1986 for details on standard potato starch phosphate contents). The phosphate content of the amylopectin potato starch was stated as 0.86mg/g.

All buffer salts (analytical grade) were provided by Fisons, U.K. and stored at ambient. For both the buffer and non-buffer systems distilled, deionized water was used as the solvent throughout. The pH 7.0, 0.1M phosphate buffer (8.66g/L $Na_2H.PO_4$ and 6.084g/L $NaH_2PO4.2H_2O$) was stored in a cold room when not in use.

2.1.2 Methods

2.1.2.1 Starch Paste Preparation - general procedure

For all procedures described here replicate preparations were made on different days. The number of replicates and the coefficient of variation for the results are given in each method section. An appropriate amount of dry starch for the concentration (generally: potato starches - 1%; corn starches - 4%; wheat starches - 4%) was weighed into small, glass Universal bottles and the weight made up to 100% with solvent. The solvent was either (i) distilled, deionized water, (ii) buffer as above, (iii) dilute NaCl solution or (iv) caseinate solution in either distilled, deionized water or buffer. In the case of potato and wheat starch only water or caseinate in water was used whereas for the corn starch study both water and buffer were used. The NaCl was only used for the potato starch.

The caseinate solution was prepared by slowly dispersing the appropriate amount of caseinate powder into the vortex of the stirring water or buffer on a magnetic stirrer (without heat). Stirring was then maintained until the powder was fully dissolved, when solutions were than transferred to volumetric flasks and made up to volume. All caseinate solutions were prepared fresh and if stored (in a cold room) never used after 12 hours of storage. Screw-cap lids were then placed onto the Universals, followed by Nesco film and aluminium foil (to prevent evaporation from the bottles). The contents were then vigorously shaken to disperse the starch powder. In the cases of those starches not easily dispersed by manual shaking (the corn starches in high concentrations of caseinate solution for example) a short period of magnetic stirring was used (~5 minutes). When the starch had fully dispersed in the solvent, judged visually, the bottles were then subjected to heat treatment as follows.

A Techne Tempunit water bath set to 95°C was employed in all cases to gelatinize the starches. A layer of latex spheres covered the surface of the water to minimize evaporative losses The bottles were placed in the water bath for 1 hour, ensuring the water level covered the height of bottle contents. The bottles were shaken manually, until the starch had gelatinised, to prevent the starch particles settling. The time of shaking varied for each of the starches; the easily gelatinised potato starch only required ~ 1-2 minutes whereas the modified corn starch required approximately 5-6 minutes.

After 1 hour of heating the bottles were removed from the bath and, after standing for approximately 5 minutes at ambient, were rapidly cooled in cold water for a short period. The standing period prior to analysis never exceeded 10 minutes. After analysis the bottles were resealed and stored at ambient for a maximum of 24 hours or in a cold room for up to 48 hours. This procedure was modified slightly for the retrogradation investigations on native corn starch; after the 1 hour's heating the bottles were transferred to a 60°C water bath to equilibrate before being measured at 60°C on the Bohlin. These bottles were then aged for 24 hours as above.

2.1.2.2 Analysis: I. General Parameters common to all starches

(a) Rheology

A Bohlin constant stress rheometer was employed for the rheological characterisation of the starch systems. In all experiments involving pasted starch the cone-and-plate geometry (4° angle, 40 mm diameter) was selected. For the characterisation of the solubilised starch (described later) the double gap geometry was necessary to handle the much lower resultant viscosity. A constant gap of 50μm, set and checked manually with a feeler gauge before the commencement of each experiment, was used for all cone-and-plate studies. After equilibration of the rheometer water bath at the desired temperature (25.0°C or 60°C) the appropriate volume of sample was loaded onto the plate or into the cup as stated in the Bohlin Users Manual (3ml for the cone and plate geometry; 13 ml for the double gap) and the plate or bob lowered onto the sample avoiding any air incorporation. For experiments performed over long times or at high temperatures a layer of paraffin oil and a sample guard were also used in order to prevent drying out of the paste. In most cases the program was run under stress viscometry to obtain comparative viscosity measurements at 50 1/s (the "modelled" shear rate for the action of eating) for each paste treatment. However the

experiment on maize starch the oscillation mode at 1Hz was employed as detailed in the appropriate section.

(b) Swelling Volume

The starch paste was centrifuged in 15 ml graduated centrifuge tubes at 1000g for 10 minutes in a Beckman swing out centrifuge. After centrifugation the pellet, if present, was clearly visible at the bottom of the tube and the graduation allowed the pellet volume to be directly read off.

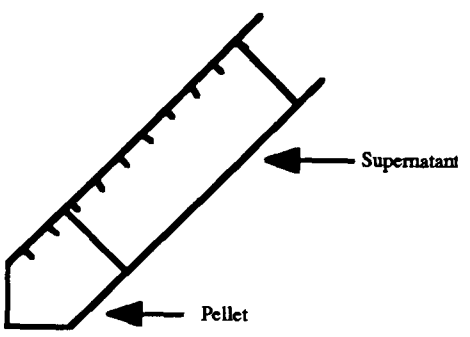


Figure 2.1.1 Centrifuged Starch Paste

The swelling volume can then be expressed in terms of the pellet volume and the total volume as follows:

$$\text{swelling volume} = \frac{\text{pellet volume (ml)}}{\text{total volume (15ml) x conc. of starch (g/ml)}}$$

Means of triplicate tests were obtained and coefficients of variation in all cases calculated to be of the order of 4%.

(c) Microscopy

In order to visualise the different starches in the presence and absence of caseinate a microscopic study was made. Pastes were prepared at starch concentrations of 1% (potato starch) or 4% (corn starch and wheat starch) in the presence and absence of sodium caseinate as desribed before. A small volume of paste (~2ml) was placed onto a microscope slide and a drop of Congo red dye pipetted into the centre of the paste. Gentle dispersion of the dye into the paste was made before a cover slip was placed over the top with viewing then made immediately on an Lietz microscope. Photographs of the slides were then taken by a camera attached to the eyepiece of the microscope using an exposure time of 2 seconds. Preparation and viewing of each sample was done in duplicate and one photograph of each test selected to show this.

2.1.2.3 Analytical Procedures Specific to the Potato Starch System

(a) Solubility

A known volume of the supernatant produced after centrifuging, as shown above, was removed by pipette and dried in an oven (at 105°C) overnight. In the absence of caseinate and at the very low levels shown in Figure 3.1.5 the solubility was calculated as follows as a mean value (±10%) of duplicate tests:

$$\text{Solubility} = \left[\frac{\text{Supernatant Volume (SN) x Solids conc. in supernatant (SC)}}{\text{total volume (15ml) x conc. of starch (g/ml)}} \right] \text{x } 100\%$$

In the case of samples which contain caseinate at higher levels the additional consideration involving the contribution of the caseinate to the amount of material in the supernatant would have to be made. The calculation is then obtained using a calibration curve for the caseinate solubility and the following equation:

$$\text{solubility} = \frac{\text{SN x (SC-C)}}{\text{total volume (15ml) x dry weight}}$$

where in this case, C represents the concentration of caseinate in the supernatant. Validity of this is uncertain since it must be assumed that all the caseinate is contained in the supernatant after centrifuging the paste, i.e. none is contained within the pellet. Performing a carbohydrate analysis may get around this problem.

(b) Intrinsic viscosity

The intrinsic viscosity of the standard potato starch both in the presence and absence of sodium caseinate was determined (over a series of at least triplicate tests as shown in the results of Figs. 3.1.12 and 3.1.14) using a Schott-Geräte capillary viscometer in a Comark N°.2 water bath set to 25.0°C. After pasting a 1% potato starch/0% caseinate and a 1% potato starch/0.1% caseinate sample in the normal way, a volume of 1ml was removed. This caseinate concentration represents a compromise between a level which gives a significant viscosity reduction effect whilst being low enough to minimize any concerns of interference with the calculation.

To each 1ml sample was added 9ml of 5M KOH. The mixture was left to stir overnight to solubulize the starch granules. Dilution's using 0.5M KOH (i.e. the final concentration of KOH in the starch samples) were then made from the stock solution to allow a range of concentrations to be run on the capillary viscometer. Viscosity measurements were made by adding 2.0ml of solution to the capillary, taking care to avoid air incorporation. When the pump tube was then placed over the larger end of the capillary and the apparatus set to "running", a flow time was determined automatically. Three flow times were made for each concentration and the mean taken. Between each run the capillary tube was thoroughly cleaned using Decon, acetic acid and distilled water and then dried using ethanol.

From the flow times we obtain the relative viscosity, η_r, by:

$$\eta_r = \frac{\text{sample flow time}}{\text{solvent flow time (0.5M KOH)}} = \frac{t}{t_o}$$

The reduced viscosity, η_{red}, is then obtained from:

$$\eta \text{ red} = \frac{(\eta_r) - 1}{c}$$

where c is the concentration. On plotting the specific viscosity against concentration the intrinsic viscosity [η] is obtained as the intercept on the y axis, i.e.:

$$[\eta] = \frac{\eta_{red}}{c} \qquad c \rightarrow 0$$

In addition, the solubilised samples were also measured on the Bohlin rheometer using double gap geometry and the viscosity values quoted for each treatment calculated as means of duplicate tests (± 5%)

(c) Hydration experiments

A series of single experiments were carried out to investigate the possibility of competitive hydration between the caseinate and the gelatinising potato starch granule. Instead of the normal pasting procedure where the starch powder is added to

water or caseinate solution and it hydrates during the course of the heating in the water bath, the caseinate solution was added to the already gelatinised starch granule paste at various times of addition ranging from 2 minutes after gelatinisation to 55 minutes (i.e. 5 minutes before the sample comes out of the water bath). The concentrations of the original starch pastes and caseinate solutions were twice those used in the "normal" experiments. To inter-disperse the two "solutions" gentle mixing was used at the time of addition, taking care not to over-shear the starch granules. The Universal bottles, as used before, were then re-covered with Nesco film and foil and left for the remainder of the one hour heating period to prevent evaporation. It was assumed that this initial concentration difference had no significant impact on the final result. To validate test results double controls at two concentrations (1% potato starch/0% caseinate, 1% potato starch/1% caseinate and 2% potato starch/0% caseinate and 2% potato starch/2% caseinate, all made as in the standard method) were also included.

As well as adding the already hydrated caseinate solutions the effect of the addition of dry powder was investigated. The addition of the correct amount of caseinate powder to the freshly gelatinized starch paste was performed by (a) vigorously stirring in the powder throughout the paste and (b) leaving the caseinate powder to rest on top of the paste and fall through the bulk of the paste at its own rate.

In addition, to take the experiment to its logical conclusion, the ultimate hydration test was also performed using only the dry powders at the final concentrations. Here the two powders were dry-mixed together before being added to the correct volume of distilled, deionized water and gelatinized as before.

(d) Dialysis experiments

In order to determine if the salt content of the native sodium caseinate was important in determining its effect on starch a 1% sodium caseinate solution was made up as before in distilled, deionized water, and then dialysed before being added to the starch. This was carried out as follows: dialysis tubing, prepared by boiling in EDTA and storing in ethanol, was cut and tied at one end to form a bag into which was poured ~3ml of the freshly prepared caseinate solution before securing the top with a knot and being then left to stir overnight in a copious volume of distilled, deionized water in a cold room. A total of four bags were prepared in this way to provide sufficient volume for testing. The following day this dialysed caseinate solution was used to prepare a 1% standard potato starch in the normal manner by heating at 95^{o}C for 1 hour with initial manual shaking. Rheological analysis was then performed on

the Bohlin to compare viscosity of the dialysed caseinate with the undialysed 1%starch/1% caseinate control run in parallel. This test was done in duplicate on different days to give viscosity means of the samples with a C.V of maximally 10%.

(e) Conductivity of caseinate

Single conductivity measurements were made on caseinate solutions in the concentration range 1-5% using a conductivity meter. Measurements were made on both "normal" and dialysed solutions.

2.1.2.4 Analytical Procedures Specific to the Corn Starch System - Retrogradation of Corn Starch Pastes

A 4% standard corn starch paste in distilled, deionized water was prepared as before and then loaded onto the Bohlin almost immediately. Cone-and-plate 4/40 geometry was used as previously described and a Jobstream program under the oscillation mode (1Hz) was employed to allow a two-stage ageing. This involved cooling the sample from 70°C to 25°C at the rate of 1°C/minute after a short thermal equilibrium time and then performing an oscillation experiment every 600 seconds over a 48 hour period. The subsequent plot of G' gave an excellent indication of the state of ageing of corn starch. This experiment was performed only once (i.e no replications) due to the time requirement.

2.1.2.5 Studies on Wheat Flours

A possible application of the work on starch/caseinate mixtures related to the Petfoods Industry. The background to this use is based on the inclusion of, generally, wheat flours into the meat emulsion of the petfood product. Excessive "damage" of the flour during the autoclaving step can lead to undesirable leaching of carbohydrate material into the surrounding "gravy", measured as percentage cook-out. This can reduce the clarity of the surrounding jelly. The objective was to ascertain whether the swelling volume reduction effects seen on some starches in the presence of caseinate could be reproduced in the flour system and hence perhaps reduce the system vulnerability to thermal damage.

Although wheat flours were the materials of interest here, to bridge the gap a brief study of wheat starch was included before the work on the flours commenced. In total six flours and a standard wheat starch were investigated in duplicate over 2 different days (i.e. to yield four results for each sample) for viscosity and swelling volume in

the manner described above. The data of Figures 3.3.1 and 3.3.2 represent the mean values, with C.V.'s of maximally 10% and 5% for the viscosity and swelling volume measurements respectively. Since wheat starch was found to produce similar pasting viscosities to corn starch the same concentration of 4% was used. For the flours a higher concentration of 5% gave this similar viscosity, due to the lower total starch content. In addition to this fundamental study carried out at Nottingham as part of this thesis an industrial investigation on the real products was also implemented using real scaled-down procedures and techniques. The outline of this industrial experimental protocol can be found in the Appendix 3.

2.2 BSA/Gelatin-Polysaccharide System

2.2.1 Materials

Bovine serum albumin (BSA) was obtained from the Sigma Chemical Company, U.K. Carrageenan, the galactomannan samples and gelatin (bloom value of ~150) were obtained from Pedigree Petfoods, U.K. The agar sample for the BSA studies was obtained from Sigma Chemical Company, U.K. whereas for use with gelatin the agar was obtained from DIFCO, U.K. The latter sample was not a pure agar, containing 21% tryptone, 10% yeast extract and 4% dextrose. The BSA was supplied in a highly purified form and produced through the "initial fractionation by heat shock" method to yield a fraction V of 96-99% purity, the rest being mostly globulins. The carrageenan was a kappa (κ) fraction, that is to say 95% is accounted for as κ-carrageenan. All samples were used as supplied without any further purification. The buffer salts (analytical grade) were supplied by Fisons, U.K. The moisture content (mean of 3 dryings $\pm$ 0.1%) of each of the above was found to be:

	% H_2O
BSA	7.84
Gelatin	10.63
Carrageenan	12.63
Agar	14.29
Locust bean gum (LBG)	10.15
Guar	11.17
Tara	8.90
High protein LBG	9.39
Low protein LBG	10.85

The protein contents of the galactomannans, as stated by the supplier, were:

standard LBG	12.0%
low protein LBG	8.1%
high protein LBG	16.9%
tara	10.5%

The polysaccharides were stored dry in sealed containers at ambient whereas the proteins were stored in a cold store. Any prepared buffer solutions (9.091g/L

KH_2PO_4 and 11.882g/L Na_2HPO_4) were stored in a cold store and made up fresh again once a period of a few days or so storage had been exceeded.

2.2.2 Methods

2.2.2.1 Gel Preparation

A reflux apparatus consisting of a small glass heating vessel and cold water condenser was used to prepare the gels. The correct weight of dry powders for the study in question (carrageenan at 2% (w/v); agar at 2%; carrageenan/LBG at 0.5%/0.5%; BSA from 0-5%; gelatin from 0-20%) was added to the appropriate volume of buffer (w/v) on a magnetic stirrer whilst being stirred and heated to boiling. This temperature was then maintained for 15 minutes. After this period the gel "solution" was then (i) poured into glass boiling tubes and covered with Nesco film and (ii) poured into cut lengths of 19mm diameter dialysis tubing, double knotted at top and bottom and outer-wrapped in domestic "Cling-Film". Both tubes were left at ambient for 24 hours. For the two systems gels were actually made in duplicate (the gelatin system) and triplicate (the BSA system) to give mean values with coefficients of variation [(S.D/mean) x 100%] of 1% (melting point measurements) and maximally 20% (the stress relaxation measurements).

In some later investigations a number of other galactomannans namely guar, tara and a high husk/high protein LBG and a low husk/low protein LBG were used in the initial dry-mixed powders in place of the standard LBG at identical concentrations and conditions.

2.2.2.2 Analysis

(a) Melting Point Determination

A Techne Tempunit heater was used to heat a transparent sided water bath, containing the 24 hour old gels, at a rate of approximately 1^oC/minute, ensuring that the water level covered the height of the gels. This is shown schematically in Figure 2.2.2. Into each boiling tube was placed a 4mm steel ball-bearing gently pushed to just under the gel surface (in order to prevent skin effects). The melting point was taken as the temperature at which the steel ball-bearing reached the base of the boiling tube in each case. This temperature was recorded by a Digitron Instruments electronic thermocouple after placing the probe into the body of the gel.

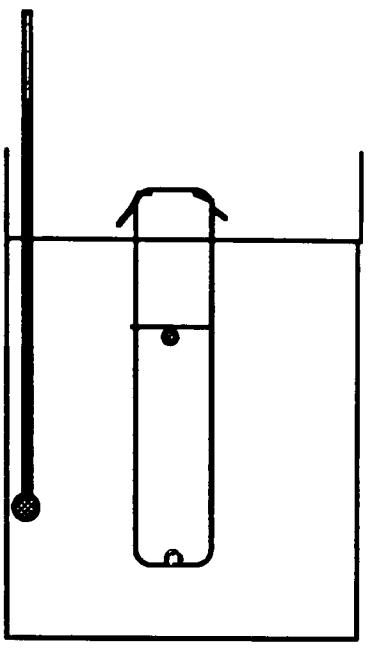


Figure 2.2.2 Melting point determination

(b) Stress Relaxation

The dialysis tubes were opened carefully without damaging the gels contained within and cylinder . samples 20mm in height were cut using a special parallel blade cutter, to always ensure parallel edges. Each test sample was cut as required and tested immediately to avoid drying out or syneresis of the sample. Large deformation stress relaxation analysis was then performed on a TA-XT2 texture analyser using a 25mm cylindrical probe under a compression of 4mm (i.e. to 16mm) with approach spedd of 2mm/s and a subsequent relaxation time of 240 seconds. The set-up is shown below.

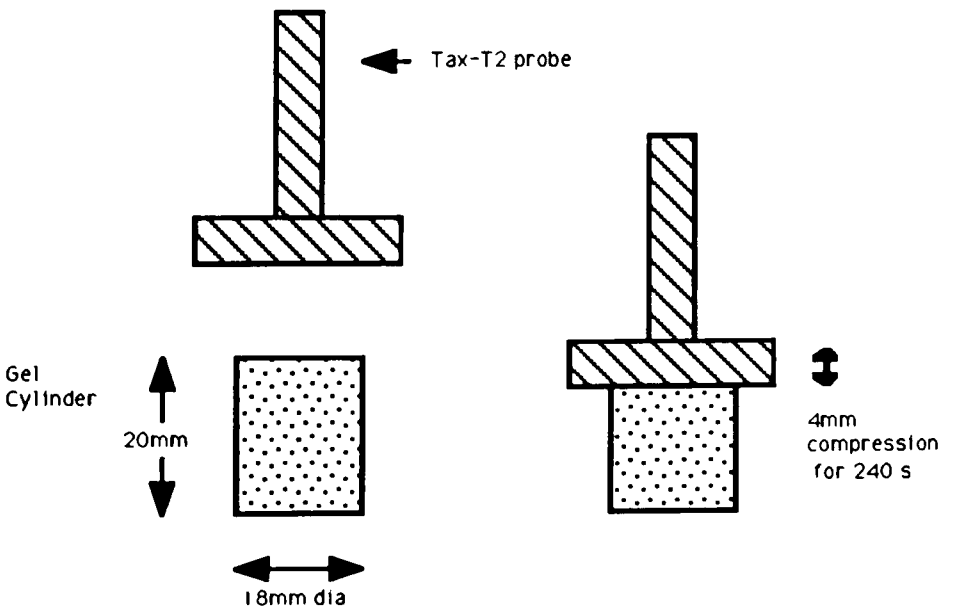


Figure 2.2.3 Stress relaxation compression test

(c) Breakstrength

Using the same apparatus as described above but employing only the "return to start" mode, cylinders of the BSA/polysaccharide gels, cut as described above, were compressed by 12mm (i.e. a distance that guaranteed rupture) and the peak force recorded that resulted in the breaking of the gel. This was done in triplicate tests with a coefficient of variation (CV) of maximally 20%.

2.3 Dried Blood Plasma (DBP) - Carrageenan/LBG System

2.3.1 Materials

Dried blood plasma (abbreviated to DBP in this thesis) was obtained from Pedigree Petfoods. The commercial name of this product was Vepro® 75, the 75 referring to its 75% minimum total nitrogenous matter content. Other components included 15% (max.) total mineral matter and 8.5% (max.) trisodium citrate. The material was stored at ambient as advised by the manufacturers and used without any further purification. The carrageenan and locust bean gum were the same as those described in the previous section. Large stocks of 0.1M KCl buffer (8.0g/L NaH_2PO_4, 5.0g/L Na_2HPO_4 and 7.46g/L KCl) were prepared, usually in 5 or 10 litre batches and mostly used in completion on the day of experiment. Any residual was stored in a cold-room. All buffer salts were supplied by Fisons, U.K. and stored at ambient.

2.3.2 Methods

2.3.2.1 Gel preparation

A large scale boil-up apparatus (vessel of 3L capacity) was used in conjunction with a water condenser as shown below. One litre of buffer was always used for each preparation with the corresponding correct weight of the protein/polysaccharide powders (w/v). The dry-mixed powders were added to the buffer and heated to 95°C with constant stirring.

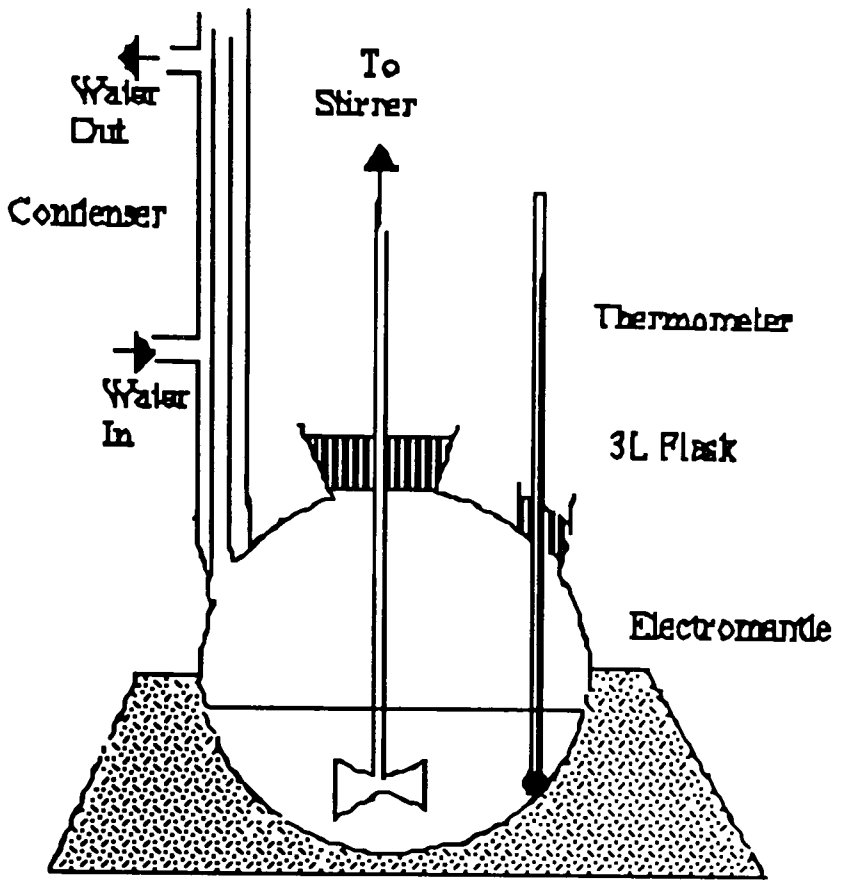


Figure 2.3.4 The "boil-up" apparatus used for the DBP/carrageenan/LBG gel preparation

Once at this temperature the solution was heated and stirred for a further 15 minutes after which time the contents were poured into 4 small (volume ~ 250ml) cans to over-flowing, hermetically sealed, water cooled and then either autoclaved or left to stand at ambient. Initial experiments utilized a static bench-top autoclave whereas later experiments employed a rotary autoclave. This latter instrument rotates the secured cans on mass (i.e. as the barrel of the autoclave rotates) at a speed of roughly 4 revolutions per minute. The option exists to either rotate whilst water-cooling or to simply remove the cans whilst hot and cool statically. Any autoclaved cans were then left overnight at ambient before testing alongside the non-autoclaved cans.

2.3.2.2 Analysis

(a) Breakstrength

The TA-XT2 texture analyser was employed as before to perform a breakstrength test. Compression of each sample whilst still in the can was made with the 25mm cylindrical probe to a distance of 10 mm penetration. This usually resulted in rupture of the gel structure and was consequently recorded as the peak force on the force-distance plot on the TA-XT2. After testing the top surface of the gel the can was

inverted and the lower lid removed to allow testing of this surface in the same manner. Overall, testing was made on triplicate samples with a coefficient of variation never greater than 15%. Since the visual observation showed interesting behaviour a series of photographs were taken of the free-standing gels.

(b) Photography

Following the observations made visually, photographs of both the statically and rotary autoclaved and non autoclaved gels were taken. This was performed on the gels still in the cans immediately after opening and also on removal from the cans.

2.4 BSA-Alginate

2.4.1 Materials

The BSA was supplied and stored as described before (previous sections). The sodium alginate was supplied by Proton Limited (Drammen, Norway) in a highly purified form and stored in a cold-store. A total of eight buffer solutions of constant ionic strength, over a range of pH from 5.0 to 8.5, were made using the compositions given in "Electrolyte Solutions" by R.A. Robinson & R.H. Stokes, 2nd edition, New York, 1959 and with buffer salts supplied by Fisons, U.K. All buffer solutions were stored in a cold-room when not in use.

2.4.2 Methods

2.4.2.1 Microelectrophoresis - Emulsion preparation

An emulsion was formed by adding BSA and alginate in equal weight ratio to the appropriate volume of desired pH buffer, so that final concentration of BSA and alginate was 1mg/ml each. For the control experiment, using BSA alone, naturally the alginate was omitted from this addition. Magnetic stirring for an appropriate period to disperse and dissolve the powders was then used. Having achieved this, the oil Di-n-butylphtalate was added to give a final oil content of 1%. An emulsion was then formed by high shearing with a Silverson mixer followed by centrifugation at 3000 rpm for 5 minutes to remove any aggregated proteins. The emulsion was then poured into the cell and left to equilibrate to cell temperature (25°C) for 30 minutes before commencing the readings as outlined below.

2.4.2.2 Analysis - Microelectrophoresis

The single value points (i.e. no replications) for each pH were performed on a Rank Brothers Mark II apparatus with a flat cell. Before commencing the experiment it is necessary to obtain values for the parameter called the stationery levels, which are a feature of the apparatus specification. The presence of the solvent in the cell causes a charging effect on the cell walls; usually such charge is negative when the solvent is water. This leads to streaming of the oppositely charged solvent near the walls and towards the appropriate electrode. This electro-osmotic streaming velocity would be constant across the cell were it not for the reverse flow phenomena at hydrostatic equilibrium, a feature of Poiseuille's Law. Thus the effect of both forces means that

the solvent is only stationery at well-defined depths in the cell - termed the stationery levels.

As a feature of the particular apparatus the stationery levels, designated s, were found from the Operating Instructions and Manual for this equipment. These levels were calculated to be:

$$S_1 = 10.86\text{mm}$$
$$S_2 = 11.61\text{mm}$$

Measuring a particle's velocity at these stationery levels is only equal to its own electrophoretic velocity. This latter parameter can be found by timing the movement of particles over a fixed distance, known through the calibration of the eye-piece graticule. Thus for each pH six timings were taken at each stationery level and in each direction of electric field. By a series of calculations we then arrive at a final value for mobility of the particle at that pH, given as

$$\text{Mobility} = V/E$$

where V is the velocity of the particle, (taken as stated above from the grid distance, which was found to be 6×10^{-5}m, divided by the time reading), and E is the electric field applied to the cell.

The electric field of the cell, E, is given by:

$$E = V/L$$

where **V** is the potential difference across the electrodes (= 40V) and L is the inter-electrode distance. This separation is itself obtained from:

$$L = KAR$$

where K is the conductivity of the solution in the cell, A is the cross sectional area of the cell and R is the resistance between the electrodes. After each timing the cell was cleaned well with water and detergent, exercising great care whenever handling the cell.

2.4.2.3 Ultracentrifugation - Solution Preparation

Using stock solutions of 4mg/ml of each component in a phosphate chloride buffer (pH = 6.5; I = 0.1M), composition of 3.080g/L $Na_2HPO_412H_2O$, 3.280g/L KH_2PO_4, 2.923g/L NaCl (after Green, 1933), single component solutions could be made by dilution with buffer to a final concentration of 2mg/ml. Combination of the two components in equal ratios then yielded the desired 1mg/ml concentration of each. Solutions were heated in screw-top jars and covered to avoid evaporative losses and heated in a Techne Tempunit water bath at the appropriate temperature (85°C or 95°C) for 1 hour.

2.4.2.4 Analysis - Ultracentrifugation

After cooling at room temperature for approximately 3 hours the solutions were loaded onto the ultracentrifuge cells and the cells assembled before measurements were made on the Optima XL-A analytical ultracentrifuge, Beckman Instruments, at 25°C using a speed of 5000 rpm. Calculations of the sedimentation coefficient at 25°C (the s_{25} value) from the 1st and 10th scans of the sedimenting macromolecules (as shown in Figure 5.2.1) were made using a digitising tablet and computer software specifically designed for this.

CHAPTER 3. RESULTS: THE STARCH-CASEINATE SYSTEM

The work described in this chapter will be divided into three sections:

3.1 Section 1: potato starch-caseinate
3.2 Section 2: maize starch-caseinate
3.3 Section 3: wheat flours-caseinate - (a) Nottingham University
(b) Pedigree Petfoods, U.K.

The initial area of interest to this study was the potato starch-caseinate interaction. This was later extended to the maize starch-caseinate system, in order to compare behaviour with another starch. The results of these two starch types are given in the first and second sections of this chapter respectively. A follow-up investigation centering on the potential for industrial applications then arose stimulated by both Nottingham and Pedigree Petfoods, Melton Mowbray as a focus of the LINK scheme. For such a study the use of wheat flours was a more realistic option The findings of both the fundamental studies by Nottingham and the applied research by Pedigree Petfoods are given in the third section of this chapter.

3.1 Section 1: The Potato Starch System

Even from visual observation it is apparent that sodium caseinate has a dramatic effect on potato starch reducing the thick, white opaque paste to a pale yellow, watery liquid on addition of even low levels of sodium caseinate. The rheological measurements confirm this. Figure 3.1.1 shows the flow curves for the control paste (1% starch) in the absence of caseinate and pasted in different levels of caseinate. In distilled, deionized water a 1% potato starch paste yields a shear-thinning, psuedoplastic paste with a high paste viscosity as would be expected for potato starch (see for example, Swinkels, 1985) but pasting in caseinate reduces this to a near-Newtonian liquid (Figure 3.1.1).

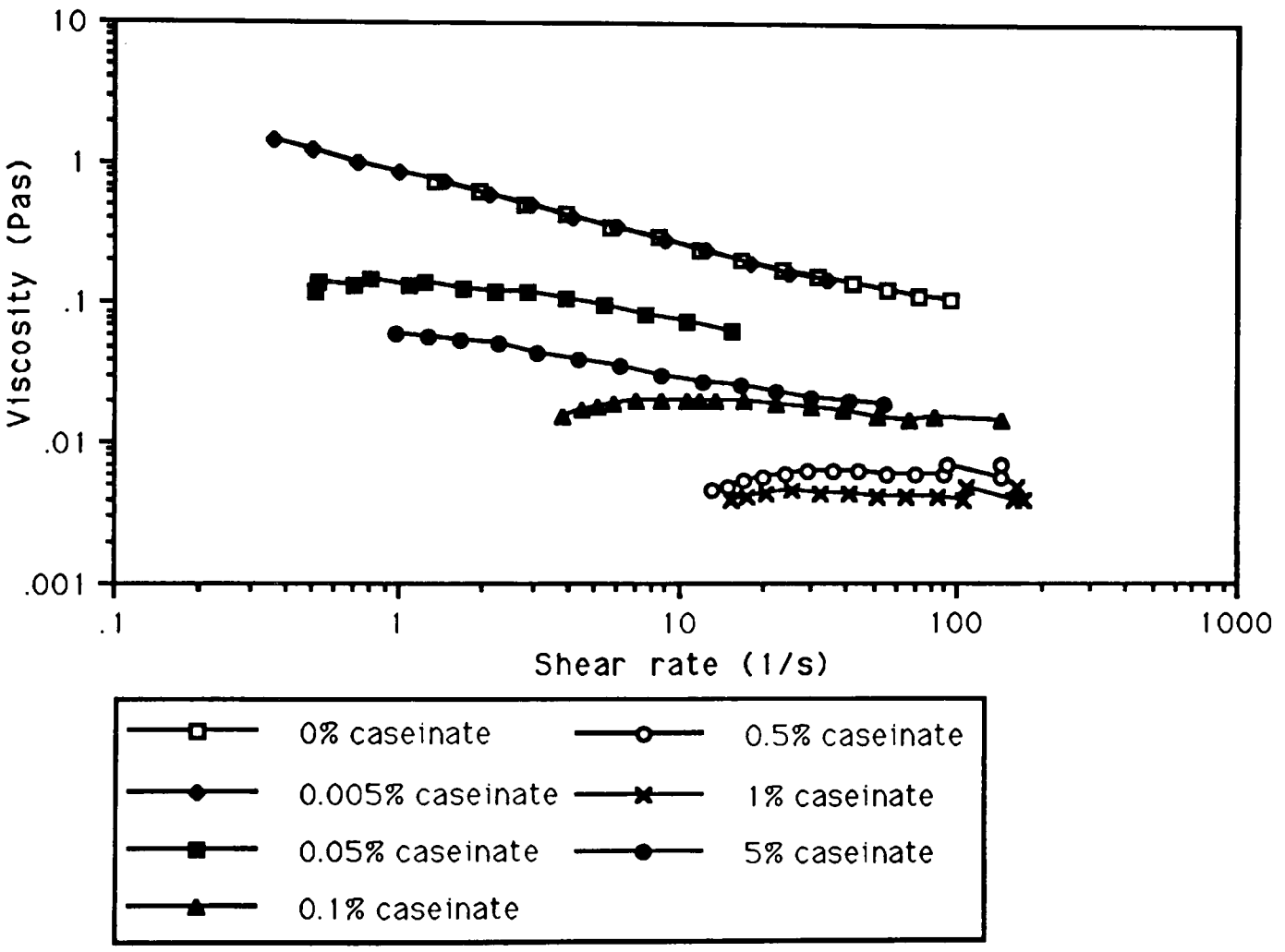


Figure 3.1.1 Flow Curves to show the effect of caseinate on 1% potato starch

Figure 3.1.2 shows the viscosity at 50 1/s for a range of caseinate concentrations. In the absence of caseinate the paste has a value of the order of 160 mPas. The shear rate value of 50 1/s used throughout the starch-caseinate system has been stated in the literature as representing the effective shear rate for the action of eating in the mouth (Wood, 1968). Therefore it is interesting that the Bohlin-determined viscosity should correlate to the mouthfeel viscosity.

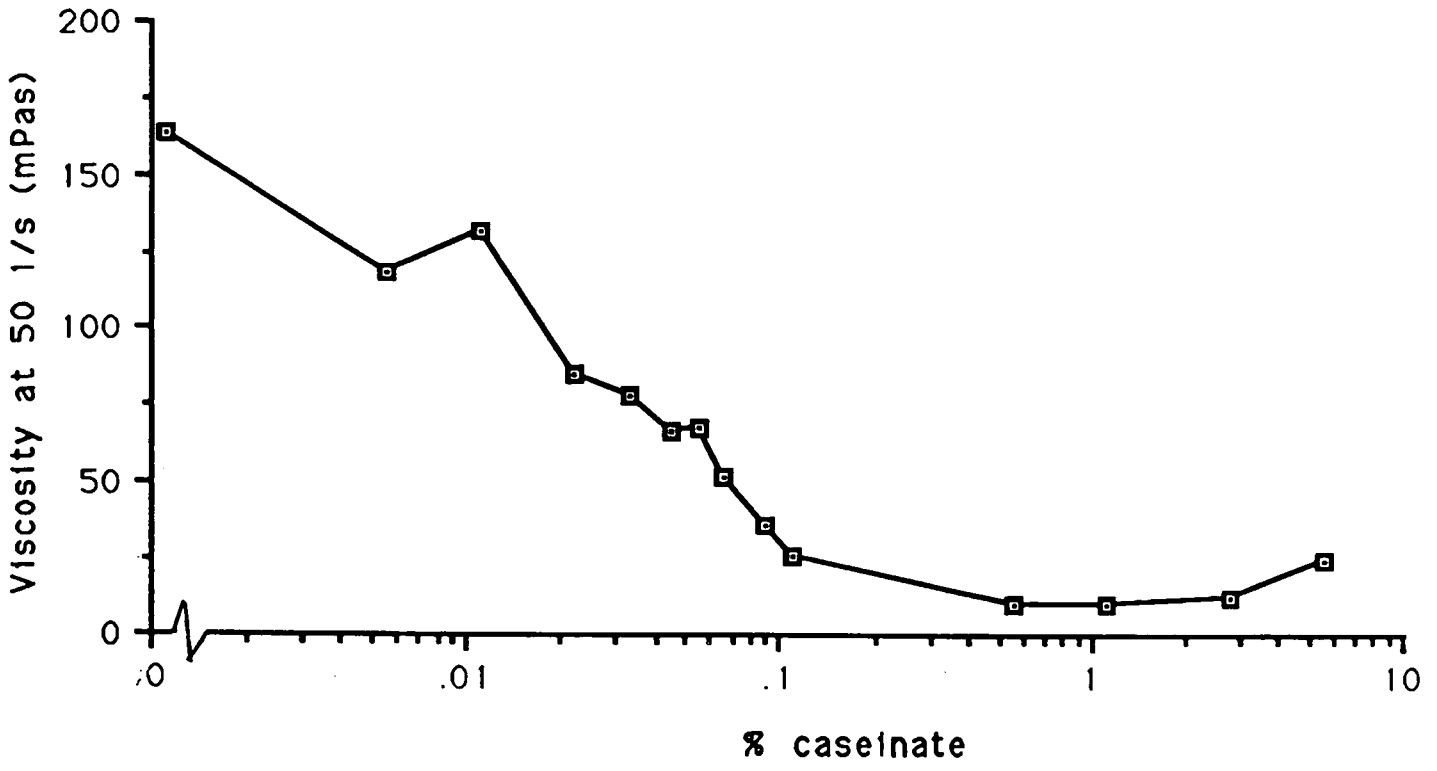


Figure 3.1.2 Effect of caseinate on the viscosity of a 1% potato starch paste

From this figure it is evident that at a level of 0.1% sodium caseinate the system undergoes an approximately six-fold loss in viscosity with a further reduction to the 1% level. It is well recognised that the viscosity of "dilute" starch suspensions is governed by the volume fraction occupied by the swollen granule (see for example, Steeneken, 1989). This therefore suggests that the caseinate reduces the swollen volume. This is confirmed by the data of Figure 3.1.3 in which swelling volume is given as a function of sodium caseinate and also by the micrographs of Figure 3.1.4.

The swelling volume of the starch granules (Figure 3.1.3) closely follows the same trend observed for the viscosity indicating the correlation between paste viscosity and the ability of a starch granule to imbibe water and swell on gelatinisation. The inclusion of sodium caseinate into the system definately reduces this ability to swell.

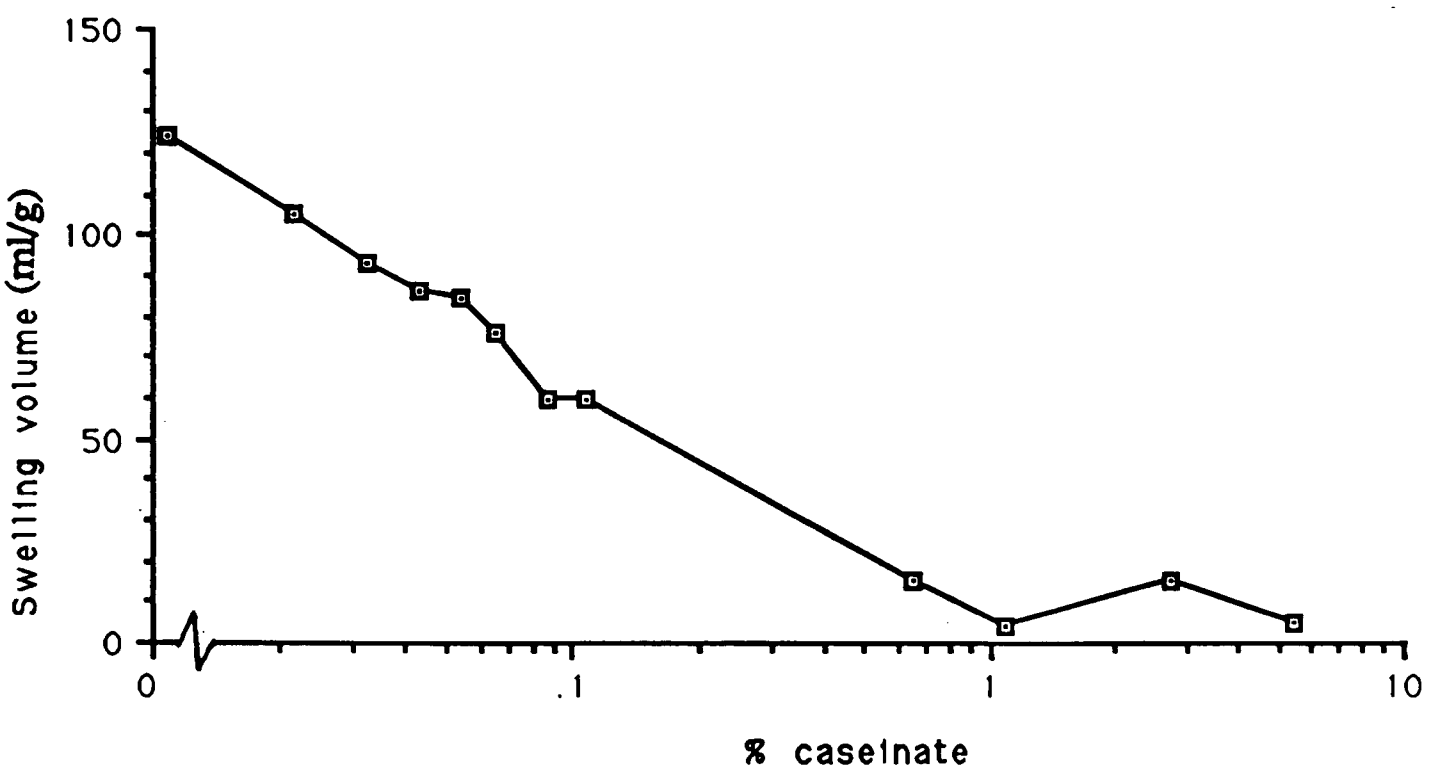
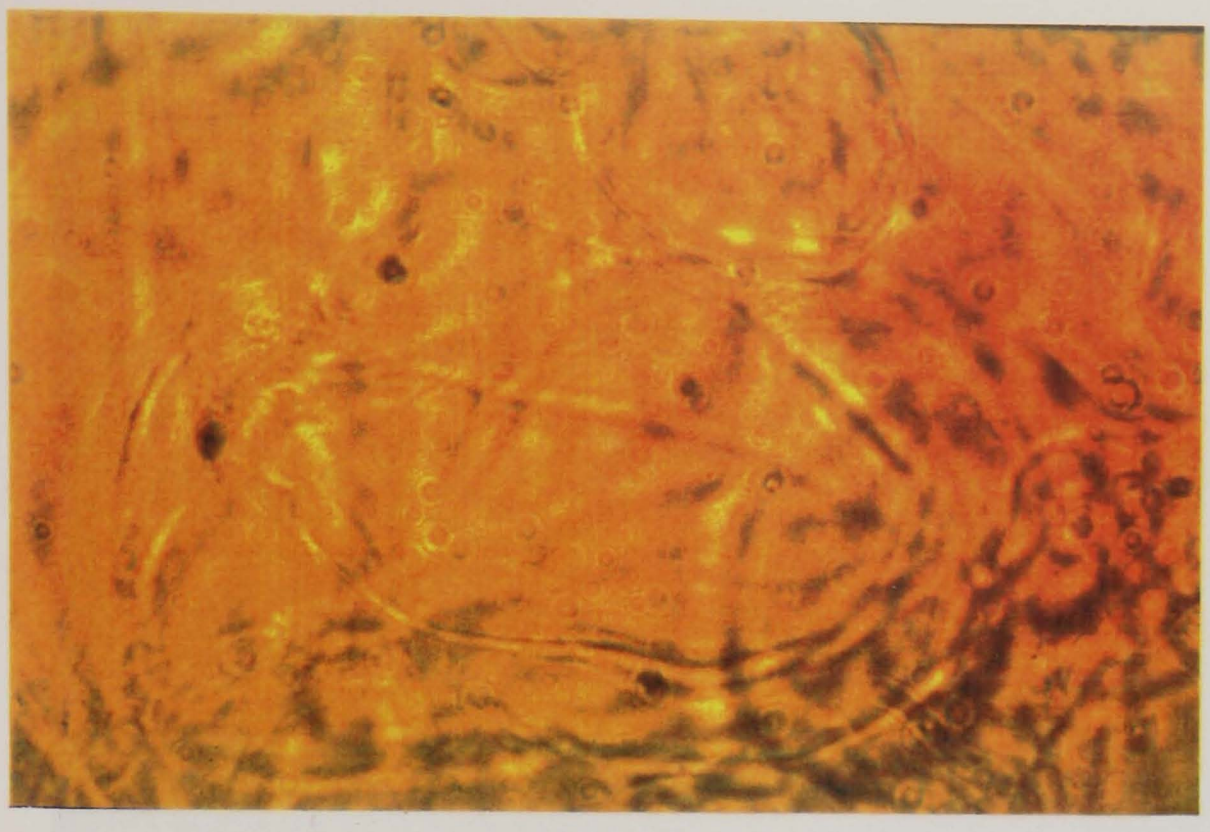


Figure 3.1.3 Effect of caseinate on swelling volume of a 1% potato starch paste

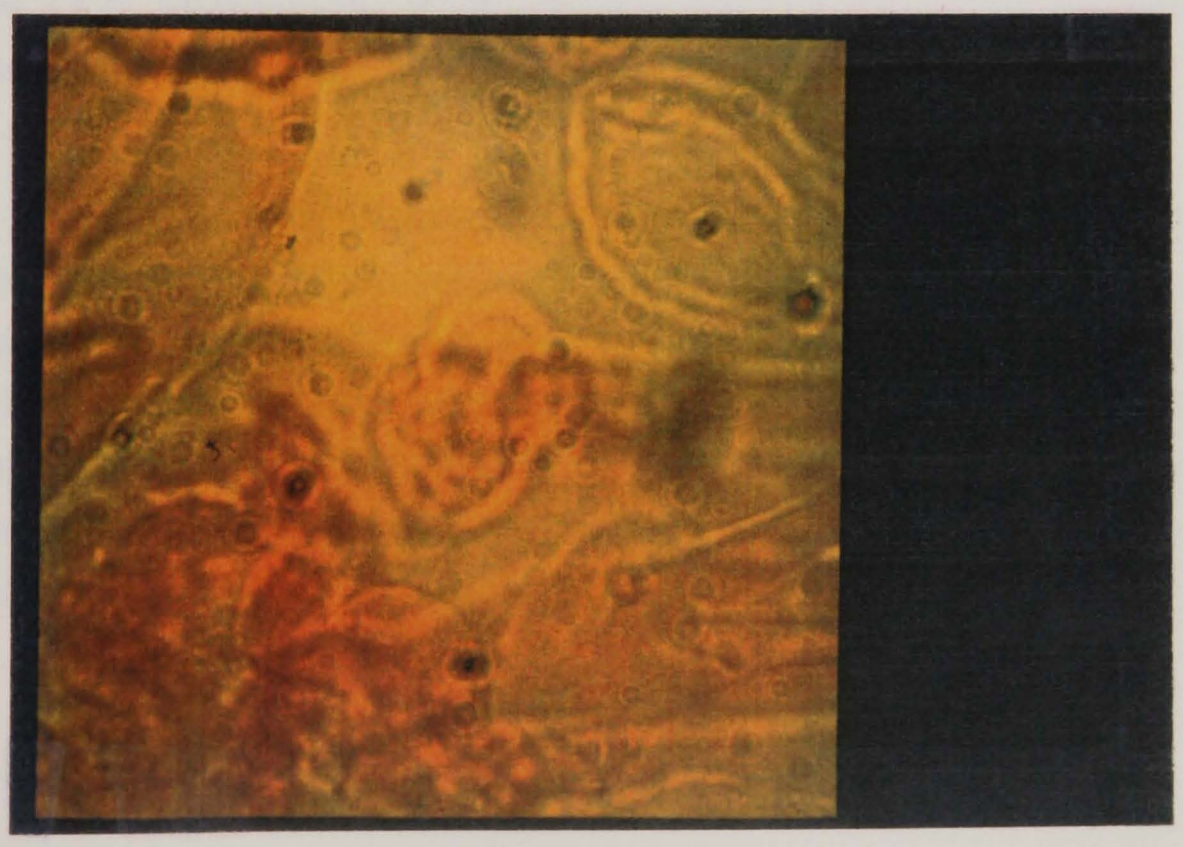
Microscopic examination at the granular level lends support to the reduced swelling. As can be seen below (Figure 3.1.4) the fully expanded, large, smooth-edged oval granule of the native potato starch (Figure 3.1.4a) is replaced by smaller, "shrivelled" granules, themselves fewer in number, in the presence of caseinate (Figures 3.1.4b and 3.1.4c).



(a)

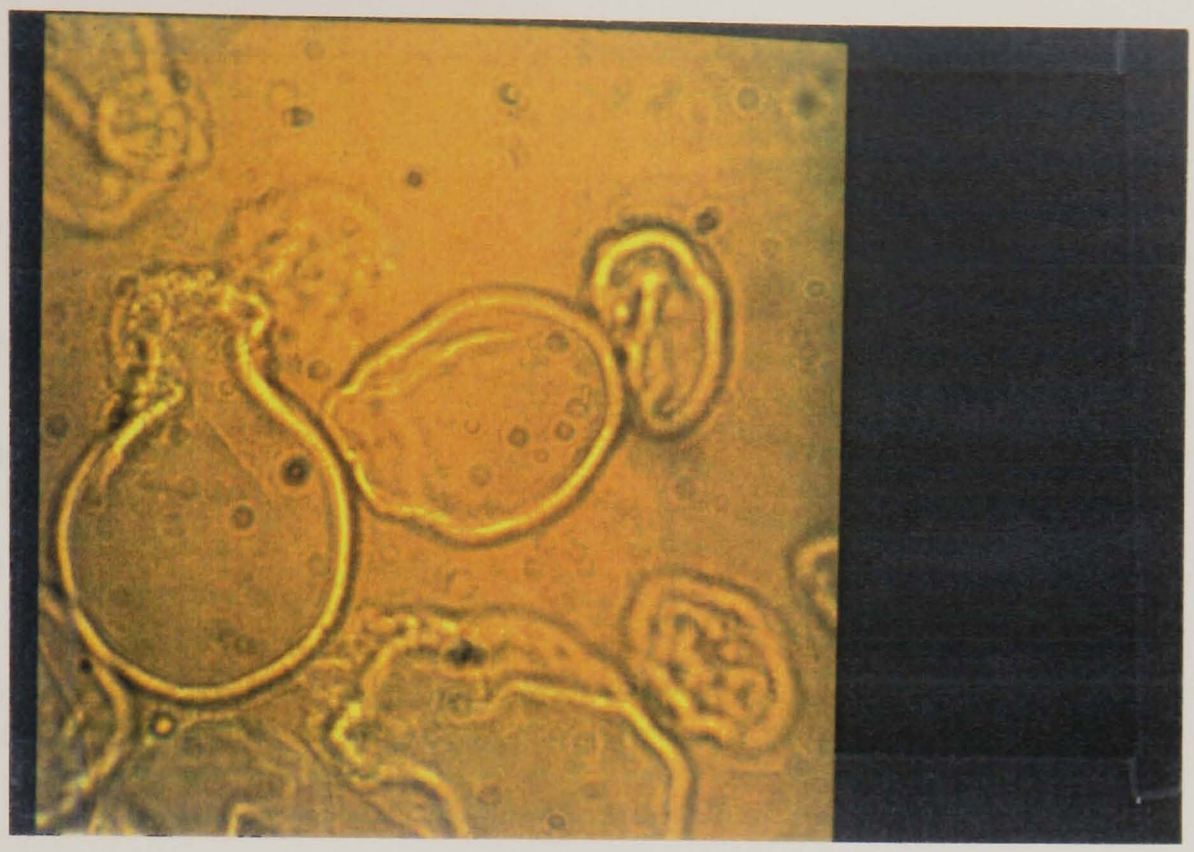
50μm

I———I



(b)

Figure 3.1.4: **Micrographs of 1% potato starch pasted in (a) distilled, deionized water and (b) 0.1% caseinate in distilled, deionized water**



(c)

50μm

I————I

Figure 3.1.4: Micrographs of 1% potato starch pasted in (c) 5% caseinate in distilled, deionized water

Some information on the leaching of the carbohydrate material into the inter-granular matrix is acquired from the solubility parameter. Assuming the swollen granules sediment out of solution and pack down into what finally forms the pellet, any inter-granular material then has to occupy the supernatant. from Figure 3.1.5 below it becomes apparent that the solubility of the system - defined as the percentage of total starch found outside the granule - increases, beit only slightly, with increasing caseinate levels.

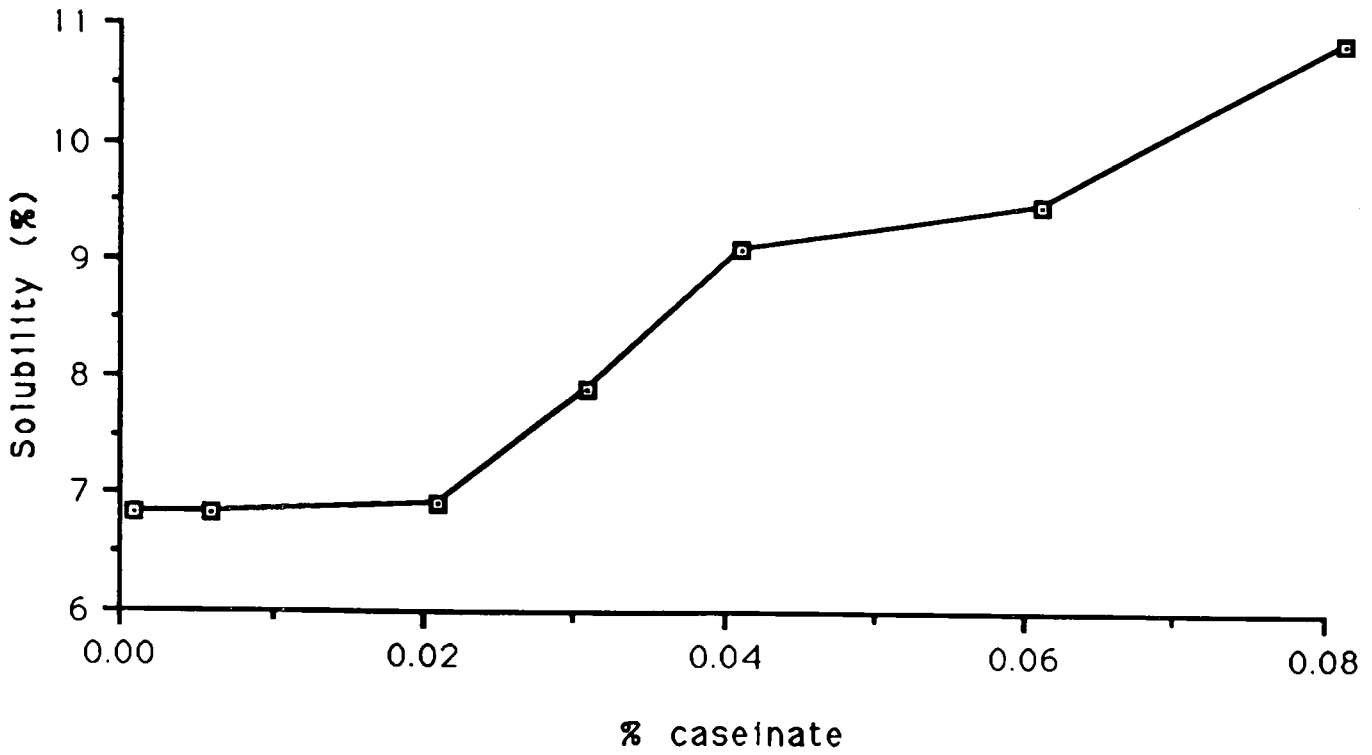


Figure 3.1.5 Effect of caseinate on the apparent solubility of a 1% potato starch paste

Figure 3.1.5 displays the amount of solids found in the supernatant expressed as a percentage of the total amount of starch in the system. This increases with increasing caseinate concentration. However if it is assumed that the caseinate is equally distributed between the granular and soluble phases then this would make a significant contribution to the reported percentage solubilities. The magnitide would be sufficiently large to suggest that Figure 3.1.5 shows that at increasing caseinate levels the amount of starch leaving the granular phase is reduced. This conclusion is tentative however as it is not clear how the caseinate partitions. In order to minimize possible errors in the solubility calculation as it stands only low levels of caseinate can be involved. Since the most dramatic effect on potato starch is seen at low levels of caseinate this is not a problem. Overall to overcome these uncertainities it would be desirable to use a method specific for carbohydrate. However it can still be said though that a reduction in swelling volume is accompanied by an increase in solubility.

Substantial reductions in the swelling volume of a number of starches on the addition of low levels of sulphite have been reported by workers form our laboratory (Mat Hashim *et al.*, 1992; Paterson *et al.*, 1994). This reduction was attributed to oxidative reductive depolymerisation (ORD) of the starch polysaccharides. This mechanism was also initially considered for the action of caseinate on potato starch observed

here. However the reduction in swelling volume found in the presence of sulphite is always accompanied by a large increase in solubility. Since this is not seen here and in addition to evidence presented further on this type of action was ruled out. Therefore the idea of an general ionic strength effect was next investigated.

To test this hypothesis sodium chloride was included in the system by using a salt solution as the solvent in the pasting procedure. It was found that the addition of sodium chloride yields a large decrease in viscosity (Figure 3.1.6). This is consistent with the results of Muhrbeck & Eliasson (1987) who found a large decrease in the viscoelastic parameters for potato starch pastes on NaCl addition.

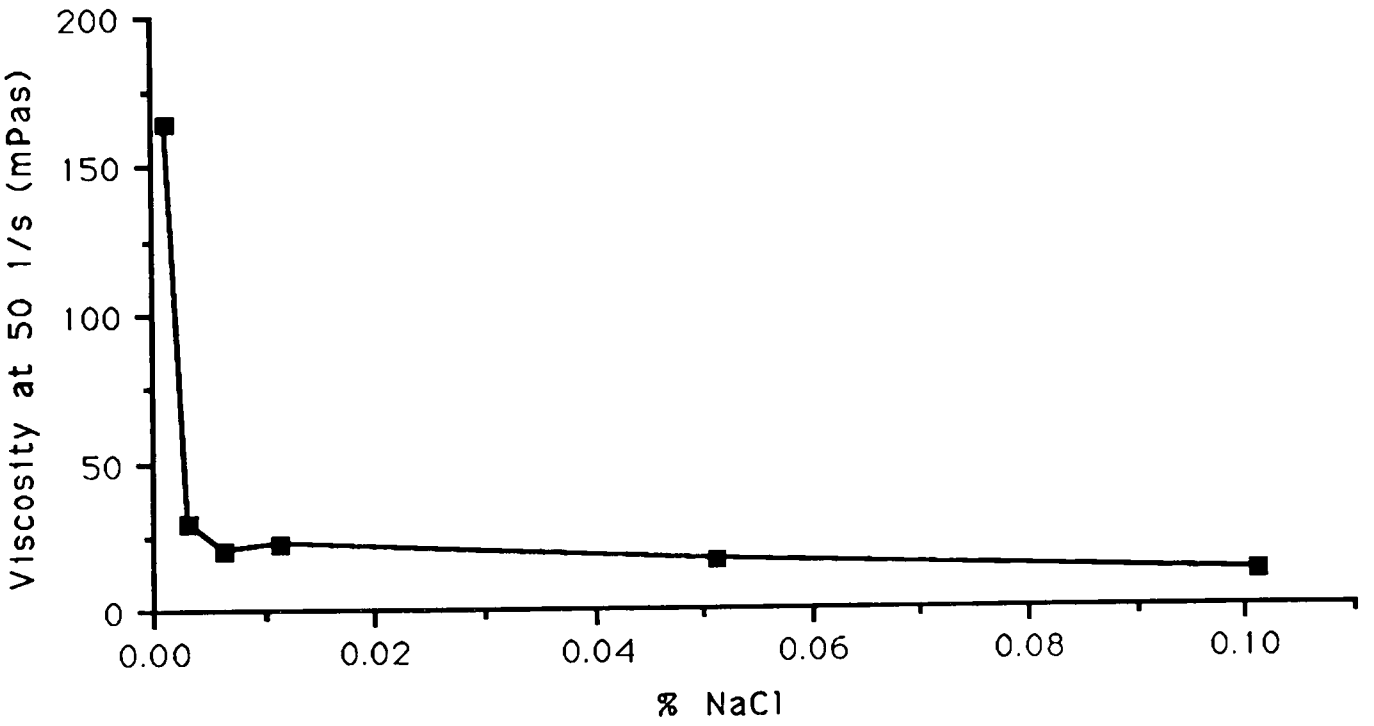


Figure 3.1.6 Effect of NaCl on the viscosity of a 1% potato starch paste

These workers related the NaCl addition to the corresponding conductivity and related the latter to the viscosity drop. Muhrbeck and Eliasson (1987) showed that it was shown that cassava starch, another root starch, does not exhibit the same behaviour in the presence of salt.

In order to assess the effect of the ions in the caseinate solution a dialysis was performed. Replacement of the "normal" caseinate solution in the pasting step for a 1% caseinate solution which had been dialysed overnight against distilled, deionized water was made. The flow curves of Figure 3.1.7 shown below indicate that the viscosity of the 1%starch/~1% dialysed-caseinate lies intermediate between the 1% potato starch control and 1% starch/1% undialysed-caseinate.

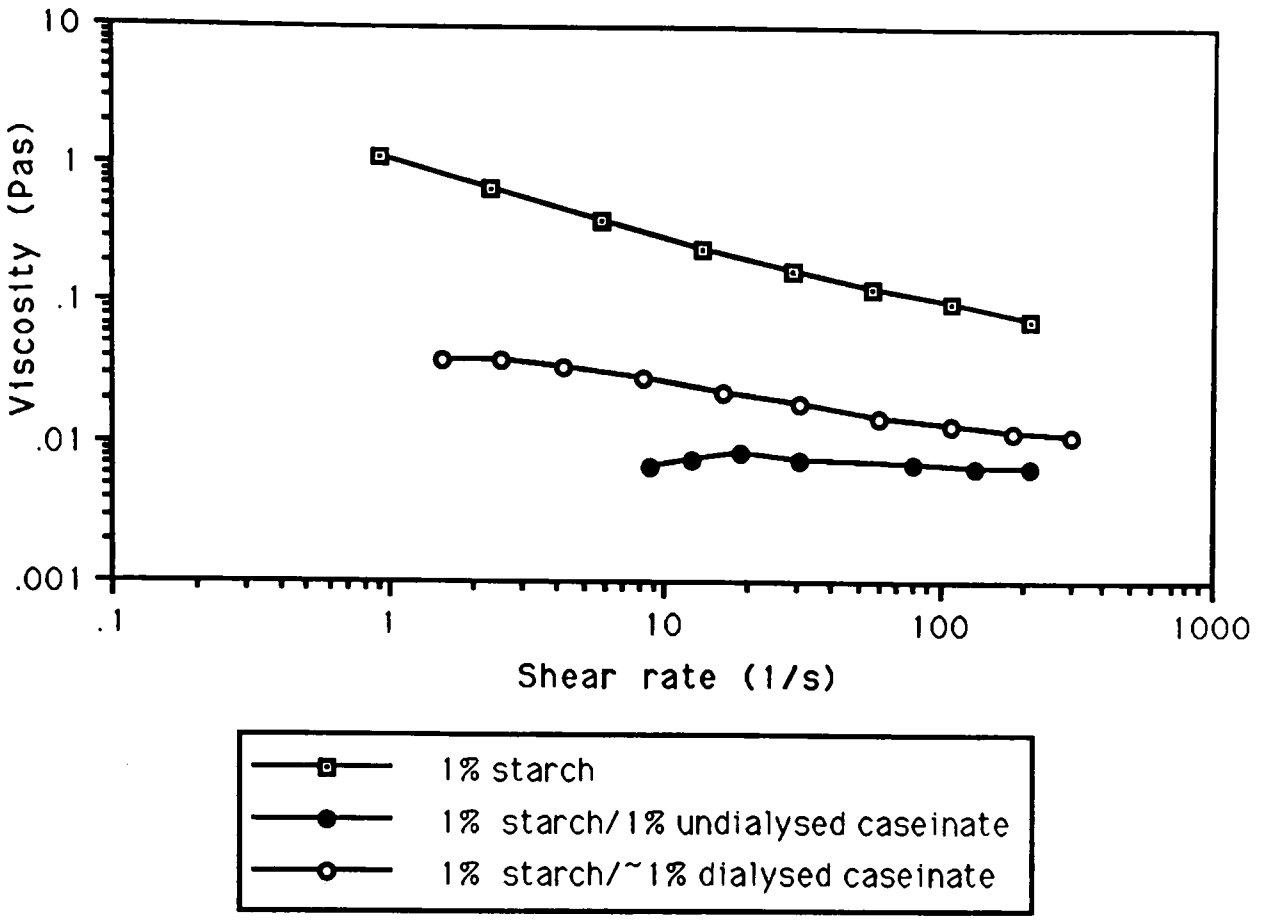


Figure 3.1.7 Flow curves to show the effect of using dialysed caseinate in the pasting procedure

This intermediate positioning may be representative of the level of ions dialysed out and possibly suggests that continued dialysis would further tend towards the properties of the caseinate-free control. Whilst having no absolute indication of the nature of the ions removed, the conductivities of the caseinate solution are shown to be reduced significantly after dialysis (Figure 3.1.8).

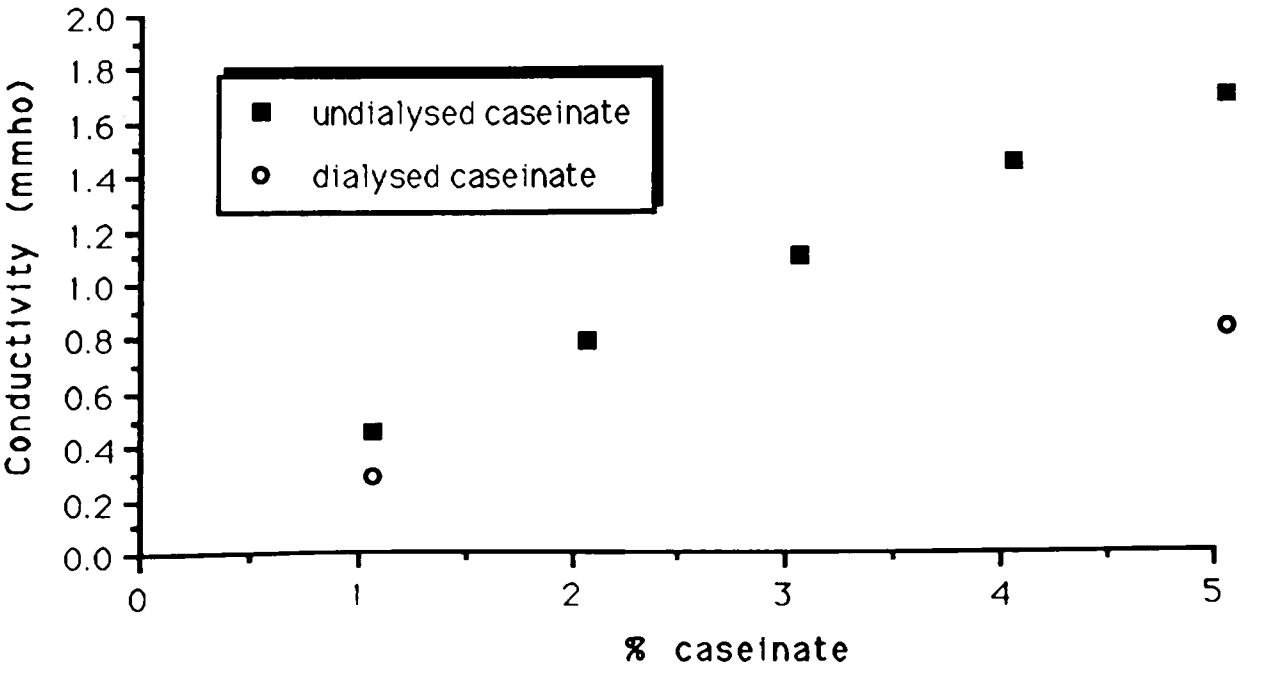


Figure 3.1.8 Conductivity of sodium caseinate solutions

From Figure 3.1.8 the linear relationship between the conductivities of the undialysed caseinate solution and the concentration is obvious; the amount of caseinate in the system is directly proportional to the ionic strength with a zero caseinate concentration almost at the origin, as would be expected for the distilled, deionized water used as the solvent (in fact the conductivity of the water was measured to be of the order of ~ μohms). The highest and lowest caseinate concentrations after dialysis show that whilst each still gives a considerable reading of conductance a significant proportion of ions have in fact been removed upon dialysis, with relatively more removed at the 5% level. This dialysis was performed over a 24 hour period and presumably continued dialysis would remove more ions. The conductivity reflects the behaviour found in the flow curves of Figure 3.1.7. This strongly suggests that the presence of ions in the caseinate solution has a direct impact on the final paste viscosity.

Another aspect important in the effect of the caseinate on the starch is the mode of hydration. Up to this point the caseinate has only encountered the potato starch when in an already hydrated state. This becomes important when considering that in the control sample the starch has unlimited access to the available water whereas in the caseinate-pasted samples a proportion of this will have been used in the hydration of the protein.

In order to assess the importance of the hydration of both components different levels of hydration of each were implemented. This ranged from adding the caseinate solution to the hydrated starch pastes at varying time intervals (to ascertain the effect of caseinate addition to an swollen starch paste), through to the addition of dry mixed powders. For the former experiment the need to maintain the final concentrations at the same level required that both the sodium caseinate solution concentration at initial addition and the starch paste concentration were double. From this consideration controls at both the desired final concentration and the double concentration initially were also included to eliminate effects due to the higher concentration. The points of addition of the caseinate solution, shown below in Table 3.1.1, were chosen to encompass the complete range of starch paste development; the addition at 2 minutes after entry into the water bath (designated t_2) involves a freshly gelatinized starch paste with the majority of time in the bath left for any interaction to take place whereas at 55 minutes the starch paste will be well established and any interaction between the starch and the caseinate will have to occur within the last 5 minutes of the heating time. Intermediary to these is the addition at 30 minutes.

However, in all of these time experiments described above the caseinate is still in a hydrated state. To reverse the procedure seen up until here the caseinate was added to the gelatinised starch as a dry powder. This was performed in two ways: (1) leaving the powder to rest on the paste surface and thus relying on diffusion and sedimentation forces to disperse the protein throughout the starch and (2) stirring the powder into the starch paste with a gentle manual action. Superceeding all of these hydration experiments is the dry mixing the powders together before hydration of either component in order to fully assess competitive hydration.

It can be seen from the flow curves of Figure 3.1.9 and more clearly from the summarized values of Table 3.1.1 that regardless of the method of hydration all produce samples of extremely similar properties and very close to that of the 1%/1% control. All tests show a massive loss of viscosity, and in all cases except the dry mixed powders, at values slightly above the control where the starch was pasted in the 1% caseinate solution. This would suggest that the optimum impact on the starch is achieved when the caseinate is in a fully hydrated form before the starch is introduced to the system. The addition of the caseinate solution at different time intervals seem to follow a trend of increasing effect with time of addition, even if only slightly. Thus the later the caseinate is applied to the paste the more it reduces the final viscosity. This is perhaps the opposite to what would have been expected, assuming that the starch will be in a more hydrated and established state before any caseinate intervention but maybe this is the cause of the effect; the larger, more swollen granule represents an easier target for any interaction. It is interesting to learn that as late as 5 minutes before the end of the experiment is sufficient for the caseinate to make an impact.

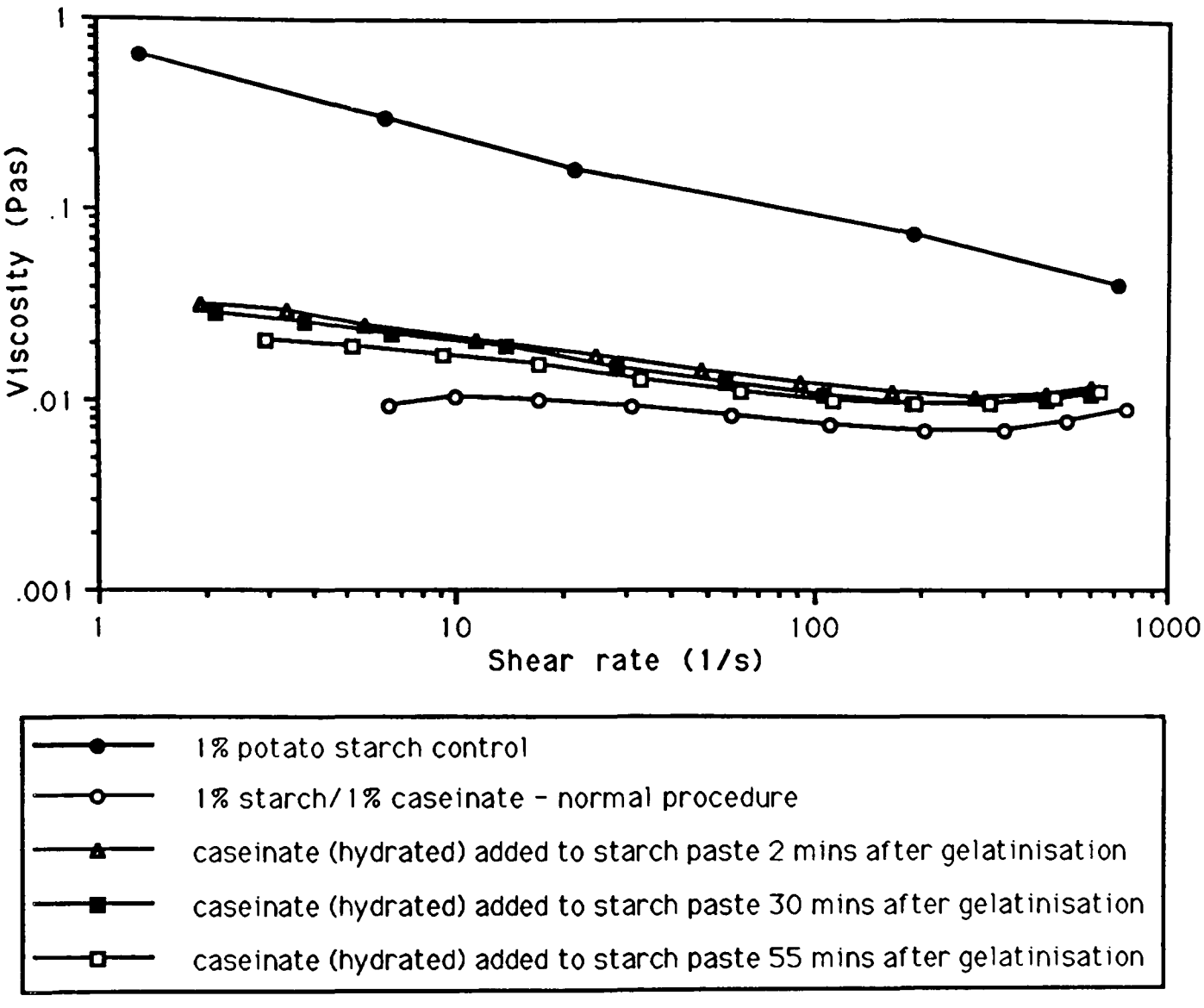


Figure 3.1.9 Flow curves to show the effect of time of addition of hydrated caseinate on the gelatinised starch paste

The addition of the caseinate as a dry powder also gives interesting results. The dry mixing of the two components gives a practically identical result to the 1%/1% control, indicative of the importance of the caseinate hydration before or at the same time as the starch. When the starch is already pasted up any addition of protein as a powder still has the effect of greatly reducing paste properties, if not to the same extent as the dry mixed powders. Any initial concern that the actual action of stirring in the powder would irreversibly destroy paste viscosity is dispelled by the results shown.

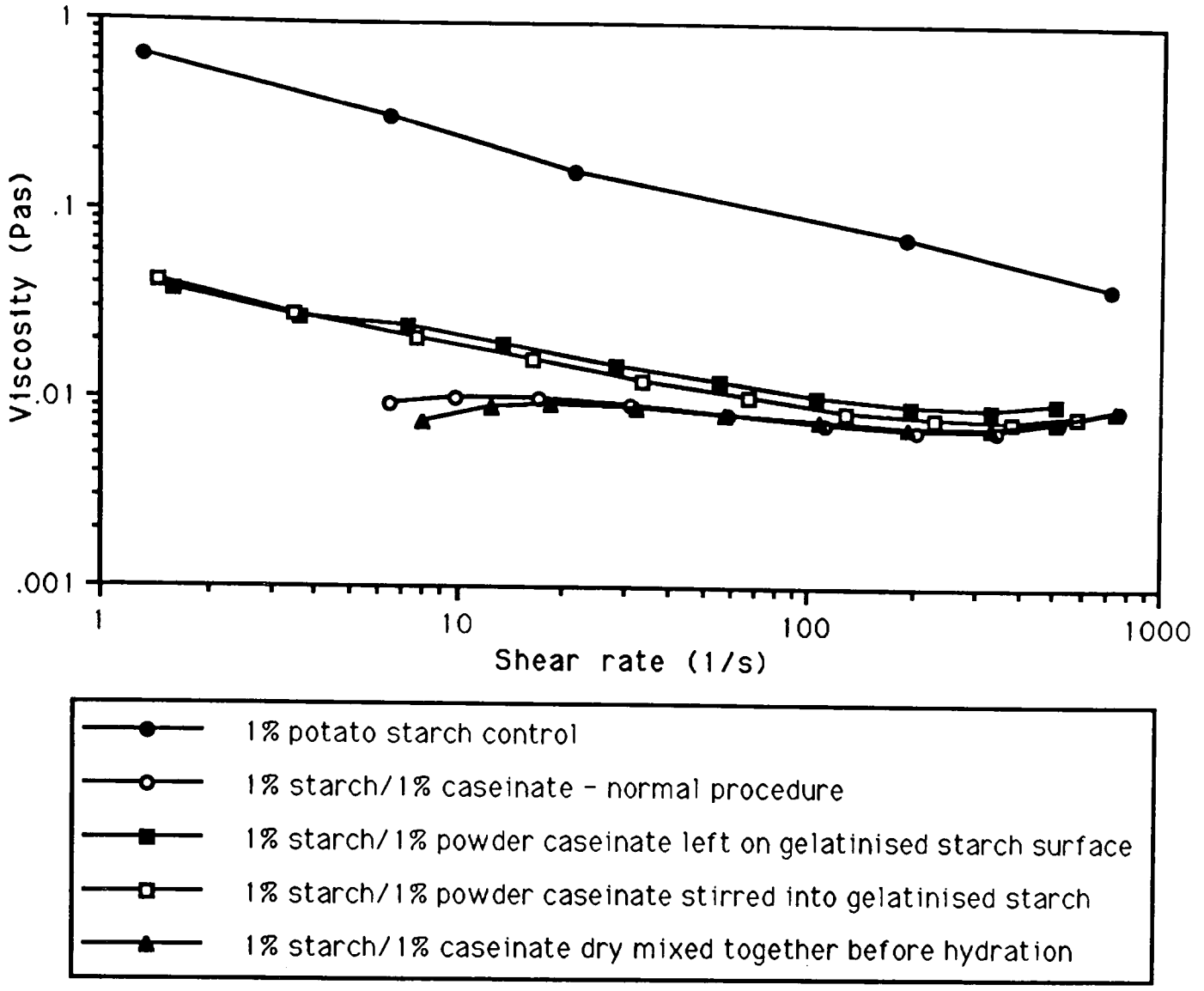


Figure 3.1.10 Flow curves of the hydration experiment: (i) addition of powder caseinate to the gelatinised starch paste and (ii) dry mixing of starch/caseinate powders before hydration

Table 3.1.1 Summarized results on the effect of hydration (see text for details of tests)

Test	Viscosity at 50 1/s (mPas)
1% control	142.8
1%/1% control	8.6
t2	15.6
t30	14.3
t55	12.8
powder stirred	13.7
powder left	12.8
dry mixed together	8.2

The preceding data strongly suggests that the origin of the effect is a non-specific ionic strength mechanism. To eliminate the possibility of any contribution to starch polysaccharide degradation from caseinate addition the intrinsic viscosity of solubilised starch was measured in the presence and absence of caseinate. The system was solubilized using the KOH method described in Chapter 2 and run on the U-tube

capillary viscometer to ultimately obtain a value for the intrinsic viscosity. This value is obtained first by plotting relative viscosity (the sample flow time/ the solvent flow time) as a function of starch concentration. Figure 3.1.11 below shows the relative viscosity of the solubilized potato starch in the absense of caseinate. The reproducibility of the three replicates is excellent.

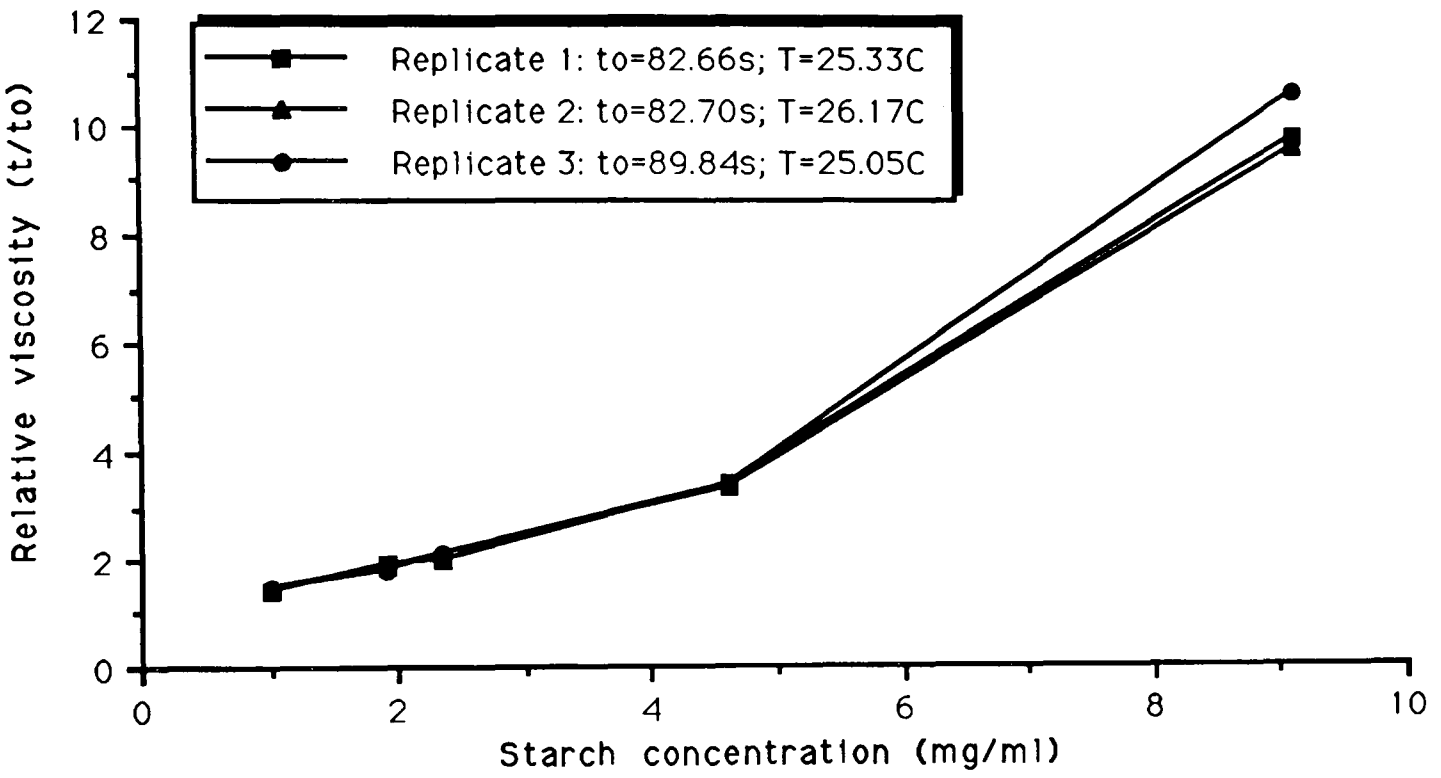


Figure 3.1.11 Relative viscosity for solubilized potato starch in absence of caseinate

By then plotting secondly the reduced specific viscosity [(relative viscosity-1)/concentration] against concentration the value of intrinsic viscosity can be obtained from the intercept at the y-axis. Figure 3.1.12 gives the intercept values as part of the equation for a straight line for the replicate with a mean value of 170 ml/g.

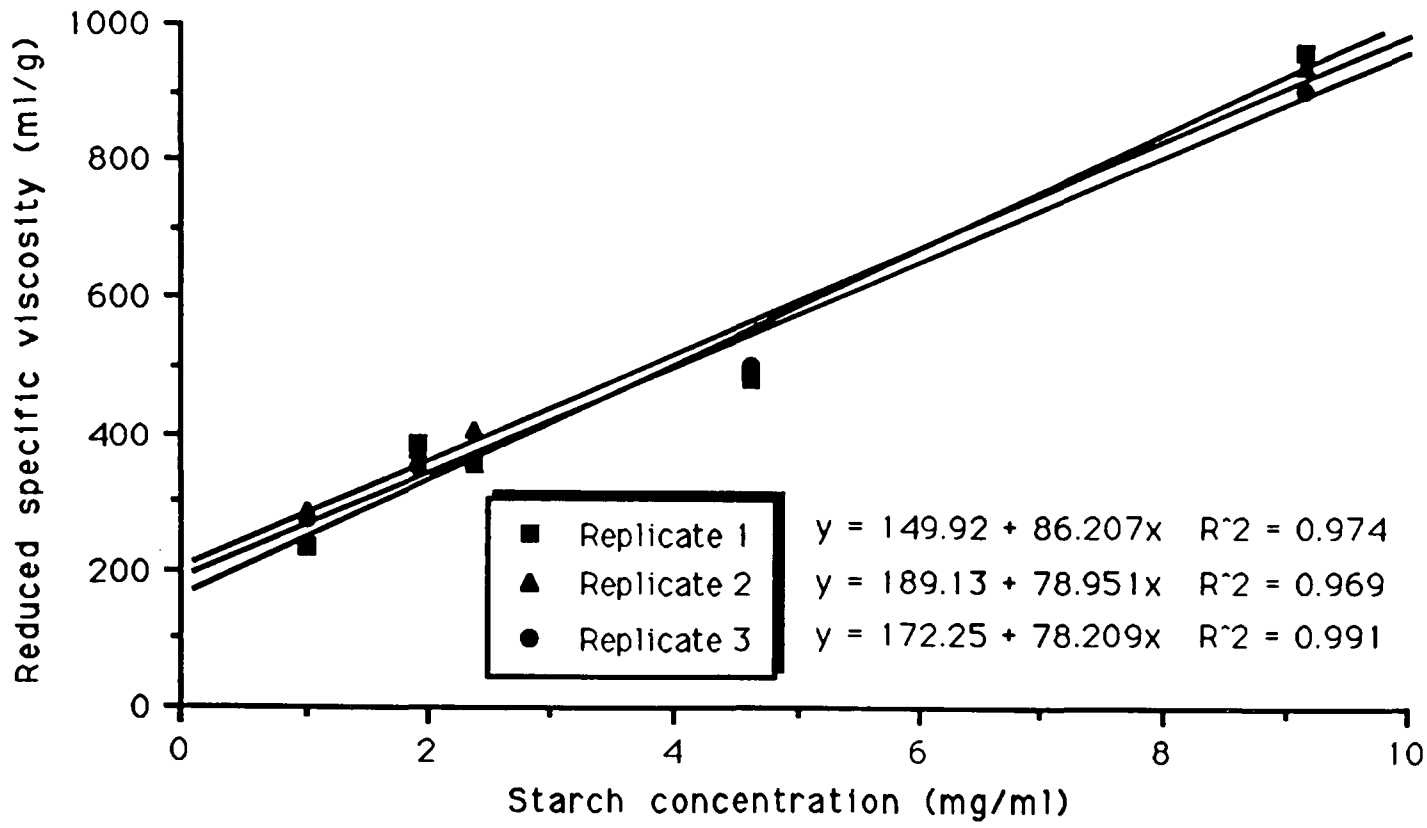


Figure 3.1.12 Reduced specific viscosity for potato starch in absence of caseinate

To repeat the procedure with a solubilized potato starch pasted in the presence of caseinate a level of 0.1% caseinate was designated as optimal to use. This represented a compromise based on the fact that significant effects on potato starch are seen at concentrations down as low as 0.1% yet maintaining the caseinate low enough to avoid the question of excessive caseinate contribution to the flow times. The relative viscosity of four replicates are given in Figure 3.1.13

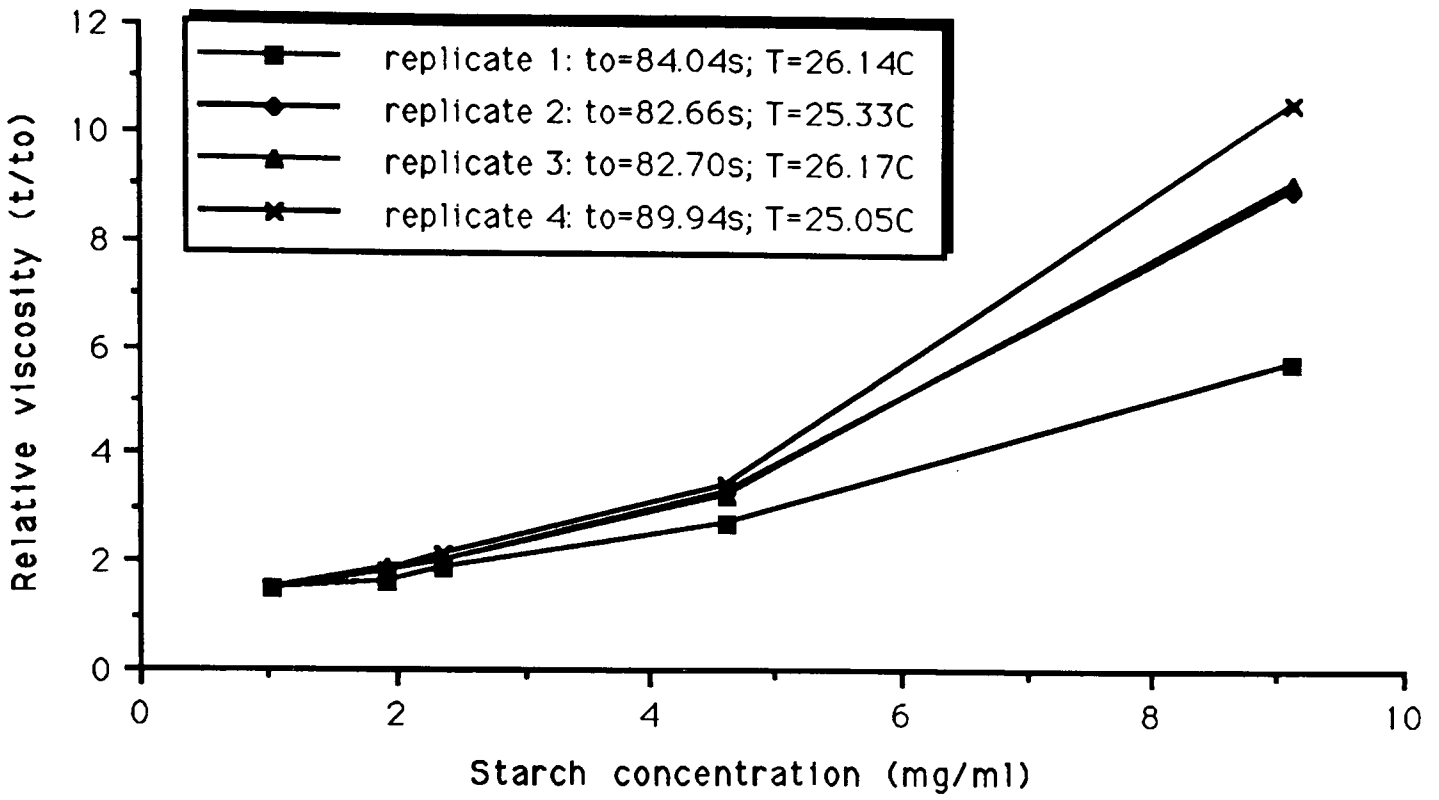


Figure 3.1.13 Relative viscosity for potato starch pasted in 0.1% caseinate

From these results it is apparent that the reproducibility of the replicates is poorer especially at the highest concentration of starch. From this data it could be argued that the 1st replicate should be rejected on the basis of the low value at the highest concentration but from the reduced specific viscosity data plotted below it is seen that the intercept of the straight line is in good agreement with the other values.

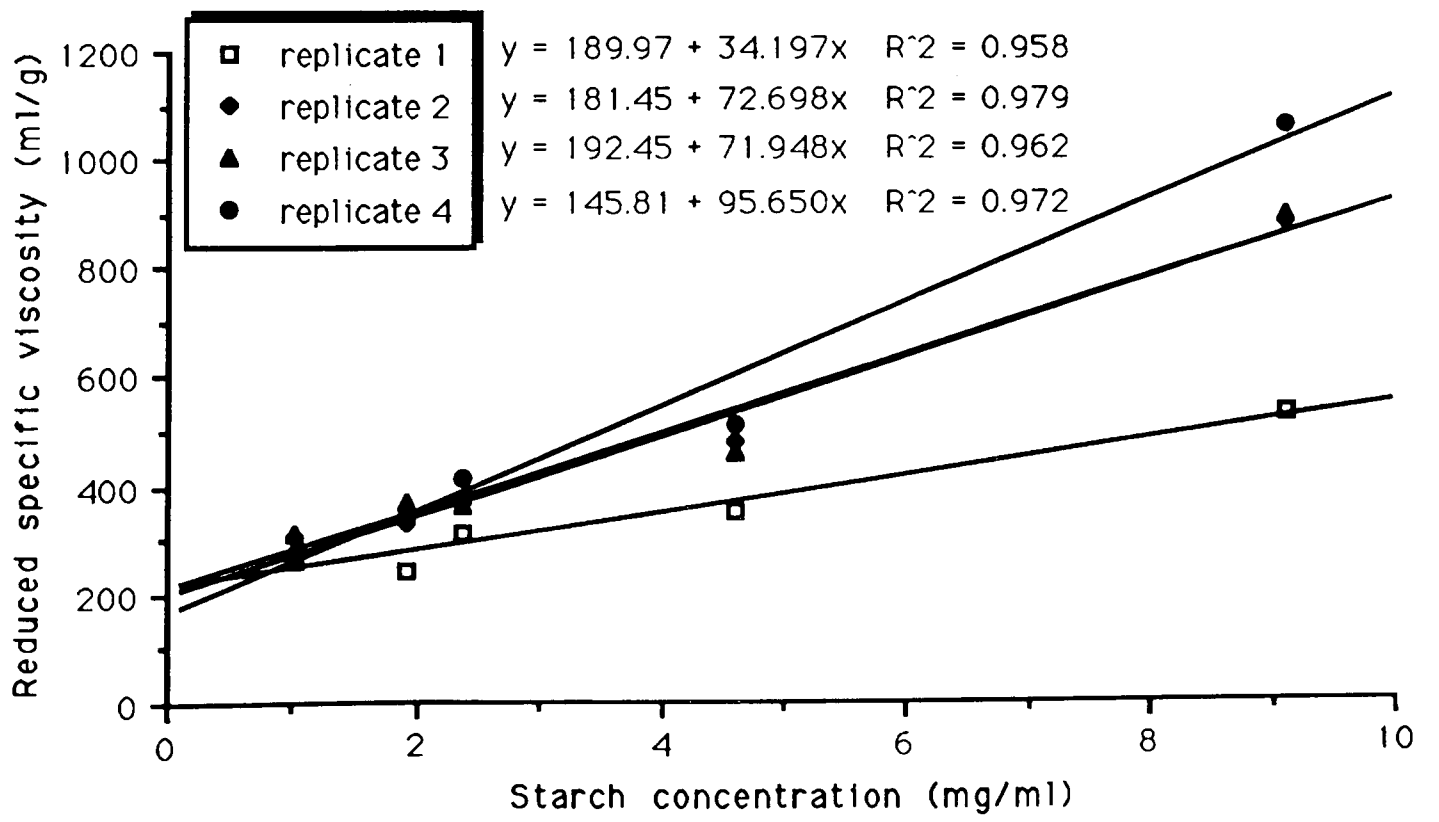


Figure 3.1.14 Reduced specific viscosity for potato starch pasted in 0.1% caseinate

From these replicates a mean value for the intrinsic viscosity of 176 ml/g is obtained. From the two sets of data, both with and without caseinate, there is no significant difference in the intrinsic viscosity values between them. The range of values and the scatter are almost the same suggesting that the three test and four control values are in fact replicates of each other. Since the solubulization of the starch results in the destruction of any granule form and the release of the granular contents into the surrounding matrix any effect on the part of the caseinate will be detected. If the caseinate was acting through an enhanced degradation mechanism then the intrinsic viscosity of the solubulized mixture would be greatly reduced, assuming the caseinate has no direct contribution to viscosity of its own. This is not seen; the slight increase seen is in fact merely due to the experimental variability of the results and essentially indicates no real difference in result. Finally it should be mentioned that it is reasonable to consider that the contribution of the caseinate to the intrinsic viscosity would be expected to be low, e.g. 0.1% caseinate gave a flow time less than 5% higher than 0.5M KOH alone.

As a check the samples were also run on the Bohlin CS rheometer using double gap geometry. Figure 3.1.15 below shows the almost indentical Newtonian flows obtained - at a shear rate of 50 1/s viscosities of 2.98 mPas and 3.29 mPas were found for 1% starch in the absence and presence of 0.1% caseinate respectively. As with the capillary viscosity measurements the starch was solubulised in 0.5M KOH.

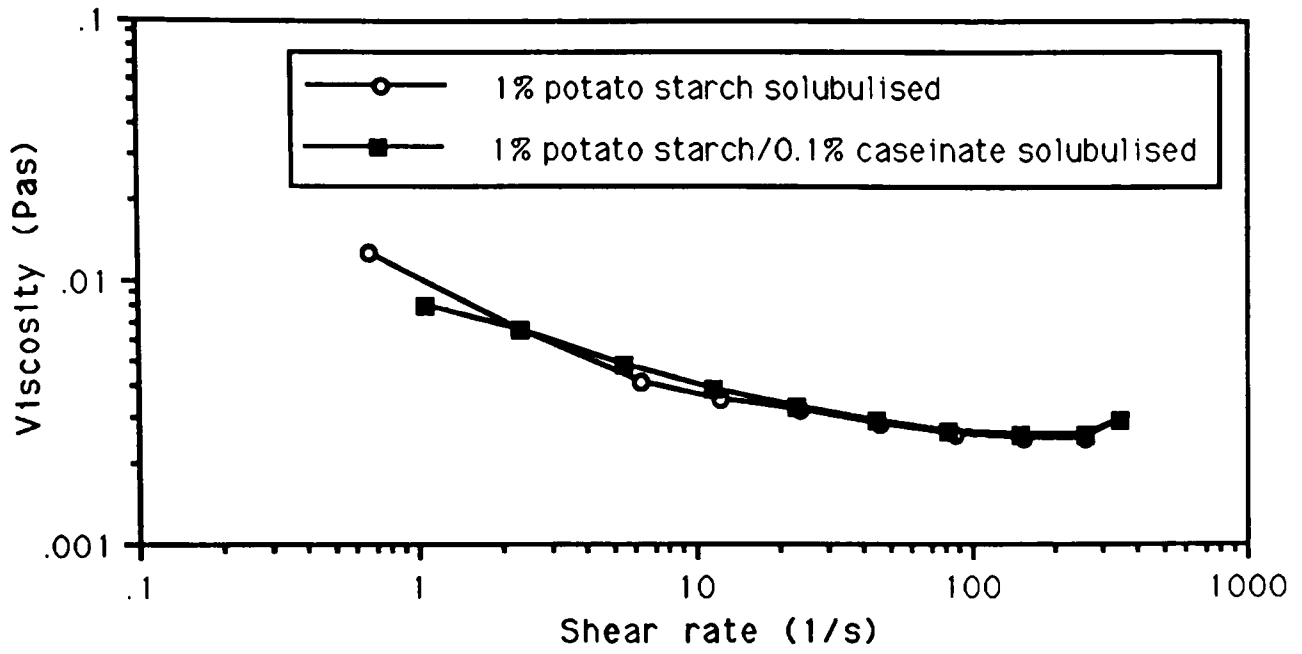


Figure 3.1.15 Flow curves of 0.5M KOH-solubilised potato starch pasted in the presence and absence of 0.1% caseinate

This further confirms the absence of any degradative effect by caseinate on the potato starch granule. Work in the literature (Mat Hashim *et al.*, 1992) indicates that a similar effect on swelling volume is seen for another starch, cassava, in the presence of very low levels of sodium sulphite. These workers postulated that the anti-oxidant sulphite caused the starch granule to undergo degradation, a process these workers termed oxidative reductive depolymerisation (ORD). However from the results of the intrinsic viscosity studies on potato starch presented here it is obvious that such a mechanism cannot be responsible. Therefore the ionic strength effect is the most likely. The interesting question then arises as to why this effect is not seen for other starches (Muhrbeck & Eliasson, 1987; Lelièvre & Husbands, 1989). Why should potato starch be so different?

One of the most significant differences between potato starch and other starches is the granule phosphate content. In the introduction the phosphate content of common starches was outlined indicating that potato starch has by far the highest level (approximately 4 times higher than corn starch). The phosphate monoester groups are covalently bonded at the C3 and C6 position of the amylopectin at a frequency of every 300 glucose units (Swinkels, 1985). This gives a polyelectrolyte character to the granule and consequently contributes to the high degree of swelling following gelatinisation in aqueous solutions.

Commercially it is possible to obtain low phosphate potato starches thus allowing investigation into the influence of the phosphate content. Figure 3.1.16 below

presents data obtained using a potato starch with a phosphate content, quoted by the manufacturer, of ~0.66mg/g (as opposed to the ~1.0mg/g for standard potato starches).

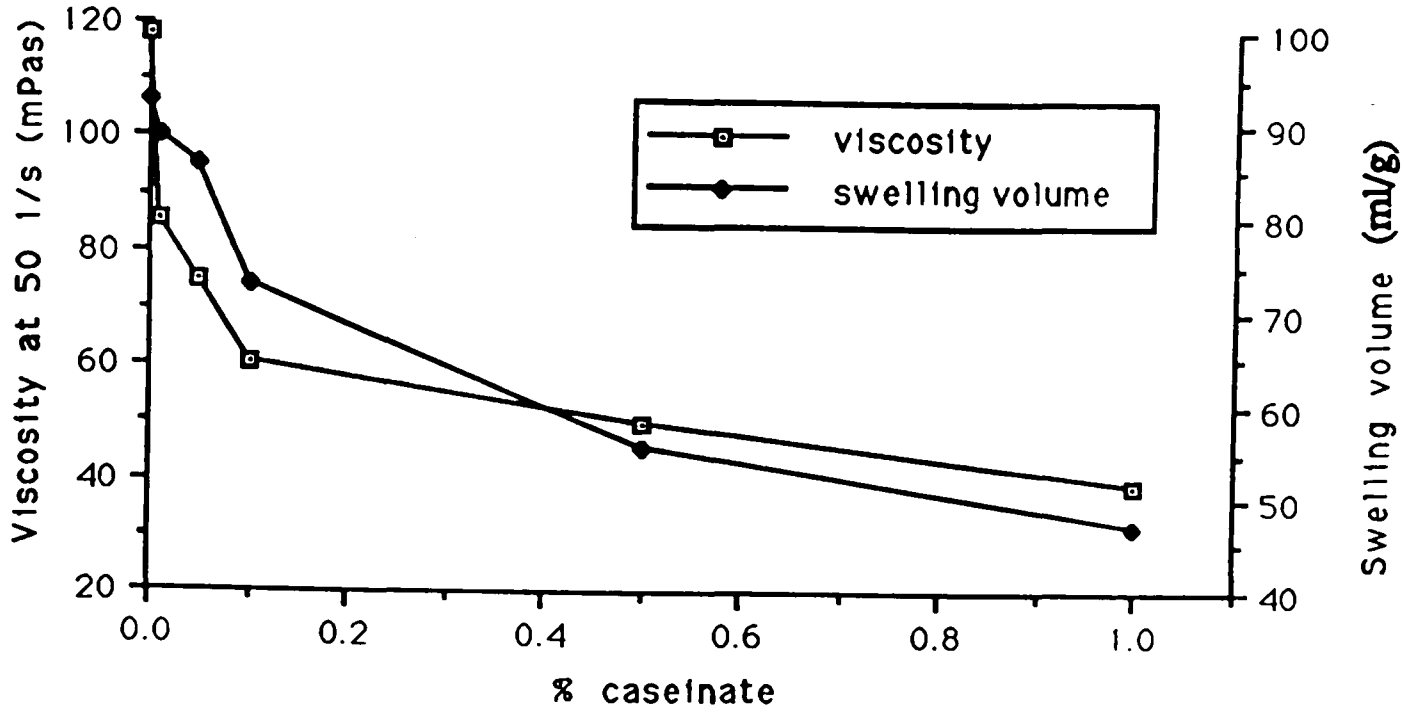


Figure 3.1.16 Effect of caseinate on viscosity and swelling volume of reduced phosphate potato starch

If the comparison is made to the reduction seen with standard potato starch then it is apparent that the phosphate content does affect the viscosity response to sodium caseinate. Replacing the standard starch with the commercially reduced sample lowers the phosphate content by ~25%. The maximum viscosity reduction seen in both the standard and low-phosphate is then different. With the standard starch the viscosity at 1% caseinate is only ~8% of the control viscosity but in the case of the lower phosphate starch the maximum reduction at 1% caseinate accounts for ~38% of the control viscosity. This discrepancy between the two must be due to the differences in phosphate content; presumably the further reduction of the phosphate would further increase the minimum viscosity. Indeed, it would be extremely interesting to see whether a potato starch with a phosphate content reduced to the level of that found in for example, corn starch, would show comparable swelling properties to other starches.

In addition to the two hypotheses stated above it has also been proposed in the literature (Hermannson, 1979) that casein forms a complex with the starch components, and more specifically, amylose. In order to investigate this hypothesis the native potato starch is replaced by one containing no (or very little) amylose. For

this an amylopectin (or "waxy") potato starch was obtained from a commercial supplier and subjected to the same treatment as before. Measurements of viscosity indicate this difference in starch composition is significant (Figure 3.1.17).

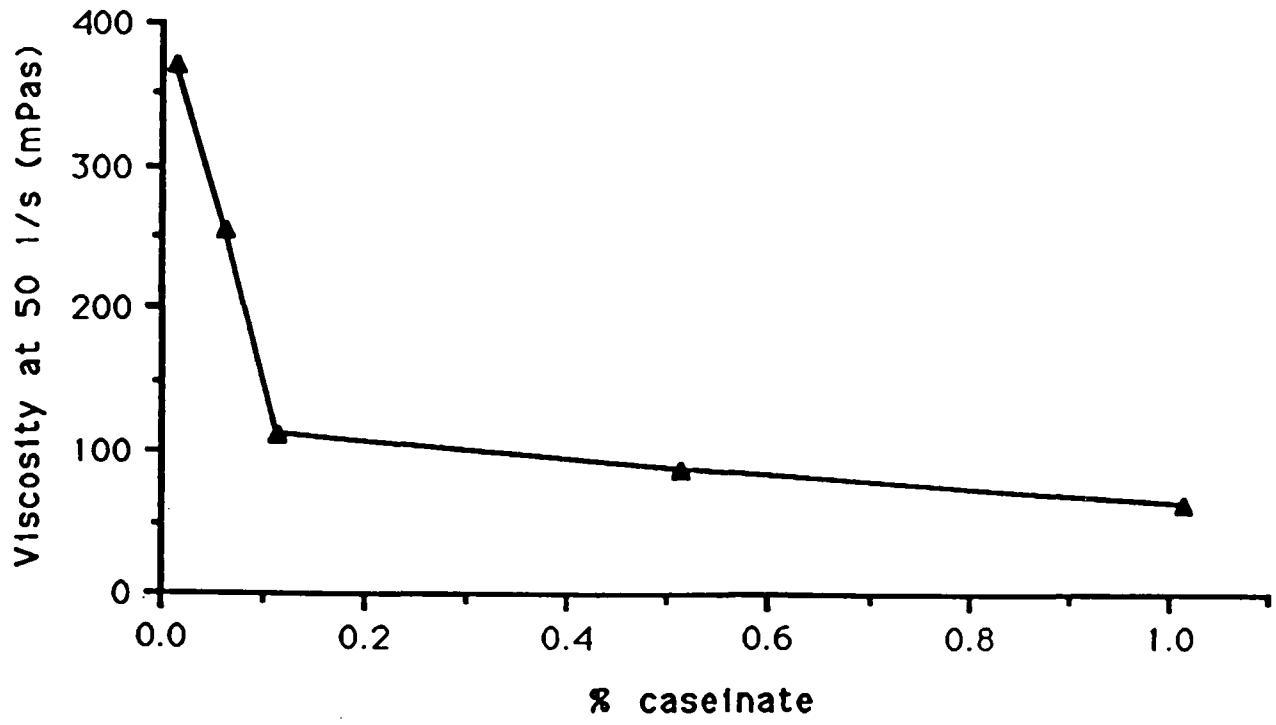


Figure 3.1.17 Effect of caseinate on a 1% amylopectin potato starch paste

The control viscosity set up initially at zero caseinate is approximatly three times greater than in the case of the standard potato starch. In the presence of caseinate a significant viscosity reduction is still observed. The minimum viscosity at 1% caseinate represents ~20% of the control value. At very high viscosities it is diffiucult to obtain swelling volume measurements; the pellet obtained after centrifugation and needed for the swelling volume calculation is not apparent. For this data the only level of caseinate yielding a readable pellet and thus calculation of swelling volume was the 1% caseinate, giving a swelling volume of 44 ml/g.

3.2 Section 2: The Corn Starch-Caseinate System

Whilst the range of experiments the corn starch-caseinate system underwent was not as diverse as for the potato starch system the actual conditions of pasting and subsequent rheological measurement varied more. Unlike the potato starch paste which was always pasted in distilled, deionized water (or as a caseinate solution in such) the corn starch pastes were also prepared in a pH 7.0 phosphate buffer. Whereas the potato starch pastes were always measured fresh at 25°C on the rheometer the corn starch samples were also measured both at 60°C and as aged pastes. Additionally a 48 hour oscillatory measurement was made on a freshly pasted corn starch to highlight the critical time period for retrogradation.

As described previously the viscosity of starch is strongly dependant on the volume fraction. Native potato starch has a swelling volume of ~120ml/100ml whereas the value for maize starch is much reduced. Since any changes in viscosity or swelling volume would be expected to be large in the region of the close packing of the granules (c* potato = ~0.6-1.0%; c* maize = ~3.5%) it is appropriate to make measurements on the higher maize concentration of 4%.

Lastly, a modified corn starch was also used to assess the differences in behaviour between this and the native corn starch. For the modified corn starch, concentrations of 1% were found to be the most suitable. To allow comparison between the different maize varieties both starch types were prepared at both concentrations.

In all cases the flow curves were fitted to the power law equation. This gave a good fit to the data in agreement with a number of previous investigations on starch (Evans & Haisman, 1979). For clarity the results are presented as the viscosity at a shear rate of 50 s^{-1}. The power law parameters are given in Appendix 1 and, as would be expected, give a strong inverse correlation between k and n.

Figure 3.2.1 below shows the effect of caseinate on the viscosity of a 4% native corn starch paste prepared in distilled, deionized water and measured at 25°C.

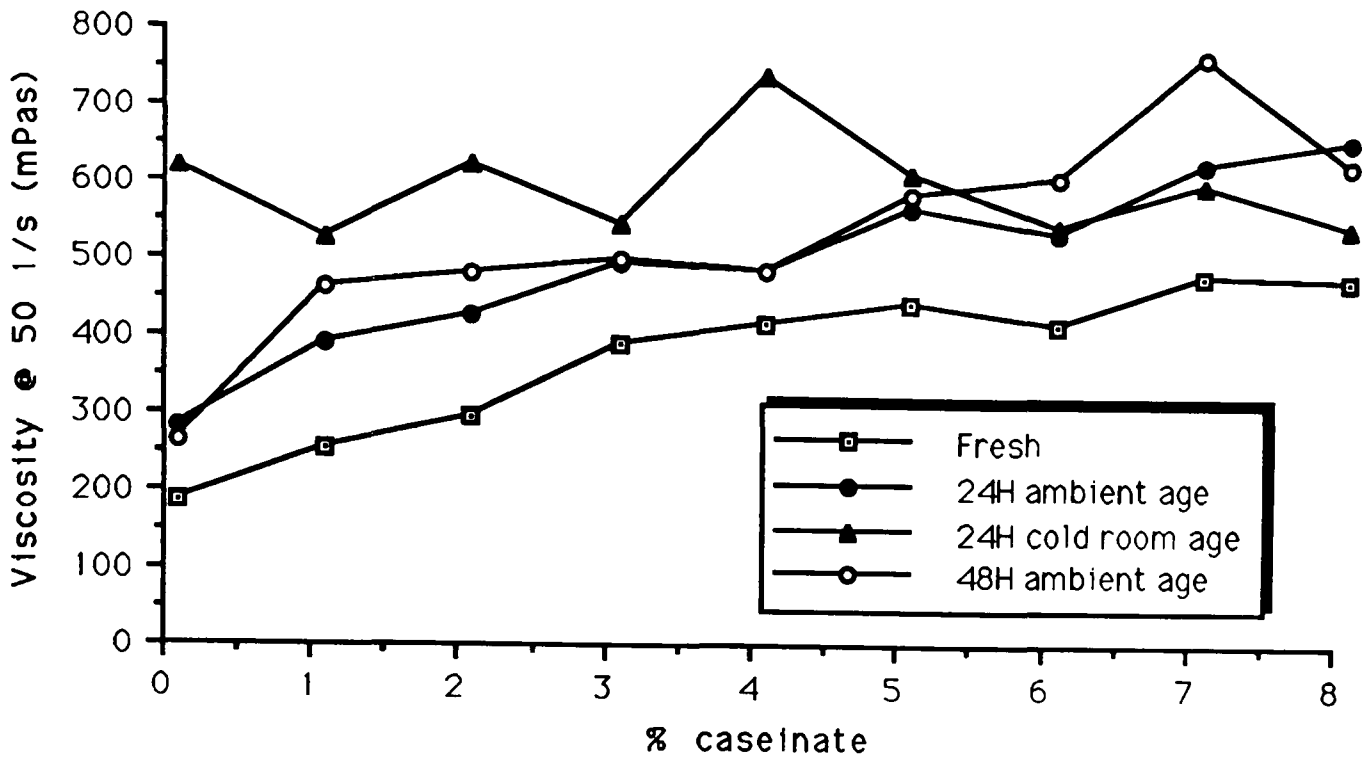


Figure 3.2.1 Effect of caseinate on the viscosity of 4% corn starch pastes prepared in distilled, deionized water and measured at 25°C before and after ageing.

The behaviour of maize starch is very different to that of potato. From Figure 3.2.1 it is apparent that there is an increase in system viscosity as the level of caseinate is increased, when prepared in distilled, deionized water. The control viscosity of the fresh paste, which is similar to that of the 1% potato starch control paste, is increased by a factor of 2-3 at maximum caseinate addition. On ambient storage for 1 day the same trend is seen but shifted to higher values. Interestingly it is also shown here that basically the full extent of retrogradation has been achieved by this time since the 48 hour ambient storage is not much farther removed from the values found at 24 hours. To investigate the effect of storage temperature the samples were stored in a cold room at 4°C for 24 hours. With this treatment much higher viscosity values are seen for the control with a greater degree of data scatter and an overall maintanence of a constant mean value over the whole caseinate concentration range.

When the same system is pasted in pH 7.0 phosphate buffer however a very different picture results. Figure 3.2.2 below shows the viscosity of the fresh treatments to be constant over the caseinate range with only a small degree of scatter. On ageing however under ambient conditions for 1 day a possible enhanced retrogradation is seen at low levels of caseinate, falling to the base level at higher concentrations. Again as with the water-pasted system there is no further retrogradation after 48 hours of storage. Cold storage for 1 day increases the viscosity of the control sample but

produces similar viscositites for the caseinate-containing concentrations as when stored at ambient resulting in an overall reduction in thickening on ageing as caseinate is added to the system.

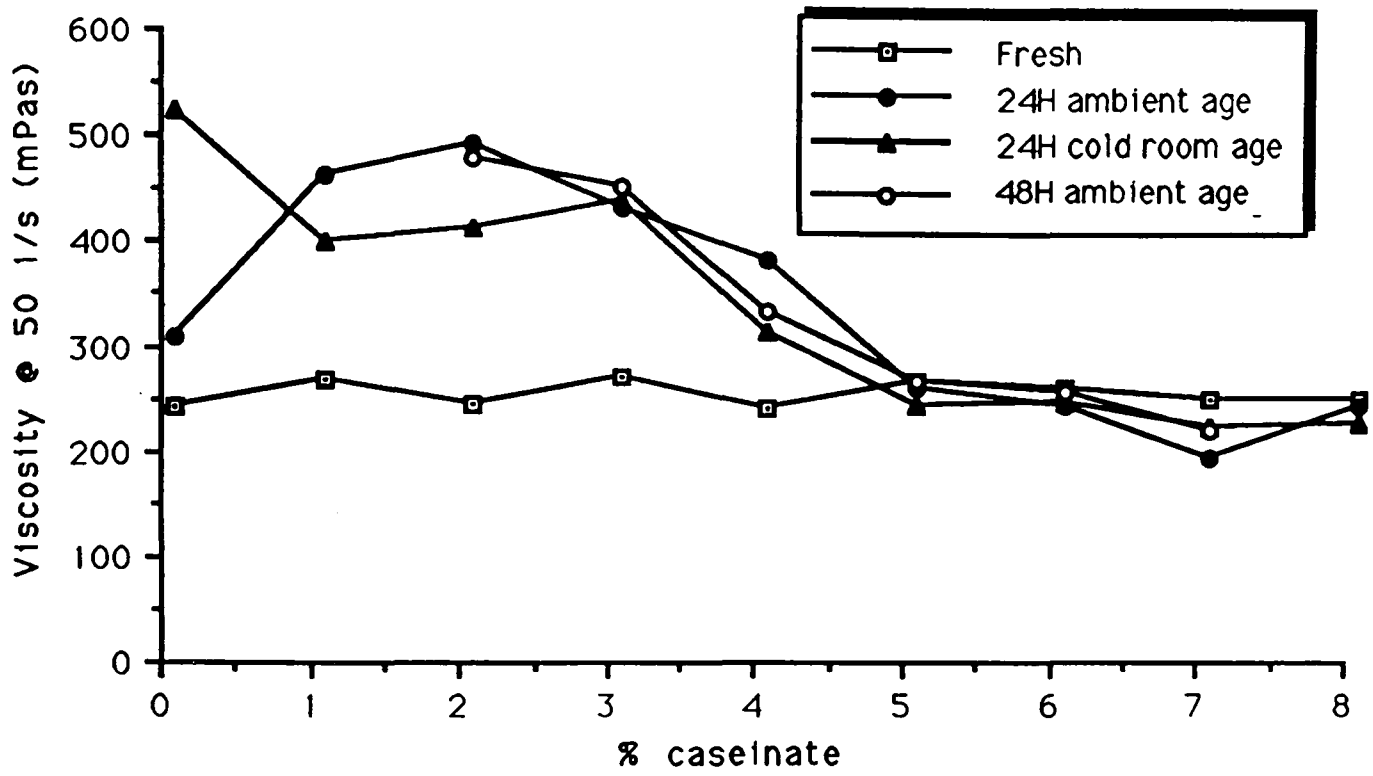


Figure 3.2.2 Effect of caseinate on viscosity of 4% corn starch prepared in pH 7.0 phosphate buffer and measured at 25°C for different ageing times and temperatures.

It is well established that in contrast to potato starch maize starch shows rapid retrogradation. This is indicated by an increase in viscosity on ageing. Retrogradation can be arrested at 60°C and therefore measurements after holding at this temperature were made. It has been reported that the ionic environment affects the gelatinisation of starches and therefore measurements were again made in both water and buffer. Since retrogradation is a factor of time and temperature a series of experiments were performed where the paste was measured at 60°C on the rheometer having been held at this temperature for a period of time. Figure 3.2.3 below show the results of this treatment.

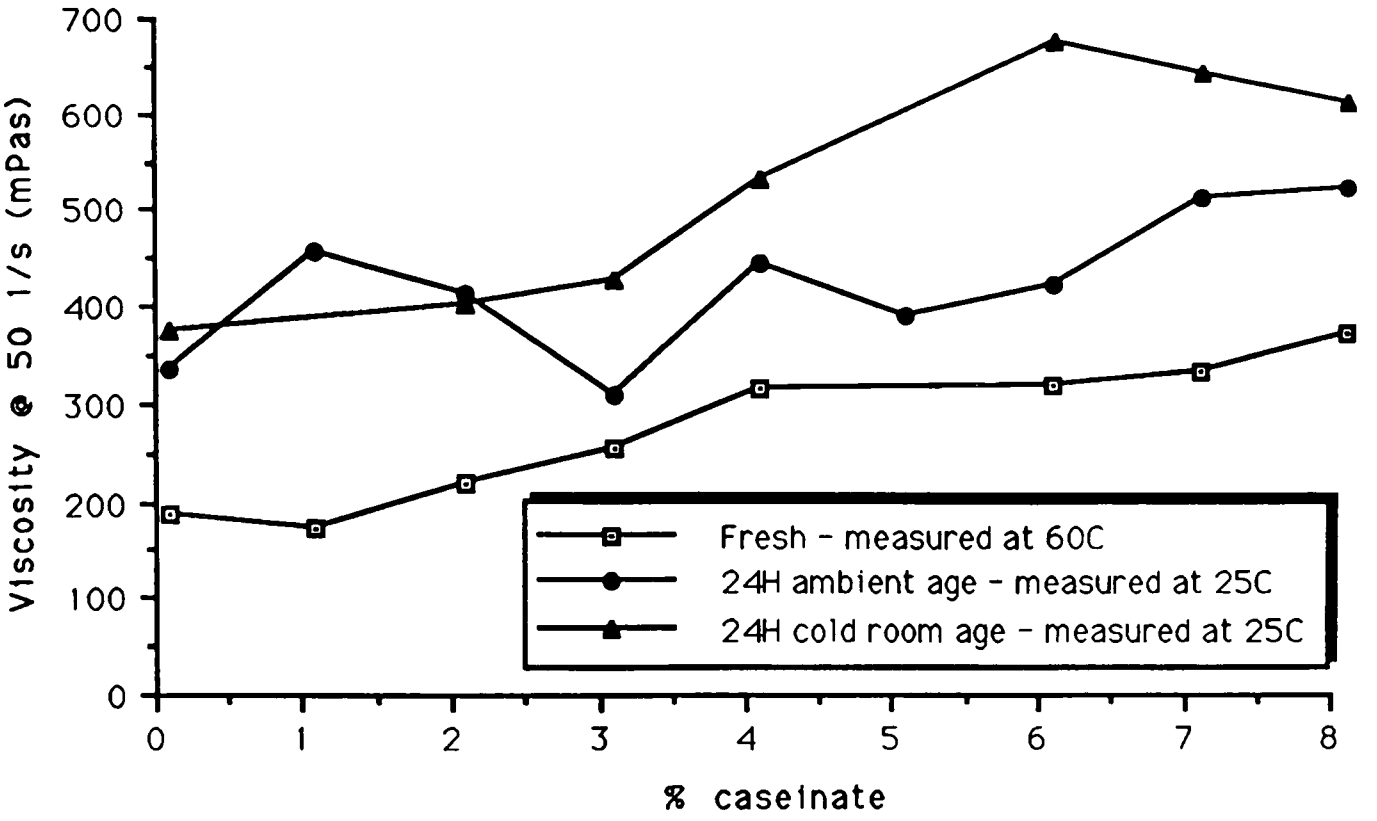


Figure 3.2.3. Effect of caseinate on viscosity of 4% corn starch prepared in distilled, deionized water and held at 60°C after normal pasting then: (a) measured at 60°C on rheometer after thermal equilibrium time, then subjected to (b) 24 h ambient storage or (c) 24 h cold room (4°C) storage with rheological measurements then made at 25°C

The fresh paste shows markedly similar values to that of the equivalent water paste measured at 25°C but the ambient- aged samples deviate slightly more away from the trend and the cold-stored samples showing much higher viscositites as expected in the light of the other data obtained. Repeating this procedure but using buffer produces the data shown in Figure 3.2.4.

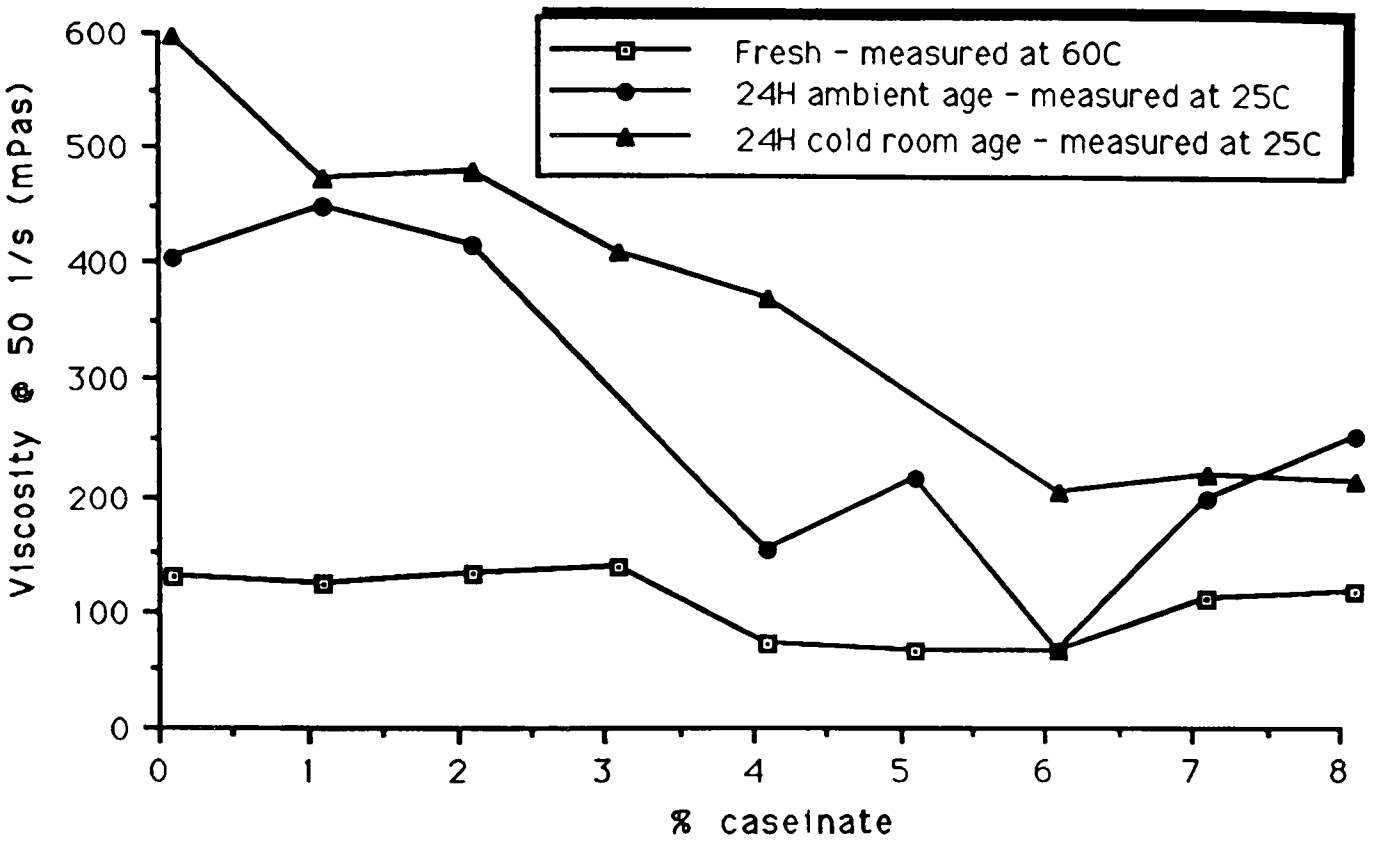


Figure 3.2.4. Effect of caseinate on viscosity of 4% corn starch prepared in pH 7.0 phosphate buffer and held at 60°C after normal pasting then: (a) measured at 60°C on rheometer after thermal equilibrium time, then subjected to (b) 24h ambient storage or (c) 24h cold room (~4°C) storage with rheological measurements then made at 25°C

Again the samples give similar results to the 25°C measured samples, but perhaps undergoing a more pronounced dip at mid-high caseinate concentrations. The peak in viscosity seen at 2% for the 25°C measured ambient aged samples is reduced to somewhat lower concentrations but is less obvious. A sharp fall to 6% is also very apparent with the ambient aged treatment again displaying similarities with the 25°C measured samples. Ageing in a cold room yields a higher control value and causes an overall reduction in viscosity as we move across the caseinate range. Reference to Figure 3.2.2 will once again highlight the similarities between the two temperatures of measurement.

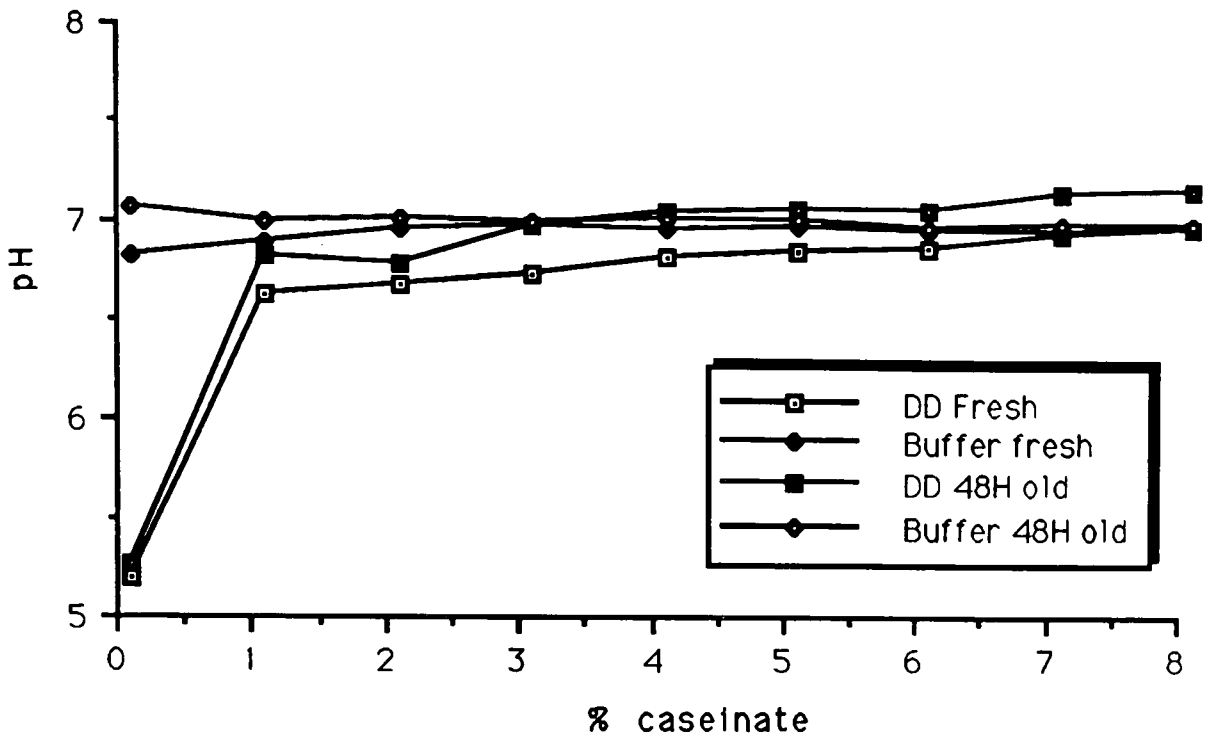
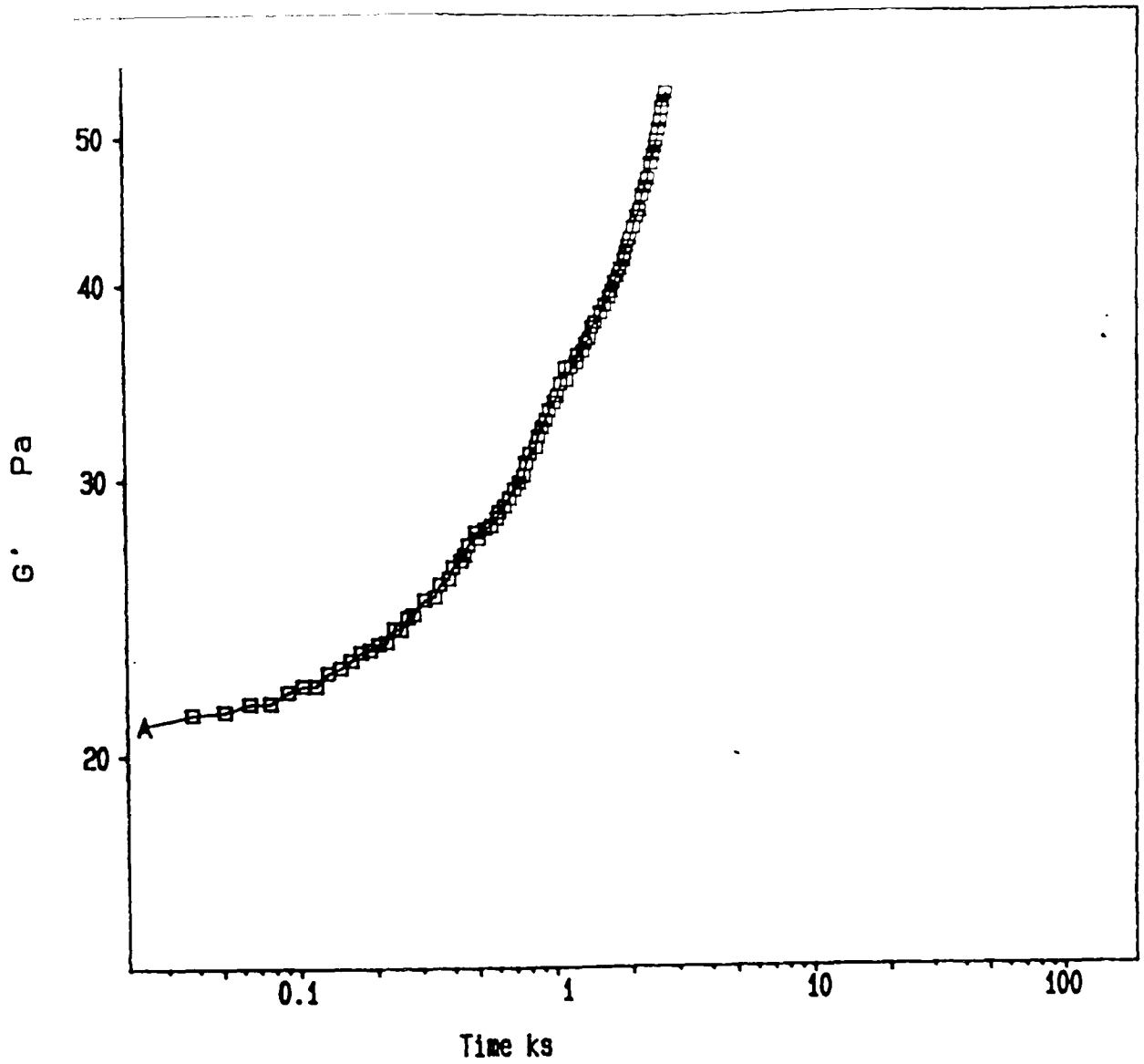


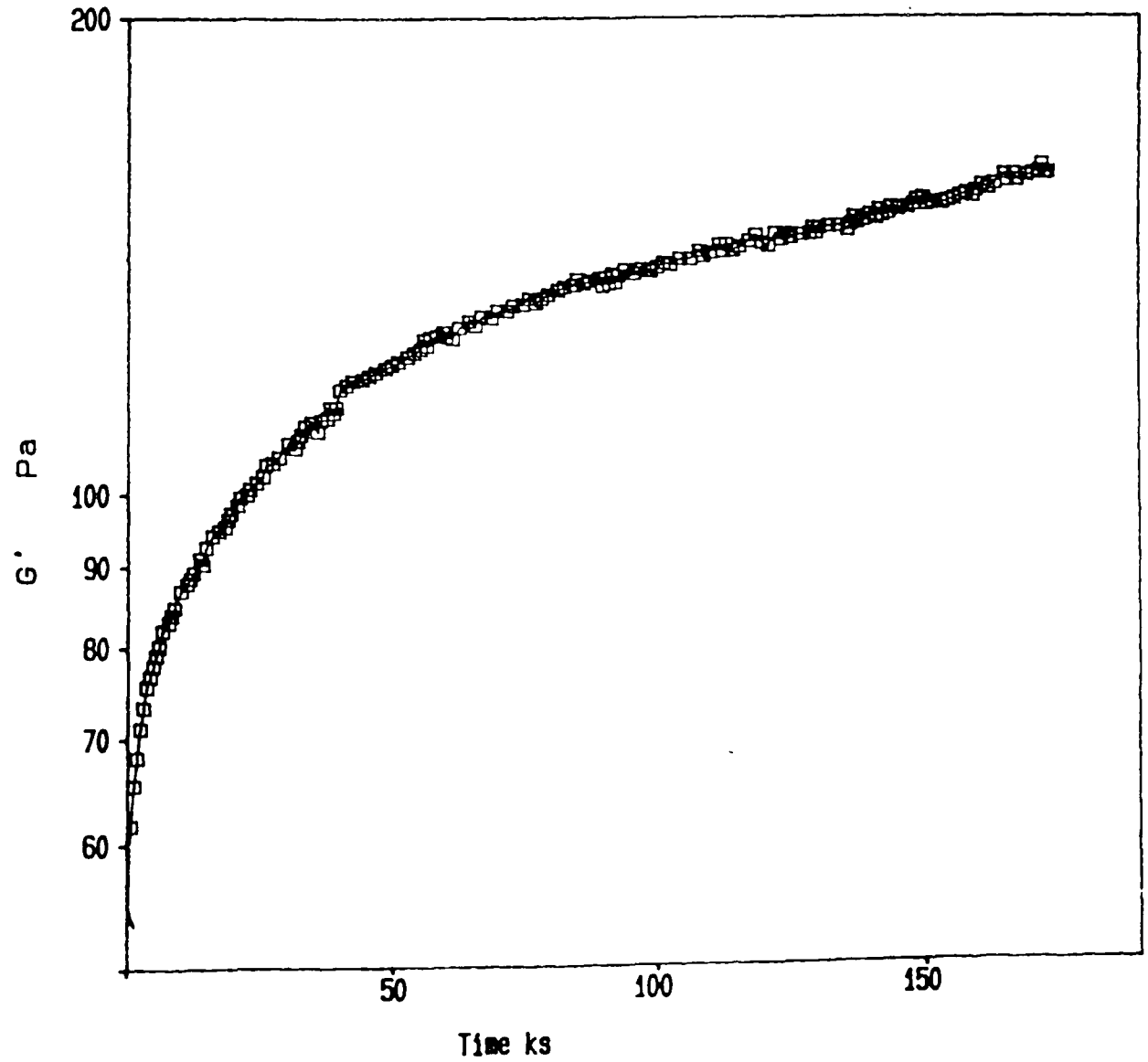
Figure 3.2.5. pH of maize starch systems - effect of solvent and ageing

The pH of all the systems used thus far are shown in Figure 3.2.5. It is apparent that whereas the pH of the buffered systems is, as expected, maintained by the buffer the water pasted systems strive to attain netral pH on the addition of caseinate.

Since it has been suggested from the previous figures that the retrogradation of corn starch is almost achieved after 24 hours an experiment monitoring the dynamic behaviour of corn starch pasted in the normal way in water was performed, in order to establish this retrogradation behaviour. A Job Stream program on the Bohlin CS rheometer was employed to bring about a two stage temperature control - the first causing a controlled cooling of the paste from 70°C to 25°C at a rate of 1°C/minute with the second maintaining temperature at 25°C for a remaining time totalling ~48 hours. Figure 3.2.6a and 3.2.6b show the results of the first and second stage ageing respectively.



(a)



(b)

Figure 3.2.6a. Ageing profile of a 4% corn starch paste in water as given by G' under an oscillation of 1Hz. (a) Temperature reduced from 70°C to 25°C at rate of 1°C/min and then (b) Temperature held constant at 25°C for remaining time

In an attempt to assess if the effects seen thus far could be repeated with a **modified corn starch** pasted in the same manner the following series of results are now presented. Figure 3.2.7 below shows the viscosity of a 1% modified corn starch ("Colflo 67" supplied by National Starch) pasted in both water and buffer. For this figure and all subsequent figures involving modified maize starch of this section ageing is performed at ambient temperatures and all measurements made at 25°C.

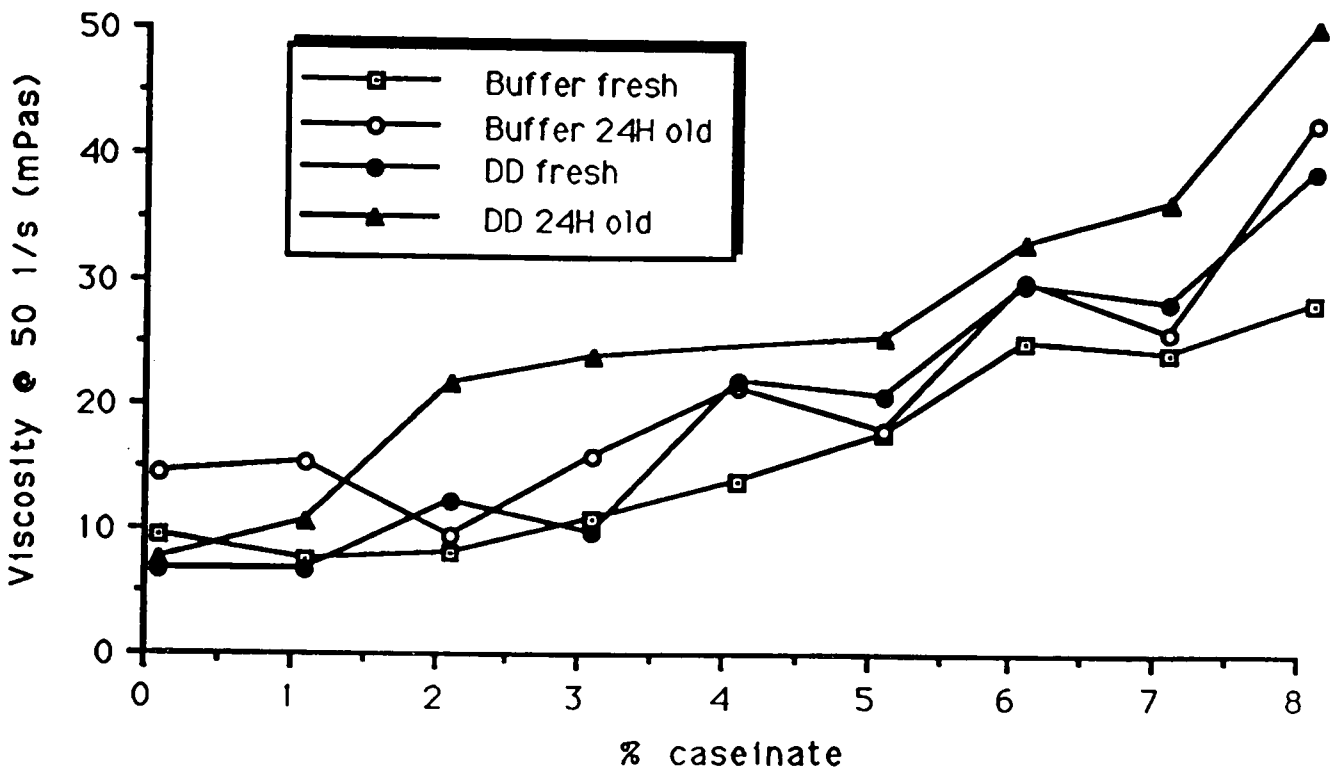


Figure 3.2.7 Effect of caseinate on 1% modified corn starch prepared in buffer and distilled, deionized water as fresh and aged samples.

From this Figure it is apparent that all systems, in both solvents and as both fresh and aged samples, give similar results. However when pasted at 4% to provide direct comparison with the native corn starch data it can be seen from Figure 3.2.8 (whereby the 1% data points are also on the graph to illustrate the absolute and relative differences between the concentrations) that the different behaviour between the solvents is once again seen, if not as convincingly, i.e. pasted in water an increase occurs on adding caseinate but pasted in buffer causes an reduction in viscosity, (ignoring here the last - possibly sporadic - data point).

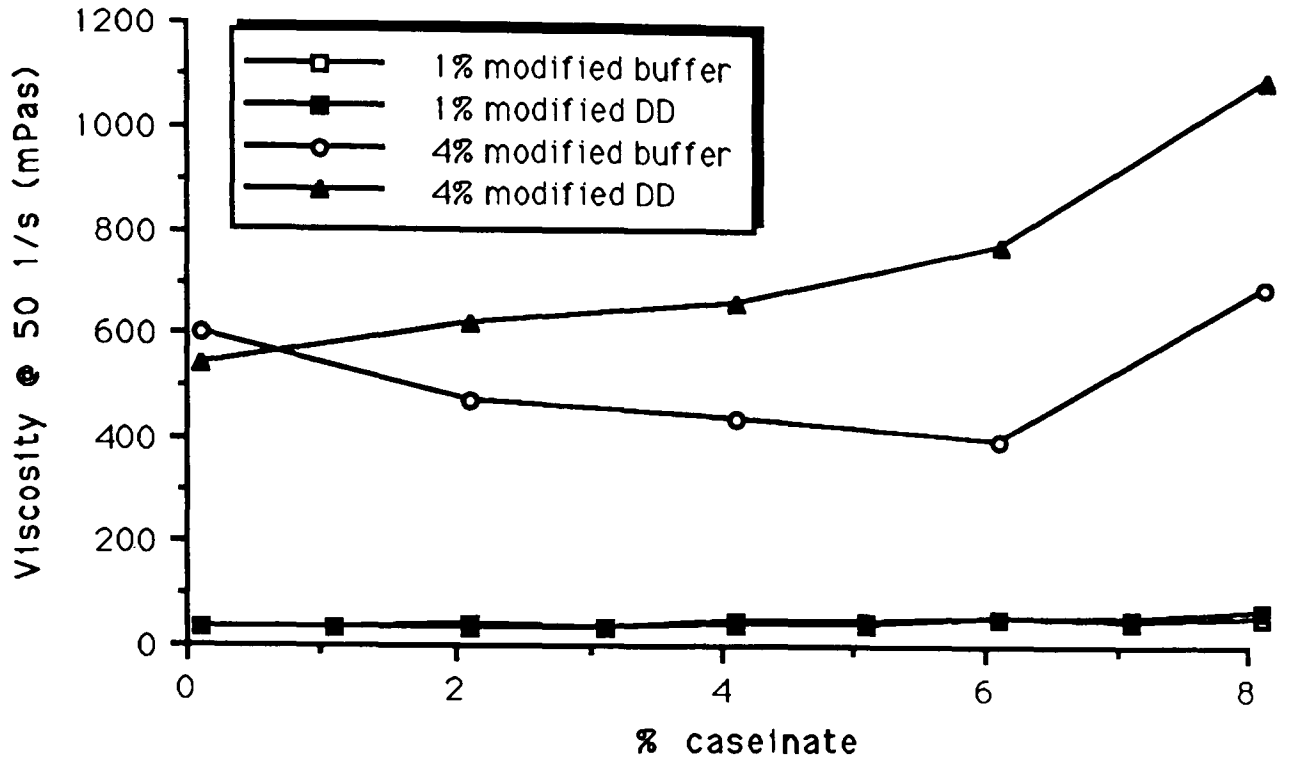


Figure 3.2.8 Effect of caseinate on viscosity of a fresh 4% modifed corn starch paste prepared in buffer and distilled, deionized water with comparison to that data previously presented at 1%.

As part of this comparison Figure 3.2.9 below shows the viscosities of 1% standard corn starch pasted in both solvents and as fresh and aged samples. The startling feature of this plot is the distinctly different behaviour shown by the aged buffer sample to the other samples. This different behaviour was shown before with the 4% standard corn starch samples on ageing in buffer.

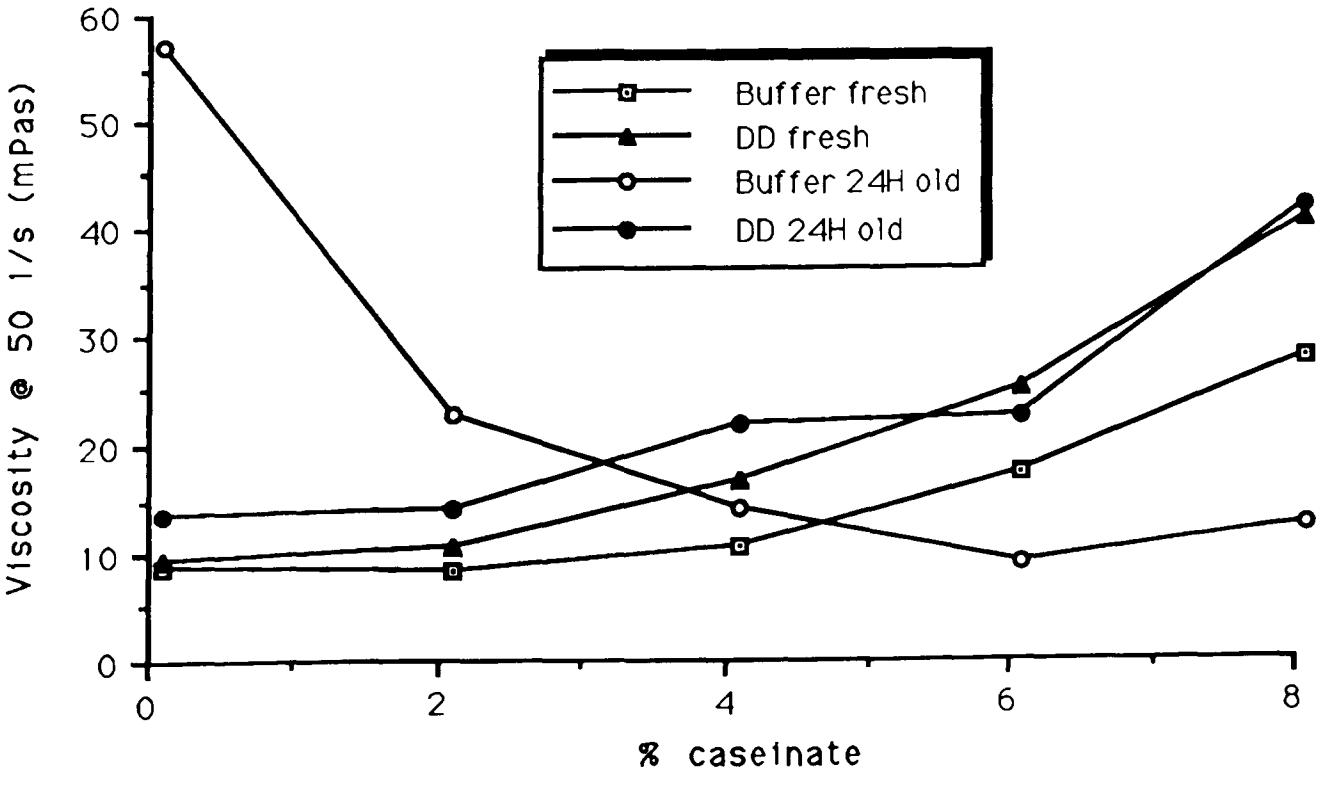


Figure 3.2.9 Effect of caseinate on the viscosity of 1% standard corn starch pastes in buffer and distilled, deionized water as fresh and aged samples

Measurement of the swelling volumes for these systems are given by the following figures. The 1% modified corn starch as fresh samples are shown in Figure 3.2.10. The type of solvent obviously greatly effects swelling behaviour; the control values being very different and the subsequent response to caseinate addition following opposite trends.

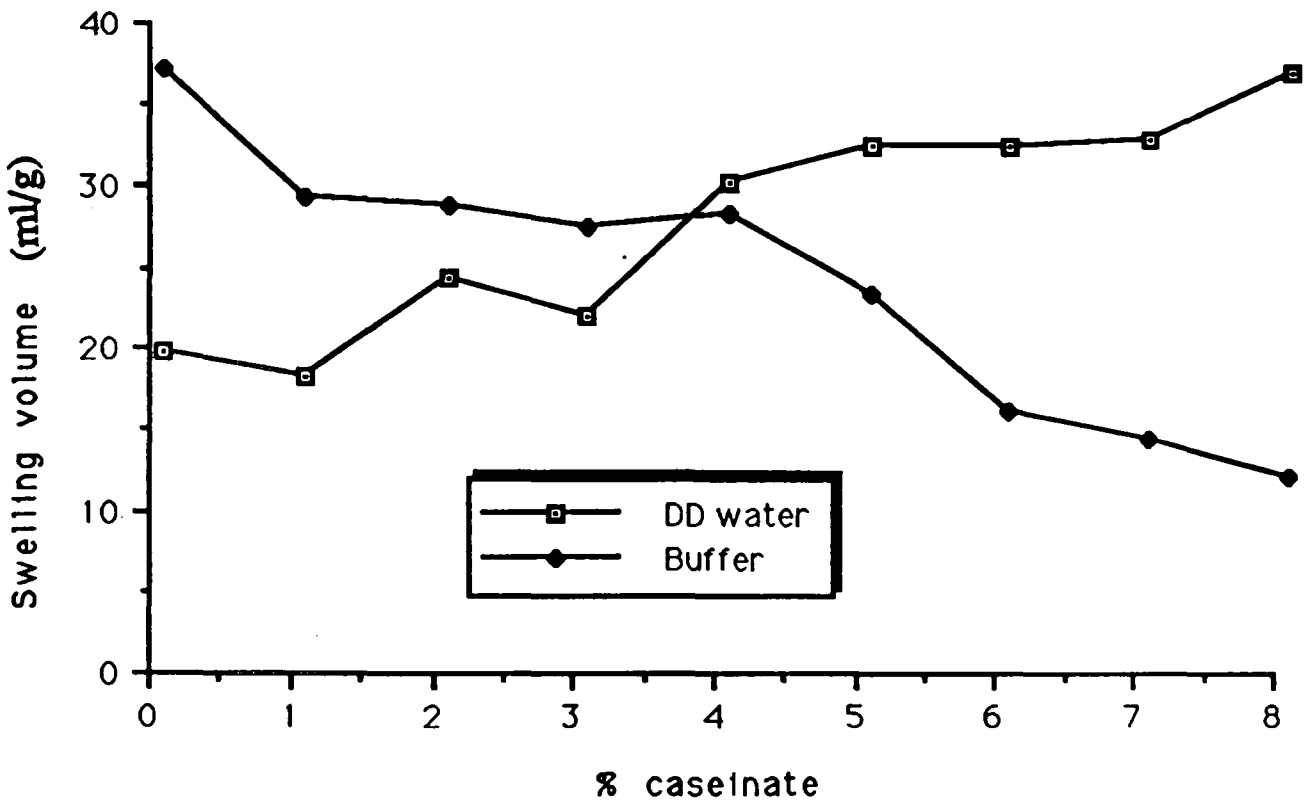


Figure 3.2.10 Effect of caseinate on the swelling volume of 1% modified corn starch prepared in buffer and distilled, deionized water

By comparison to the 4% modified samples it is seen (Figure 3.2.11) that the same trends are still apparent but significance decreases as the starch concentration increases, i.e. the differences between absolute values are minimized at higher starch concentrations.

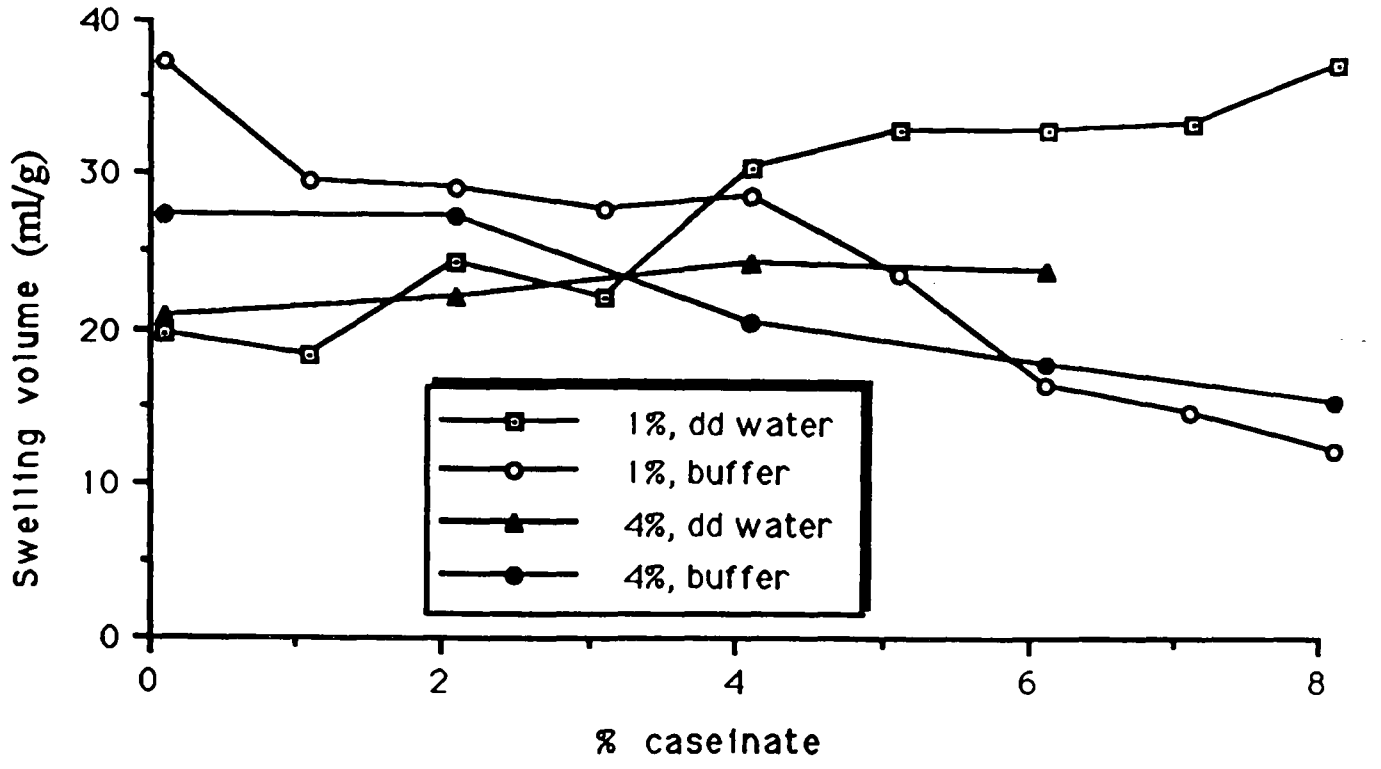


Figure 3.2.11 Comparison between the effect of caseinate on swelling volume of 1% and 4% modified corn starch paste; all samples fresh and measured at 25°C

By now comparing the behaviour of the standard and modified corn starches at the same concentration in both solvents (Figure 3.2.12) it is realized that the 1% standard corn starch behaviour closely mimics that of the 4% modified corn starch shown in Figure 3.2.11 above, as differences across the caseinate range are minimized at this concentration of starch.

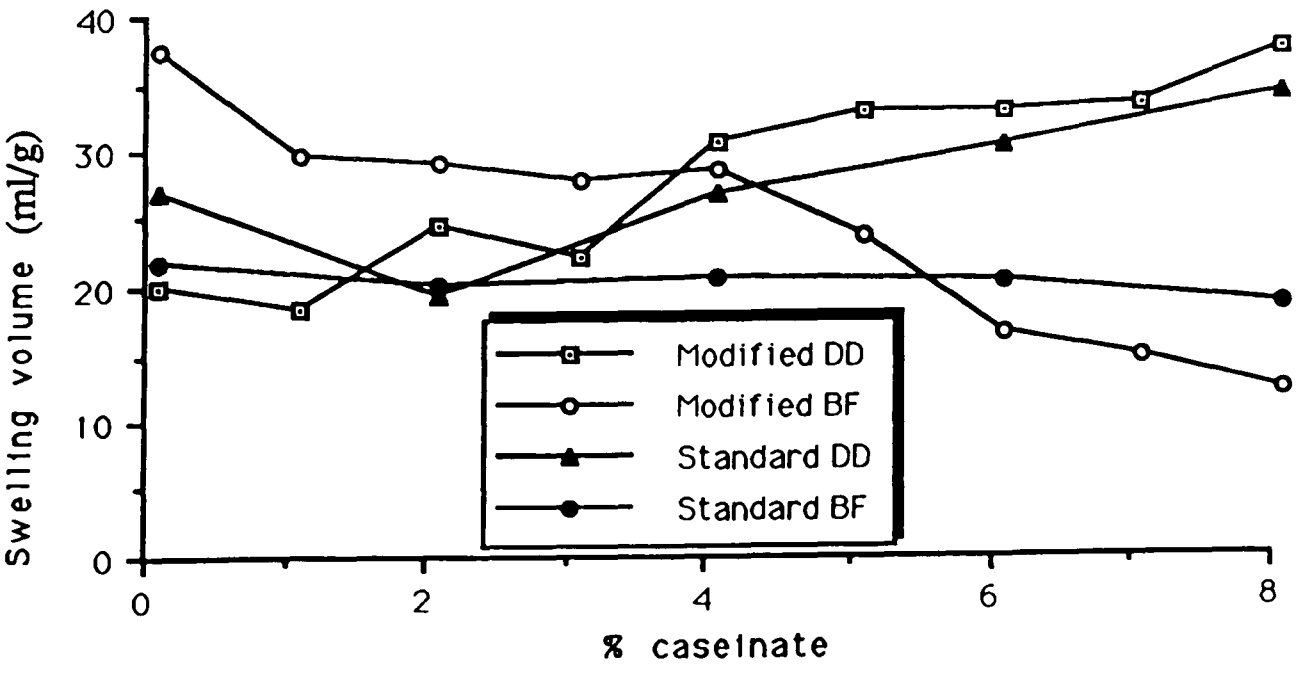


Figure 3.2.12 Comparison between the effects of caseinate on 1% modified and 1% standard corn starches prepared in buffer and distilled, deionized water. All samples fresh and measured at 25°C

3.3 Section 3: The Wheat Starch/Flours-Caseinate System

This short section gives the results of the investigations into the effect of caseinate on the viscosity and swelling volume of a range of commercial wheat flours. From the interesting results seen with the starch-caseinate systems up to this point industrial interest was stimulated in applying such effects to real food applications. In addition to the fundamental studies, using the same techniques as for sections 1 and 2, which I carried out at Nottingham, on-site work was also performed by Pedigree Petfoods of Melton Mowbray, U.K on real systems.

The initial hope from an industrial point of view was that damage to the starch present in the gravy of a petfood gel system could retain the starch polysaccharides within the granule. If the caseinate could retain starch polysaccharides within the granule in certain cases, could it do the same to the starch found in wheat flour, added to a petfood product, and thus reduce the damage on autoclaving? The results of the studies I performed are shown below. Figure 3.3.1 shows the viscosity as a function of caseinate concentration for the whole range of flours at 5% and wheat starch at 4% as previously employed for the corn starch studies.

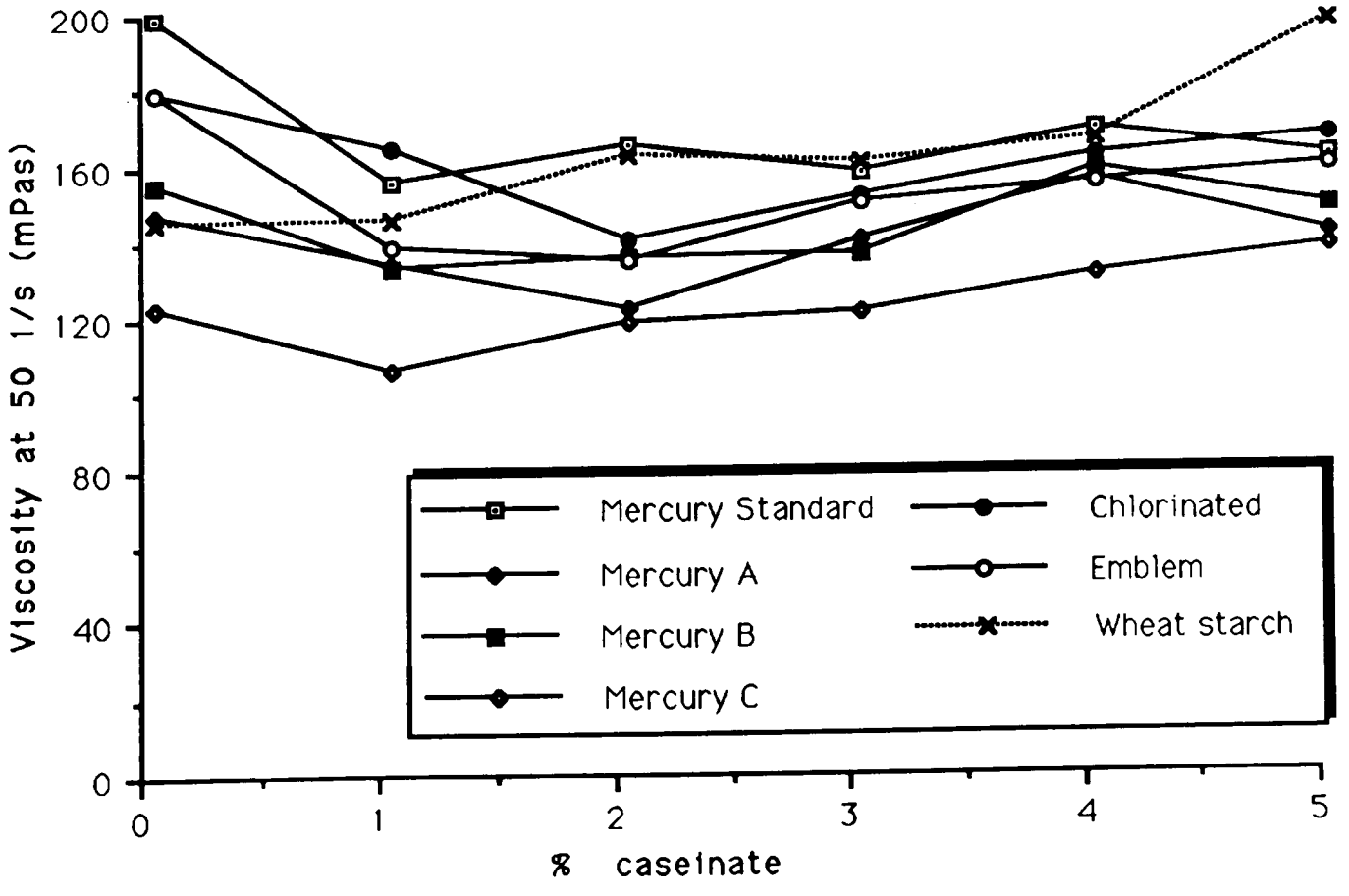


Figure 3.3.1 Effect of caseinate on the viscosity of 4% wheat starch and 5% wheat flours pasted in distilled, deionized water.

It is shown from Figure 3.3.1 that in most cases the viscosity of the flour systems at 5% caseinate deviates little from the control (zero caseinate) value. In 5 out of 6 of the flours the viscosity in the presence of maximum caseinate is slightly reduced. In the case of the 4% wheat starch the viscosity increases on caseinate addition to give a value at 5% caseinate 50% higher than the control. This is similar to the effect observed with native maize starch in water, except the magnitudes of increase are much reduced. The effect on the swelling volume is shown in Figure 3.3.2.

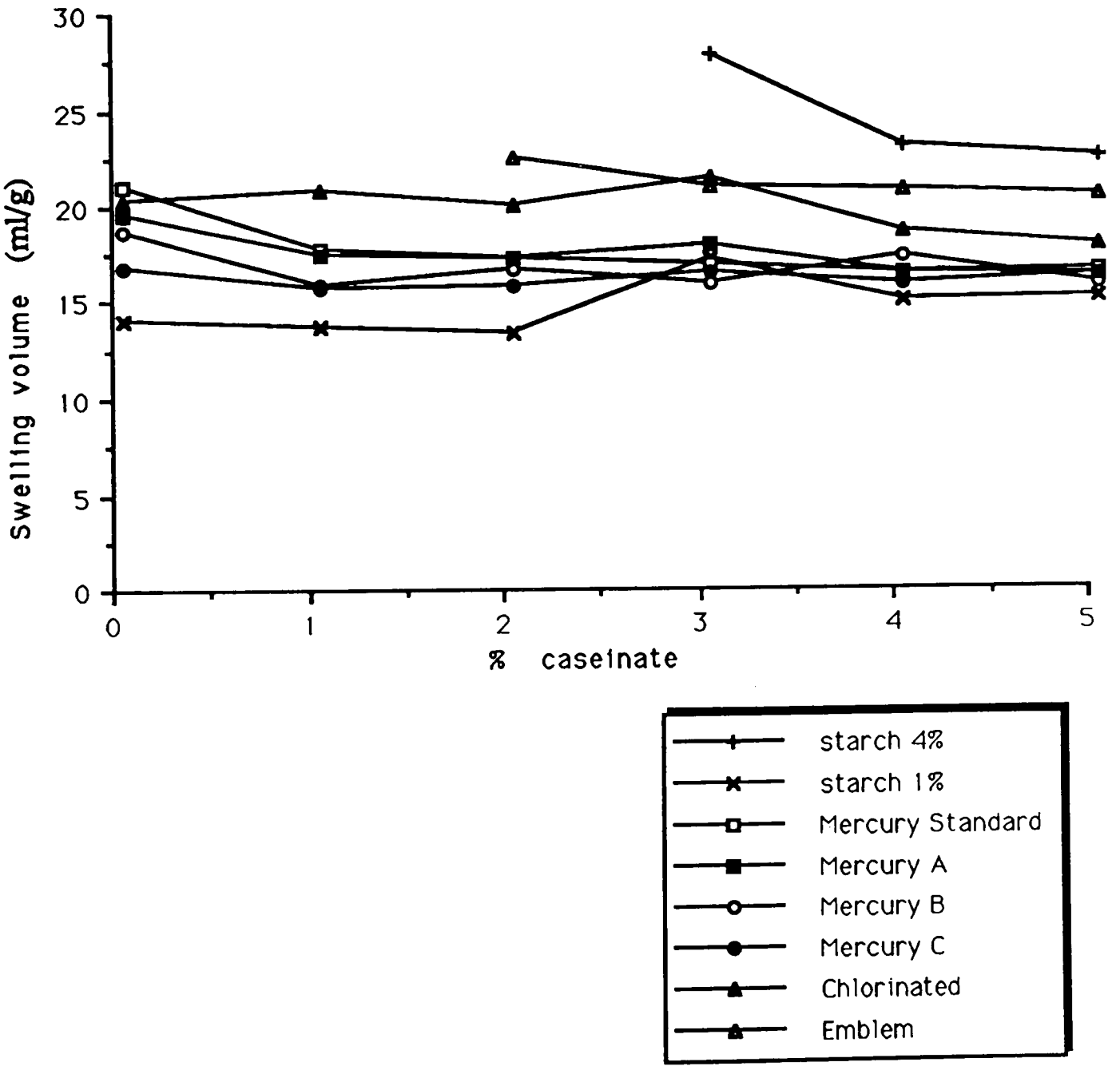


Figure 3.3.2 Effect of caseinate on the swelling volume of wheat starch (pasted at 1% and 4%) and wheat flours (pasted at 5%) in distilled, deionized water

From this Figure it is evident that little effect is seen on swelling volume of the wheat flours on caseinate addition. This would then agree with the lack of any response on viscosity seen in Figure 3.3.1. Overall the swelling volume of the 1% wheat starch paste is also unaffected, perhaps showing a slight tendency to increase. However, at 4% wheat starch the swelling volume is apparently reduced; levels of 3% caseinate

are required before a granular pellet can be measured. This response is most similar to that seen with 1% modified maize starch in buffer which also shows a reduction in swelling volume whilst giving a ~50% increase in viscosity at 5% caseinate. In the latter case it was suggested that the caseinate kept the starch polysaccharides in the granular phase and thus reduced the degree of swelling whilst the viscosity of the caseinate contributed to the overall system viscosity. However for this to apply it was also a requisite that the buffer overwhelmed the otherwise entropically unfavourable tendency to phase seperate. Since the solvent used in the wheat starch preparations was distilled, deionized water it is unlikely this mechanism would apply. Since the flours contain starch at approximately the same level (~80% starch content) one would perhaps expect the same effect to be seen (overlooking the effect of the other flour components at this stage), which is indeed not. It may be that ideas applicable to maize starch cannot be directly translated to wheat starch. Microscopy of both the wheat and maize starch systems in water and caseinate at this concentration was inconclusive and the micrographs are shown in Appendices A1 and A2.

The work of Pedigree Petfoods (as reported by Pedigree Petfoods) is shown in Appendix 3 as a brief report. Chunk strength was reported to decrease on caseinate addition and this be correlated to the small reduction in viscosity I found for most flours, even though the flour concentrations used are different. Overall however it was concluded from this investigation that insignificant effects were observed and no further product application pursued with these levels and conditions.

3.4 Discussion of Chapter 3

Sodium caseinate exhibits an effect on starch, the nature and extent of this effect is dependant on the type of starch and, within starch types, dependant on the solvent used. As a consequence the underlying mechanisms are different for different starch types.

Potato starch undergoes a massive viscosity and swelling volume loss when pasted in even very low levels of caseinate. Neither parameter recovers to original values with higher levels of caseinate addition. The effect on the swelling volume can also be observed microscopically. It is now believed that the action of caseinate on potato starch is brought about through an non-specific, ionic strength effect. The evidence for this is as follows:

(a) Additions of low levels of sodium chloride to potato starch also bring about a similar dramatic loss in viscosity.

(b) Dialysing the sodium caseinate before its use in the pasting step produces a paste with rheological properties intermediate between that of the potato starch alone and that of the mixture with undialysed caseinate. The degree of loss of ions on dialysis is reflected in the solution conductivity.

(c) Using a potato starch with a ~25% reduction in phosphate content reduces the extent of viscosity and swelling volume loss. The viscosity of the standard potato starch pasted in 1% caseinate is ~8% that of the control value; for the reduced phosphate this corresponding viscosity represents ~33% of the control value. Although the loss of paste viscosity on caseinate addition is not prevented totally it is reduced. Using a starch with an even lower level of phosphate would perhaps prevent viscosity loss to a greater extent.

Such a significant effect on paste properties on electrolyte addition was not observed for other starches, for example it was not seen for maize starch in the work of this thesis or in the studies on cassava starch by Muhrbeck and Eliasson (1987). Paterson *et al.* (1994) also showed little effect of NaCl on wheat, rice, maize, cassava and sago starches but significant swelling volume and viscosity reductions with potato starch. Doublier *et al.* (1994) have also studied the effect of caseinate addition on the behaviour of wheat, potato and tapioca starches. In contrast they report a large decrease in the volume fraction occupied in the paste by the swollen starch granules and in the concentration of starch macromolecules solubilised in the continuous phase. Surprisingly only in the case of tapioca starch did this reduction in swelling

volume produce a decrease in apparent viscosity; in the other two cases an increase in the swollen volume was reported.

The reason for the unique behaviour of potato starch is almost certainly the polyelectrolyte character of potato amylopectin due to the significant proportion of esterified phosphate groups on the amylopectin chain. As a result the granule expands into a more extended conformation as a result of electrostatic repulsion between like charges. Any increases in ionic strength of the solvent medium will repress the polyelectrolytic effect (Yang, 1961). This can be increased to the point where the polymer acts as if were uncharged. Another way of understanding this phenomenom is in terms of the Donnan effect (Donnan, 1911). With such highly charged biopolymers it can be shown (Tanford, 1961) that at the equilibrium such as arises here small, mobile ions are unequally distributed between a polymer phase and the surrounding solution. Such an uneven distribution of co- and counter-ions will cause an osmotic pressure difference between the polymer phase and the surrounding solution causing the former to swell. At low ionic strengths the osmotic pressure will be large. Indeed for low ionic strengths and/or small macromolecules of high charge density the osmotic pressure can be dominated by the Donnan effect (Dickinson & Stainsby, 1982). Reducing the number of charges on the molecule, as in the case of the low phosphate potato starch, will reduce the polyelectrolyte character of the starch. It was known as long ago as 1938 (Katz *et al*, 1938) that the gelatinisation temperature of potato starch is dependant on added salts. From their studies they concluded that the swelling could be enhance or retarded, that is the gelatinisation lowered or raised, depending on the type of anion used. This anion specific behaviour is probably due to solvent effects and is different from the phenomena described here.

In addition to establishing the effect of the caseinate on potato starch it is also shown that the caseinate exerts a marginally stronger effect on the viscosity of the potato starch when hydrated before or at the same time as the starch. However additions of caseinate to the starch in a dry form and at very advanced stages of pasting still produce almost the same massive loss in viscosity. Adding the caseinate in both hydrated and dry powder forms to the gelatinised potato starch at different points during the pasting procedure still yields a massive viscosity loss. This value is only marginally higher than that of the standard procedure. Dry mixing the powders, i.e. allowing for competitive hydration, produces a value identical to that of the standard procedure. It therefore appears that the already swollen granule can be deswelled by caseinate addition. It would be interesting to see if this process is completely reversible by inducing swelling by dialysing out of ions from a gelatinised starch system.

A number of other interaction mechanisms, which have been shown to apply to other starches and reactants in the literature, can be ruled out for this study on the basis of experimental evidence obtained from this thesis. Any possibility of the underlying mechanism stemming from an oxidative reductive depolymerisation (ORD) is eliminated by the fact that solubulising the potato starch granule after pasting in the presence and absence of caseinate yields no difference in the intrinsic viscosities. The steady shear viscosities of the solubulised samples are also not significantly different. If the caseinate was indeed acting by degrading the polysaccharides from within the starch granule - such as the action of sulphite on starch (Mat Hashim *et al.*, 1992) for example - then the intrinsic viscosity of the solubulised sample would be markedly lowered in the presence of caseinate. This is not observed for this study; the two values are basically identical within the limits of experimental error.

Other mechanisms postulated in the literature include the amylose-casein interaction (Hermansson 1979). This has again been ruled out by the use of a "waxy" (amylopectin) potato starch in place of the standard starch. A significant viscosity loss is again observed with this starch in the presence of caseinate. At 1% caseinate the viscosity is only 20% that of the control paste.

The mechanism described for potato starch is not applicable to maize starch, either the standard or modified varieties. Maize starch does not contain any significant phosphate (Swinkels, 1985) and thus factors such as Donnan effects are not important here. Standard and modified forms showed little difference in responses to caseinate and these will be discussed below separately. It was apparent that the use of different solvents gave different reponses. The main features of this study are that:

(a) The **4% standard** maize starch in **distilled water** undergoes an enhanced viscosity on increasing levels of caseinate which is paralleled by an increase in swelling volume. Retrogradation is unaffected by caseinate addition and simply follows the fresh curve. On ambient ageing for 1 day normal retrogradation is apparent as the same trend is merely shifted to higher viscosities. It is evident that maximum retrogradation has been reached by this time as the lack of any further viscosity increase at 48h indicates. Ageing at colder temperatures produces more paste thickening highlighting the time-temperature relationship of retrogradation.

(b) The **standard maize** starch in **buffer** does not undergo an enhancement of paste viscosity on caseinate addition with no change in swelling volume. At high caseinate

levels it would appear that retrogradation is inhibited since there is no viscosity increase on ageing.

(c) Reducing the concentration of the **standard maize starch to 1%** clearly emphasises the difference found between the solvents on ageing the starch preparations. A 1% standard starch in the absence of caseinate retrogrades to a much greater extent in buffer but undergoes the same retrogradation protection on increasing amounts of caseinate.

(d) When an attempt is made to prevent much of the retrogradation of the **standard 4% maize starch** by **maintaining elevated temperatures (60°C)** on cooling the (i) unaged samples in water show little differences except that lower viscosity values are found for the 60°C sample at high caseinate levels. On cold-room ageing of the sample differences become more apparent perhaps as a result of more intense retrogradation over shorter time scales. (ii) in buffer the same trends are evident for both normal cooling and elevated temperature cooling, only the differences between fresh and aged samples are exaggerated. The fresh paste produces a much lower viscosity overall at the 60°C treatment perhaps due to the reduced development of the paste at this temperature, or as a direct function of higher temperature of measurement. On ageing the protection against retrogradation is more pronounced.

So why should the choice of solvent affect the response to caseinate? It is suggested that in distilled, deionized water the starch behaves the same whether caseinate is present or not. The granule contents leach and possibly form an interpenetrating network with the caseinate phase. The granule "ghosts" are interspersed throughout this network. With the standard water system the granule undergoes gelatinisation in the normal way with the protein simply contributing an effect of its own to the paste viscosity. Hence overall viscosity increases as caseinate concentration increases. From the pH measurements (Figure 3.2.5) it is apparent that caseinate buffers, on first addition, against distilled, deionised water to produce a pH value of around neutral. Thus the pH of the buffer and distilled water systems are very similar. It therefore appears that the reason for the different observations must be ascribed to the ionic strength differences. This idea is supported by the different control values also obtained. The viscosity of the buffer-pasted system is slightly higher than for the maize starch pasted in water but it could be argued that this is merely due to experimental variation. If, however, this *is* a real effect then one reason for this may be that anions absorbed from the buffer could possibly affect the swelling of the granule (Oosten, 1982) and, in this case, reduce it from the value found in distilled, deionized water. Strongly hydrated ions, i.e sulphate (present in this buffer as Na

salts) tend to increase the order of the water stucture and as a consequence these ions can increase gelatinisation temperature (and therefore reduce swelling as is seen here). The converse is also true; any ions at the opposite end of the Hofmeister series tend to break up the water structure and decrease the gelatinisation temperature (Gerlsma, 1970; Collison, 1968).

There is conflicting information in the literature on the influence of caseinate on maize starch. It has been reported that caseinate addition increases the swelling and viscosity of maize starch in distilled water (Lelievre & Husbands, 1989) and this is consistent with the results presented in this thesis. In contrast Hermansson (1979) reports a decrease in viscosity of maize starch (amongst a wide range of starches) on caseinate addition in a variety of solvent media. This patented work was interpreted in terms of complex formation between caseinate (or casein) and the starch polysaccharides outside of the granule. The granule is then emptied of its contents in an attempt to compensate for the inbalance in the equilibrium between soluble starch components on either side of the granule. It was suggested that the number and size of complexes increased with reaction time.

The different behaviour in buffer compared to water that is indicated in my results may go some way to resolving the apparent conflict between the work of Lelievre & Husbands and Hermansson. Rather than interpreting the results in buffer in terms of complex formation I would suggest that it is possible to understand this in terms of phase seperation between caseinate and the starch polysaccharides. At high salt concentrations phase seperation between a polyelectrolyte e.g. caseinate and a non-polyelectrolyte is encouraged because the ions swamp the unfavourable entropy decrease that would occur as a result of the uneven distribution of counter-ions in the phase seperated system.

A consequence of this would be that starch polysaccharides are maintained in the granular phase at high caseinate levels for the buffer system. Since they are incompatible with caseinate they will be unlikely to leave the granule. The effect of this will be to reduce retrogradation since there is no possibility of interaction between amylose released from the granule. The lack of change in viscosity on caseinate addition may reflect a slight decrease in swelling volume, which is compensated for by an increase in the viscosity of the continuous (non-granular phase) with increasing caseinate. A reduction in swelling volume on caseinate addition could also be the reason for Hermansson's results. It is possible that under conditions where the biopolymers are incompatible, swelling of starch will be

inhibited at high concentrations of casein(ate) where the phase seperated hypothesis is most appropriate. It would be fair to add that although the majority of the examples in the Hermansson patent relate to measurements in high salt concentrations there are a couple of examples that also determine the viscosity reduction in distilled water. It is possible that the caseinate employed in their work was particularly susceptible to phase seperation, perhaps because of its ionic content or poor solubility.

The increase in viscosity and swelling volume in water found here and by Lelievre & Husbands (1989) could be explained by caseinate penetrating the swollen granule and possibly complexing with the starch polysaccharides as indeed suggested by Hermansson (1977) only enhancing swelling by electrostatic or osmotic effects. Alternatively the presence of low levels of ions associated with the caseinate could, through water structuring effects, influence the gelatinisation behaviour of starch. It is important to appreciate that this is very different from the phase separation ideas invoked to explain the influence of caseinate in buffer. The accompanying swelling volume increase of the 1% standard maize in water most likely occurs as a result of some caseinate involvement with the starch polysaccharides (possibly the Hermansson (1977) amylose-caseinate complex is important here) or the sedimentation of the protein into the "granular" pellet.

In many food products it is more desirable to use modified starches with functional properties tailored to the end-use. Replacing the native starch with a modified maize starch and employing the same experimental procedure gives essentially similar differences between the two solvents when the starch concentration is 4%. When the starch concentration is 1% the effect of caseinate on viscosity is the same in water and buffer. The explanation is almost certainly that at low maize starch concentrations the contribution of the granule to viscosity will be low because the volume fraction occupied by the granular phase will be much lower than the close packing fraction. The swelling volume measurements suggest a volume fraction of ~0.30 for 1% maize starch where spheres close pack at a volume fraction of 0.6-0.7 (Rha & Pradispesena, 1986). The rheology is therefore dominated by the continuous caseinate phase and a similar viscosity increase will be seen in both solvents.

A schematic representation of the postulated difference between the buffer and the water systems is shown in Figure 3.4.1.

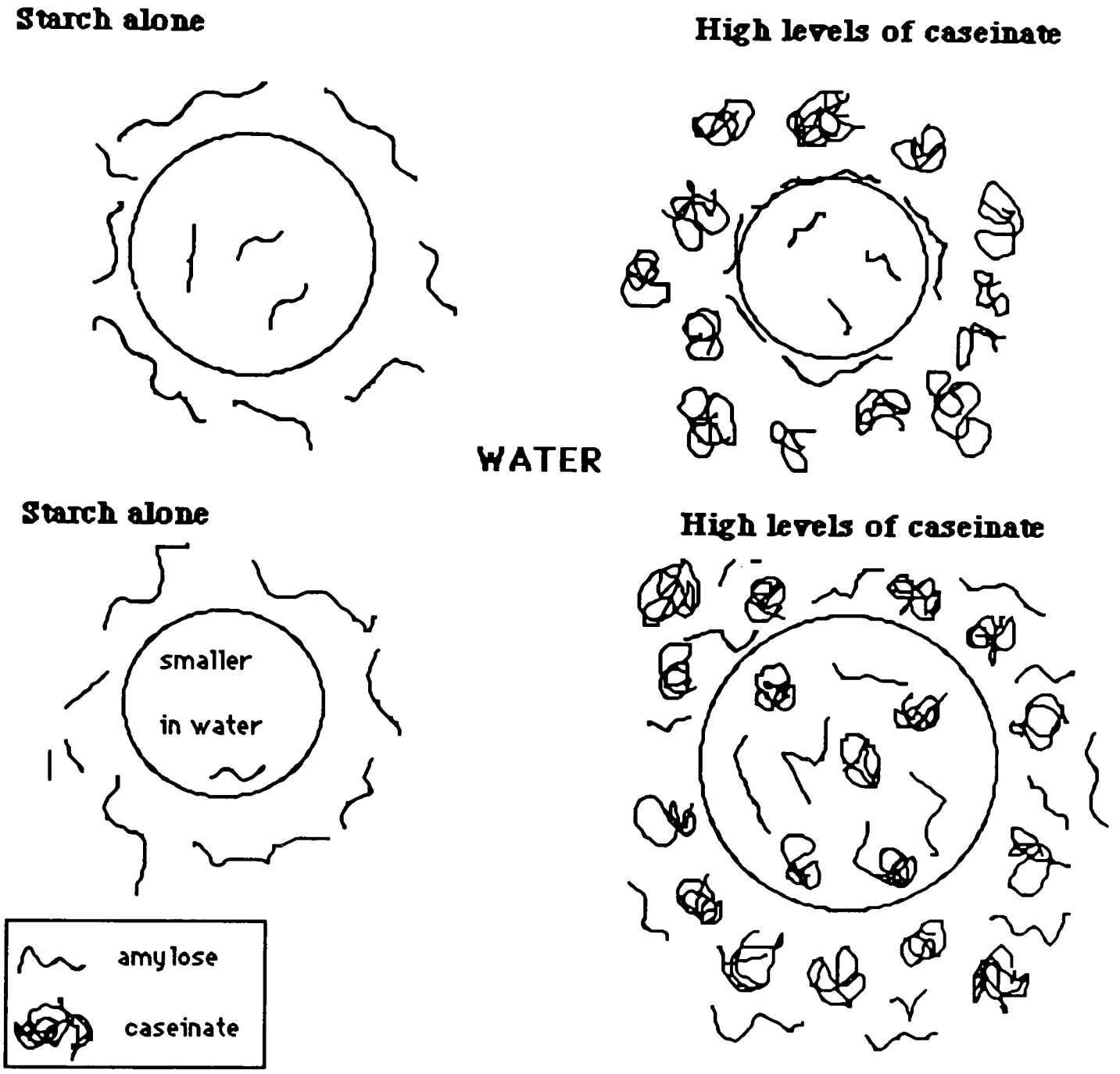


Figure 3.4.1 Schematic representation of the proposed maize starch-caseinate systems in buffer and water. The reduced swelling shown for the control buffer system may be speculative but is based on the assumption that the results seen are not merely a product of experimental variation.

CHAPTER 4: RESULTS OF CARRAGEENAN-LBG-AGAR-PROTEIN SYSTEMS

This chapter consists of four related sections. The first two sections, as well as utilizing the same experimental techniques and mode of analysis, also involve the same three polysaccharide systems at the same concentrations: 2% carrageenan, 0.5% carrageenan/0.5% locust bean gum (LBG) and 2% agar. The only major difference between the two sections is the protein employed. In the first section this is gelatin whereas in the second it is BSA. The third section centres on dried blood plasma systems. This protein is industrially relevant and available sufficiently cheaply to allow large scale experiments to be carried out. Photographs of the interesting phenomenon found are included. The fourth and last section of this chapter stems from the observations found on the carrageenan/LBG-blood protein interaction shown in sections 2 and 3 and essentially involves the search for an explanation of this. It was postulated that the "interaction" with blood plasma involved the LBG components rather than the carrageenan as previously thought. To test this a number of alternatives for the LBG in the mixture were searched out and utilized.

4.1 Section 1: Gelatin-Carrageenan/LBG/agar

This section describes the results of the interactions between gelatin and a number of polysaccharides, all submitted to the same experimental procedure to yield gels and measured as described in chapter 2. A significant part of this work was performed by an ERASMUS student (C. Le Bon) working under my direction. The polysaccharides used totalled three, each at specific concentrations: both carrageenan and agar were used alone at levels of 2% whilst the third system consisted of carrageenan in combination with locust bean gum (LBG) at levels of 0.5% each. As described in the first chapter galactomannan polysaccharides do not gel alone but merely form viscous solutions. When however LBG is used in combination with κ-carrageenan (a blend used frequently in commercial gelling applications) synergistic gel formation occurs and hence allows for the lower levels of individual polysaccharides employed in this study. For the carrageenan containing systems a 0.1M KCl phosphate buffer (pH 7.0) was employed as the presence of potassium ions favours κ-carrageenan gelation whereas the agar gels were prepared in water.

The properties of the gels were characterized by both their melting point and the parameters derived from the stress relaxation experiment. The simplest property to describe first is the melting point. This is shown below in Figure 4.1.1.

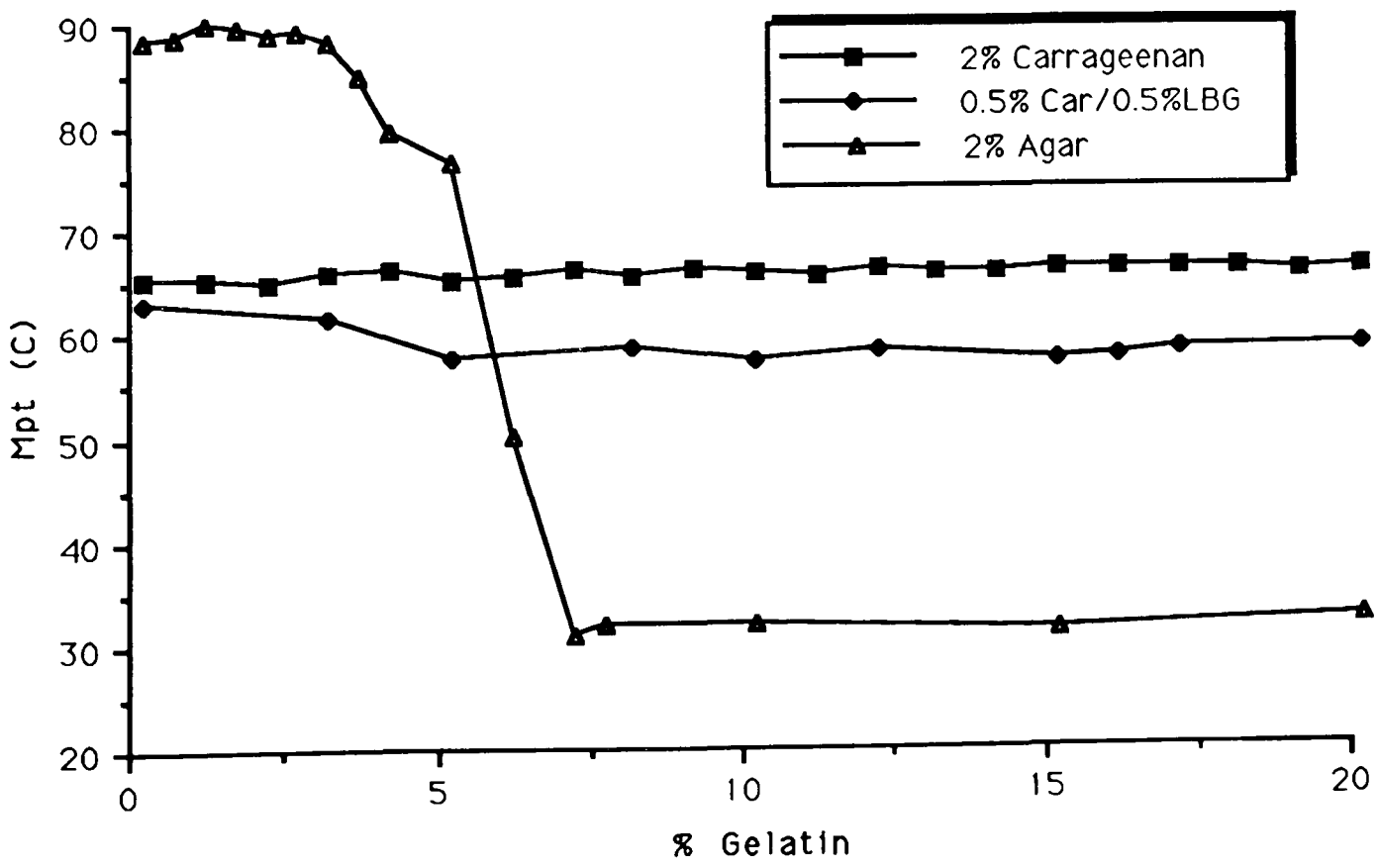


Figure 4.1.1. Effect of gelatin on the melting point of carrageenan, carrageenan/LBG and agar gels. The melting point of 20% gelatin alone is 30.8°C

It is immediately evident from Figure 4.1.1 that, on the addition of gelatin, two distinctly different responses are exhibited by the polysaccharides. Unlike the carrageenan gels, where no real significant change is seen over the gelatin concentration range, the agar gel undergoes a massive fall in melting point at a critical limit of gelatin inclusion. Up to about 4% gelatin the melting point of the agar-gelatin gel is consistent with that found for the agar gel alone - high values of ~90°C. However above a gelatin concentration of 7% the melting point resembles the characteristic melting point of a gelatin gel, that is to say, melting much lower, in the thirties. Between these two extremes there is a transition covering a gelatin concentration range of 4-7%. This has been reported also in the literature by several workers and is discussed at the end of the chapter. It is thus suggested here that the agar-gelatin gel exists in two states depending on the concentration of the protein component. At low gelatin concentrations the gel consists of a continuous agar phase with gelatin inclusions whereas at high protein levels the gelatin becomes the continuous phase. From the shape of the above plots phase inversion occurs with gelatin concentrations in the range 4-7%.

Considering the results of the stress relaxation experiments now it can be seen from Figure 4.1.2 that the carrageenan-containing systems follow almost the same path; an initial slight decrease in F_0 on adding low levels of gelatin but soon changing to a steady increase in F_0 with increasing gelatin concentration. This can perhaps be accounted for by the disruption to the carrageenan network on first addition of gelatin and up to levels before which the gelatin can lend a contribution to the combined gel strength in its own right, seen at about 5% on Figure 4.1.2. The carrageenan and carrageenan/LBG gels both attain final F_0 values, at maximum gelatin addition, which are greater than that found for the gelatin-free gels. Thus overall it can be said that the addition of gelatin enhances the gel strength of carrageenan and carrageenan/LBG gels.

In the case of the agar, which is one of the best gelling polysaccharides initial decrease in F_0 on the first additions of gelatin is again seen but for this gel the decrease is a great deal more significant, F_0 falling by a factor of about 6 on the addition of 5% gelatin. Recovery of gel strength (since the F_0 parameter measures the "resistance" of the gel cylinder to initial compression and therefore can be used as a indicator of gel strength) is not indicated from this figure until levels exceeding 10% gelatin are achieved. Even so the original gel strength is never regained; the final F_0 value at maximum gelatin addition is still only ~ 2/3 of the gelatin-free value. The F_0

data further support the melting point data suggestion of an agar-gelatin phase inversion at 5% gelatin.

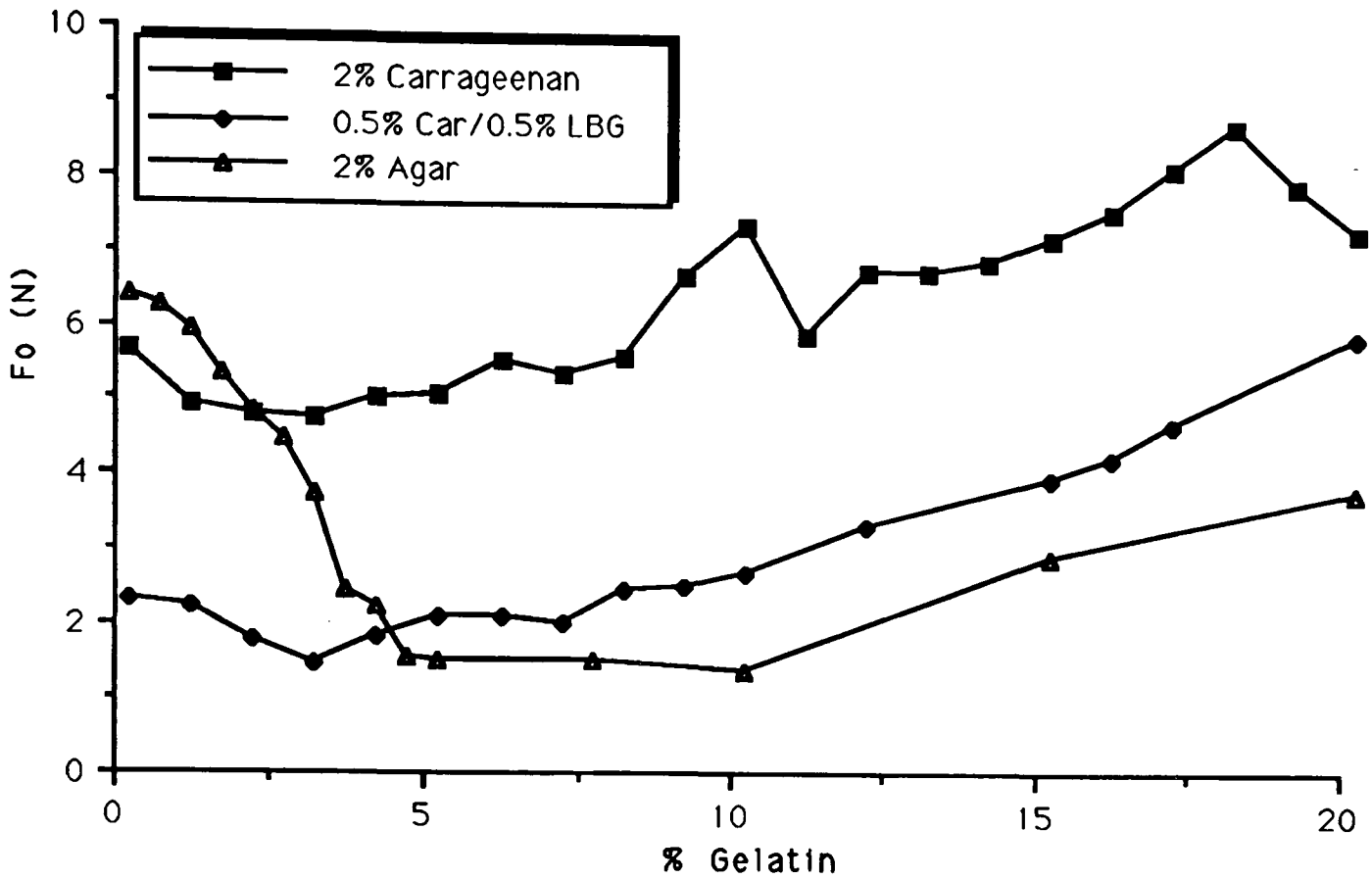


Figure 4.1.2. Effect of Gelatin on the Fo value of Carrageenan, Carrageenan/LBG and agar gels. Fo for 20% gelatin alone is 3.47 N.

The second parameter obtained from the stress relaxation experiment to be discussed is the k_1 constant shown in Figure 4.1.3. This parameter represents the reciprocal of the initial slope of the normalised force-time plot, i.e. a low value of k_1 implies that the initial stress is relaxing rapidly. From Figure 4.1.3 the responses of the three gels are once again shown to be different. The carrageenan gel, although showing a good deal of scatter at the low end of gelatin concentrations, basically gives an overall slight increase in its k_1 values with increasing gelatin addition. For the carrageenan/LBG gels this increase is more significant and the plot smoother. The reasoning for this is perhaps based on the more elastic gel of carrageenan to begin with whereas the carrageenan/LBG gel occupies a control of less than 50% of this value. The final values of k_1, at maximum gelatin levels, for both carrageenan gels is approximately the same and much lower than that attained by the agar gel. This latter polysaccharide gives a less easy to explain plot with the presence of a definite discontinuity at 3-3.5% gelatin. Up to the 3% gelatin there is a great increase in the k_1 parameter to many times the value for gelatin alone but at a gelatin addition of 3.5% the value falls significantly. Beyond this level there is an even greater increase to a final value higher than both the carrageenan gels. Why this discontinuity should arise

is uncertain; perhaps from the findings of the other plots the idea of a phase inversion is reinforced but occurring at a slightly lower gelatin concentration of 3% as opposed to the 5% threshold seen for the F_o data.

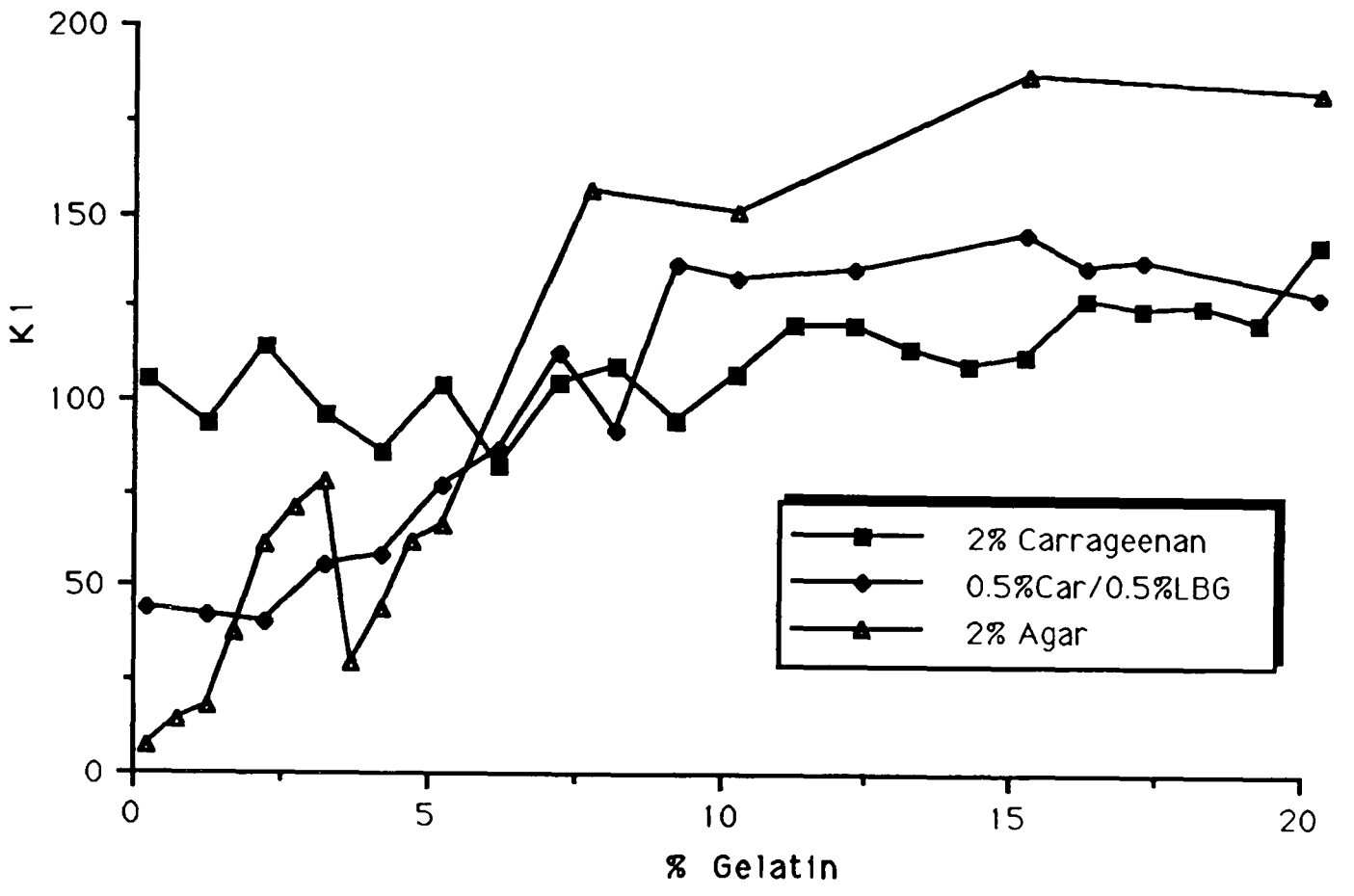


Figure 4.1.3. Effect of gelatin on the k1 values of carrageenan, carrageenan/LBG and agar gels. k1 for a 20% gelatin alone is 246.1 seconds.

The phase inversion mechanism of the agar gel is further supported by the second constant parameter, k_2. Whereas the carrageenan gels undergo a gradual but definite increase to a 20% gelatin value approximately twice that of the control, the agar gel has basically two plateaux at different gelatin concentrations with a period of sharp increase between them. The first plateau, occurring over the 0-2.5% gelatin concentration range, represents the agar-continuous phase with the second plateau region, existing at 7.5-20% gelatin, representing the gelatin-continuous region.

This parameter gives an indication of the elastic character of the material; the more solid a material the higher its corresponding k_2 value will be. This is derived from the fact that the reciprocal of k_2 measures to what extent the stress decays during relaxation, i.e. $1/k_2$ for an elastic solid approaches zero and indicates the reluctance of the stress to relax, with the converse for liquid character also applying. Hence for the systems shown in Figure 4.1.4 addition of gelatin to the carrageenan gels merely increases the "solid character" at a rate related to the level of gelatin addition but at most only doubling the value. In the case of the agar gel this degree of "solidness"

shown at the control (zero gelatin) level is much lower than for the carrageenan and carrageenan/LBG gels and remains so for the gelatin levels of the first plateau described earlier. However final values at 20% gelatin indicate a more than six-fold increase in k_2 which can only be suggestive of the predominantly gelatin gel, following the afore mentioned phase inversion.

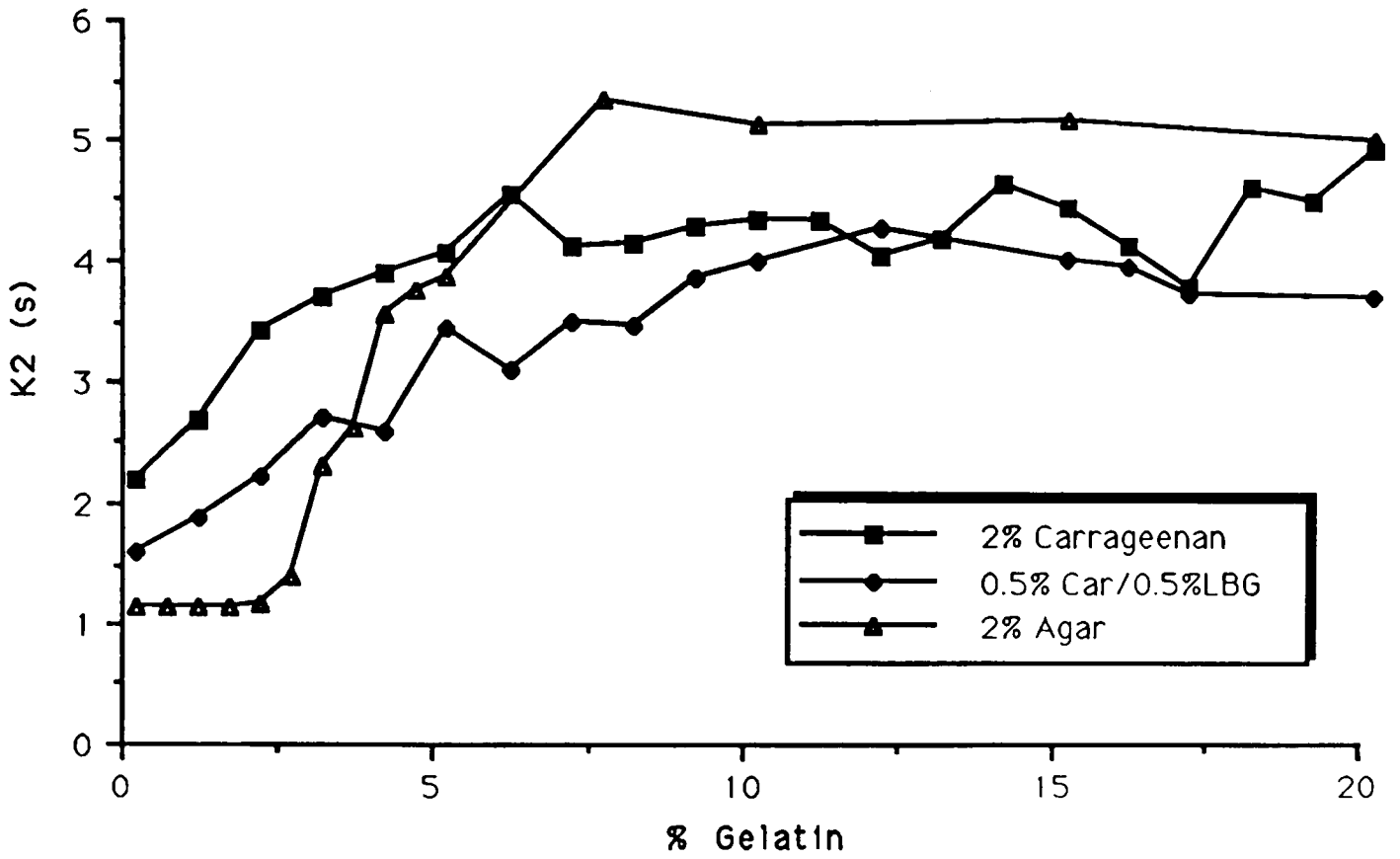


Figure 4.1.4 Effect of gelatin on the k_2 value of carrageenan, carrageenan/LBG and agar gels. k_2 for 20% gelatin alone is 5.83

The last stress relaxation parameter to describe the gels is the asymptotic modulus, E_a, which is derived using contributions from the initial force, F_0, and the k_2 value as described in chapter 1. This value assigns real numbers to the otherwise merely empirical data. Since this modulus can provide information on the internal structure of the gel, the sharp peak seen at 3% gelatin for the agar gel further supports the ongoing suggestion of a phase inversion mechanism between the agar and gelatin. This is, once again, not seen with either of the carrageenan containing gels, instead just showing a steady increase in E_a with gelatin concentration with small amounts of scatter present within each curve.

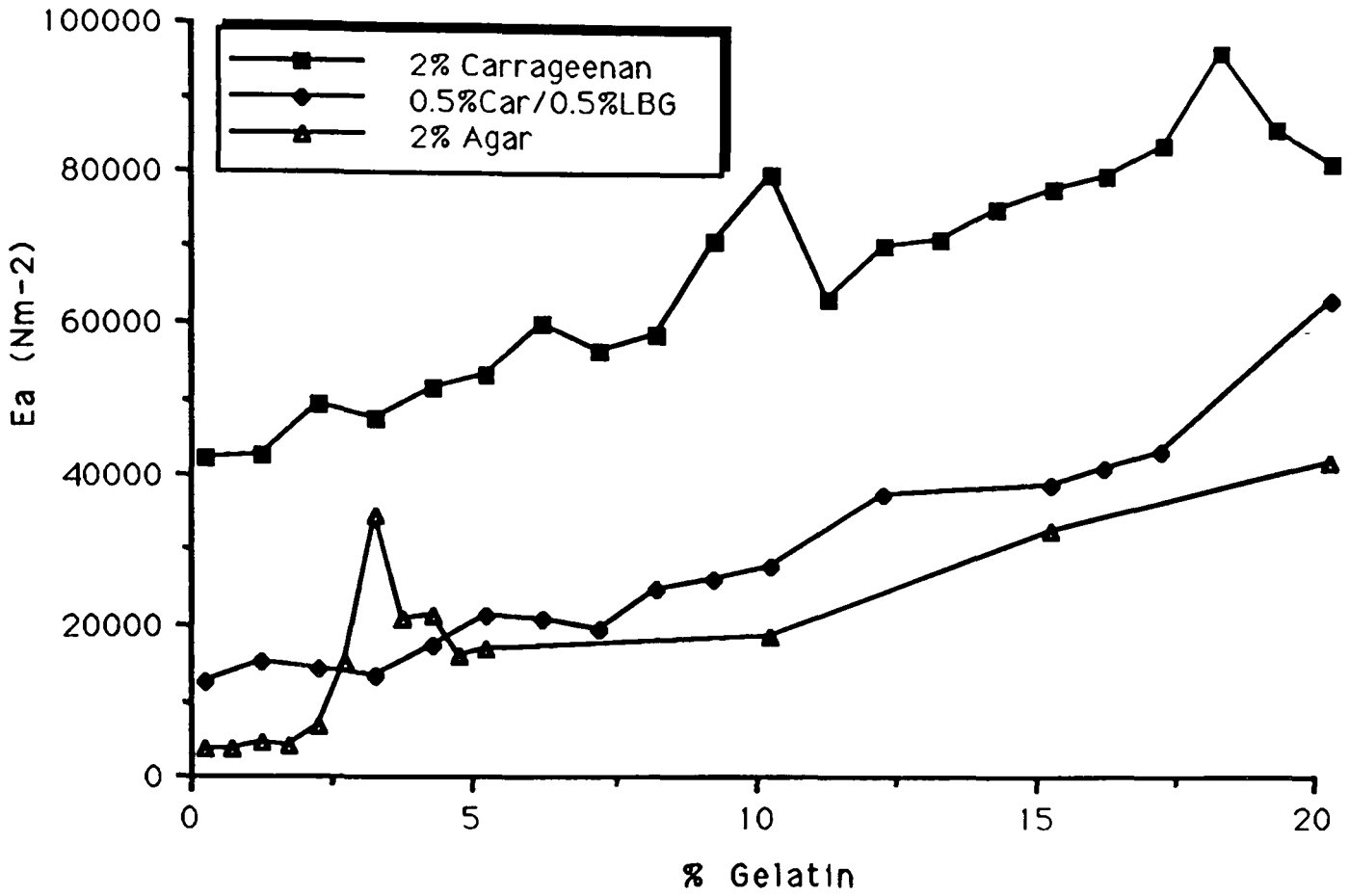


Figure 4.1.5. Effect of gelatin on the asymptotic modulus, E_a, of carrageenan, carrageenan/LBG and agar gels. E_a for 20% gelatin alone is 40490 Nm^{-2}.

4.2 Section 2: BSA-Carrageenan-LBG-Agar

Using the same techniques and analysis as for the previous section the interactions between the polysaccharide gels (2% carrageenan, 0.5% carrageenan/0.5% LBG and 2% agar) and BSA were investigated. All systems (carrageenan, carrageenan/LBG *and* agar) were prepared in the same buffer as for the gelatin studies (pH 7.0). The results of the carrageenan-containing gels with BSA can be directly compared with those of gelatin in the previous section, since all other factors were kept constant. However the conditions for the agar system differs between the two proteins. It should be noted that in addition to the solvent differences (water and buffer for the gelatin and BSA systems respectively) the source of the actual agars was unfortunately different; the analytical grade agar for the BSA preparation was purer than the "plate count" agar used inadvertently in the gelatin samples. Nonetheless in most cases the results obtained show that useful comparisons can still be made.

For practical reasons BSA was included at lower concentrations than used for gelatin due to the limitations of using a thermally gelling protein; at the maximum level of 5% BSA used the protein can just about be satisfactorily incorporated into the mixed gel. Any higher than this presents problems of uniformity in the gel. In addition to the stress relaxation studies performed for both the gelatin and the BSA systems a breakstrength test was also carried out on the BSA-polysaccharide gels. Again the TA-XT2 texture analyser was used to obtain a simple force-time plot with the first peak force registering the breakstrength of the gel.

As with the gelatin-polysaccharides system the first technique of melting point determination will be discussed. Figure 4.2.1 below shows the melting points of the three gels. The features of the three systems noted immediately are the apparent lack of any real effect on two of them - 2% carrageenan and 2% agar - but the significant increase in melting point of the carrageenan/LBG mixed gel system. Although slightly higher than the control values of the gelatin-polysaccharide systems the control gels (i.e. no BSA) give melting points as expected, characteristically high for agar and down in the 60°C's for both of the carrageenan-containing gels.

Considering the single polysaccharide components first, the agar gel undergoes a small increase in melting point to a value at maximum BSA addition ~4°C higher than the control value. The use of very low levels of BSA indicates that this increase commences at the lowest level of protein addition, 0.05%, and continues to rise slightly but steadily until 1% BSA when the melting point almost plateaus. The small

overall increase in melting point may be accounted for by the possibility of the thermo-gelling action of the BSA contributing to the heat resistance in its own right. Certainly the remarkable phase inversion suggested for the gelatin-agar gel does not occur here. The carrageenan gel undergoes an even smaller overall increase in melting point than the agar gel. At the highest level of BSA addition this is less than 2°C. In contrast the inclusion of BSA in the mixed carrageenan/LBG system has a marked effect. At the lowest level of BSA addition (0.05%) a 4% increase in melting point is observed. At the maximum level (5%) of protein addition the melting point has increased by 15°C from the control value. A possible hypothesis for the difference between the behaviour of carrageenan and carrageenan plus LBG is considered in the discussion of this chapter.

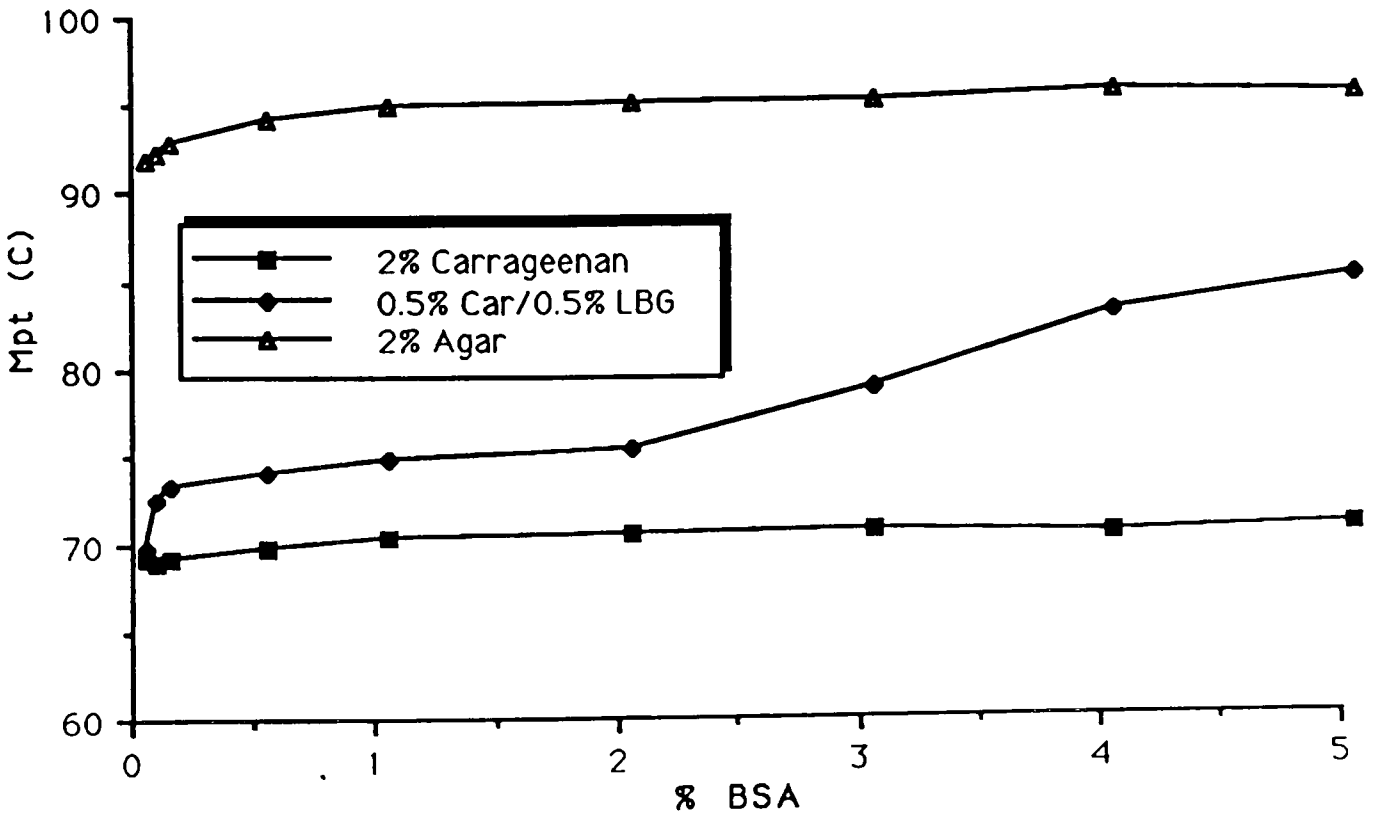


Figure 4.2.1. Effect of BSA on melting point of carrageenan, carrageenan/LBG and agar gels

The results of the stress relaxation test are next presented. The initial force, F_o, values are shown in Figure 4.2.2. For all three systems a decrease in F_o is observed on addition of the lowest level of BSA. The extent of this fall is almost identical in all cases. It is not clear whether this is a real effect or merely reflects a small "error" obtained in the control (zero protein) value. It can be seen that the control values reported for the gelatin system (Figure 4.1.2) are slightly different to those found for the BSA work. From here the F_o values of all three systems continue to decrease. Up to 3% BSA the carrageenan and agar gels follow almost identical paths again but at the higher concentration of BSA the carrageenan gel undergoes a much sharper

decrease, with a final F_o value at 5% BSA of half that of the control whereas the F_o value for the agar gel continues to continues to fall only slightly. This latter response is somewhat similar to that seen for the carrageenan/LBG gel where the initial sharp fall is followed by a slight decrease up to the highest levels of BSA. Since the F_o parameter measures the gel's resistance to initial compression the reduction in F_o observed for all gels would indicate an overall reduction in gel strength.

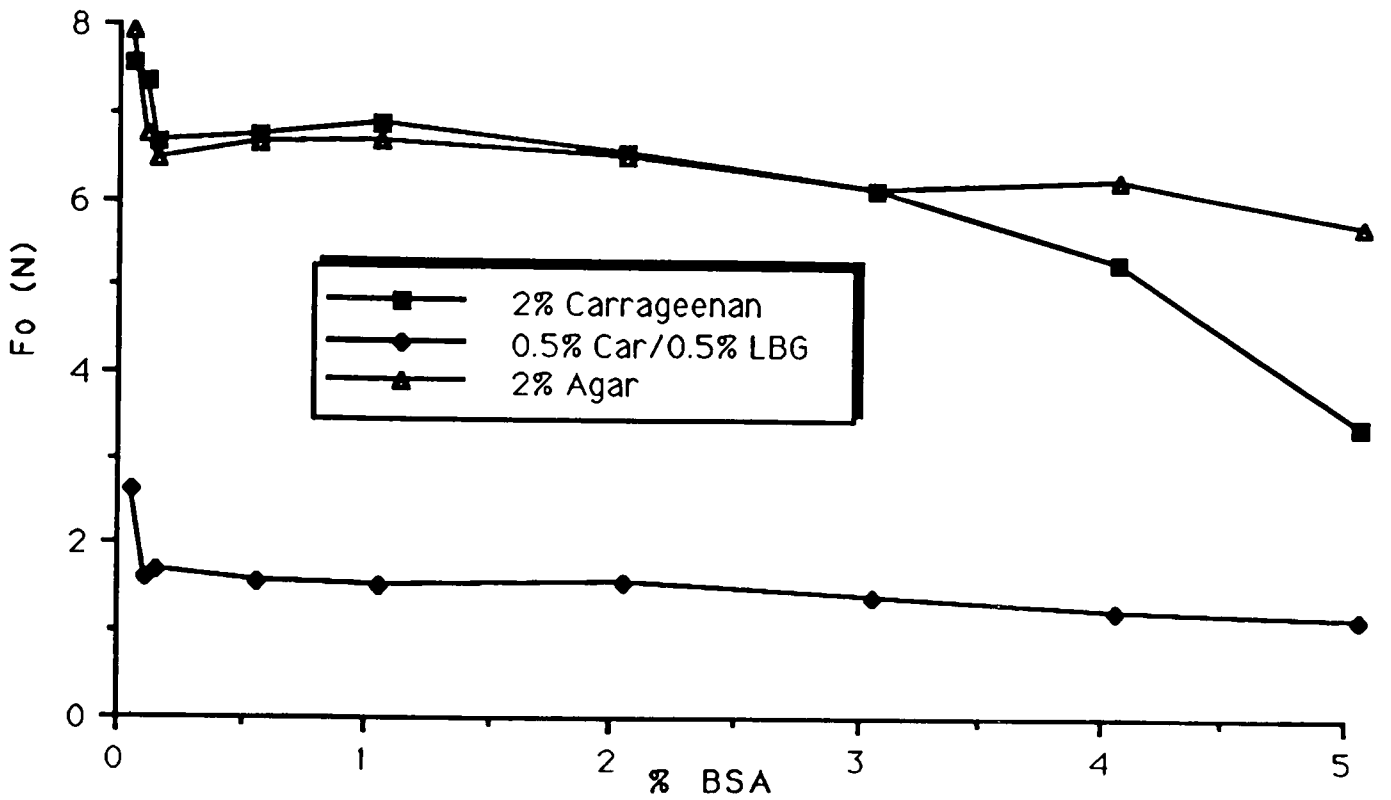


Figure 4.2.2. Effect of BSA on F_o values of carrageenan, carrageenan/LBG and agar gels

This is confirmed by the breaking force data shown in Figure 4.2.3. The breaking force was measured by simply continuing the compression at the same speed used for the initial deformation in the stress relaxation experiment to a distance which encompasses the rupture of the gel. If plotted again as a force-distance graph the first maximum peak then corresponds to that force imposed at failure, termed in this study as the breakstrength force. Reasonable correlation between the two parameters is seen when compared to the breakstrength plot of Figure 4.2.3. In all systems the breakstrength is reduced on addition of BSA but to differing extents: for carrageenan this reduction is dramatic and measurable from first additions of BSA (ultimately undergoing a seven-fold decrease at 5% BSA); for agar an overall reduction is seen but is not so great (ultimately halving); for carrageenan/LBG the smallest effect is observed after an initial decrease to 1% BSA (ultimately a three-fold reduction at 5%).

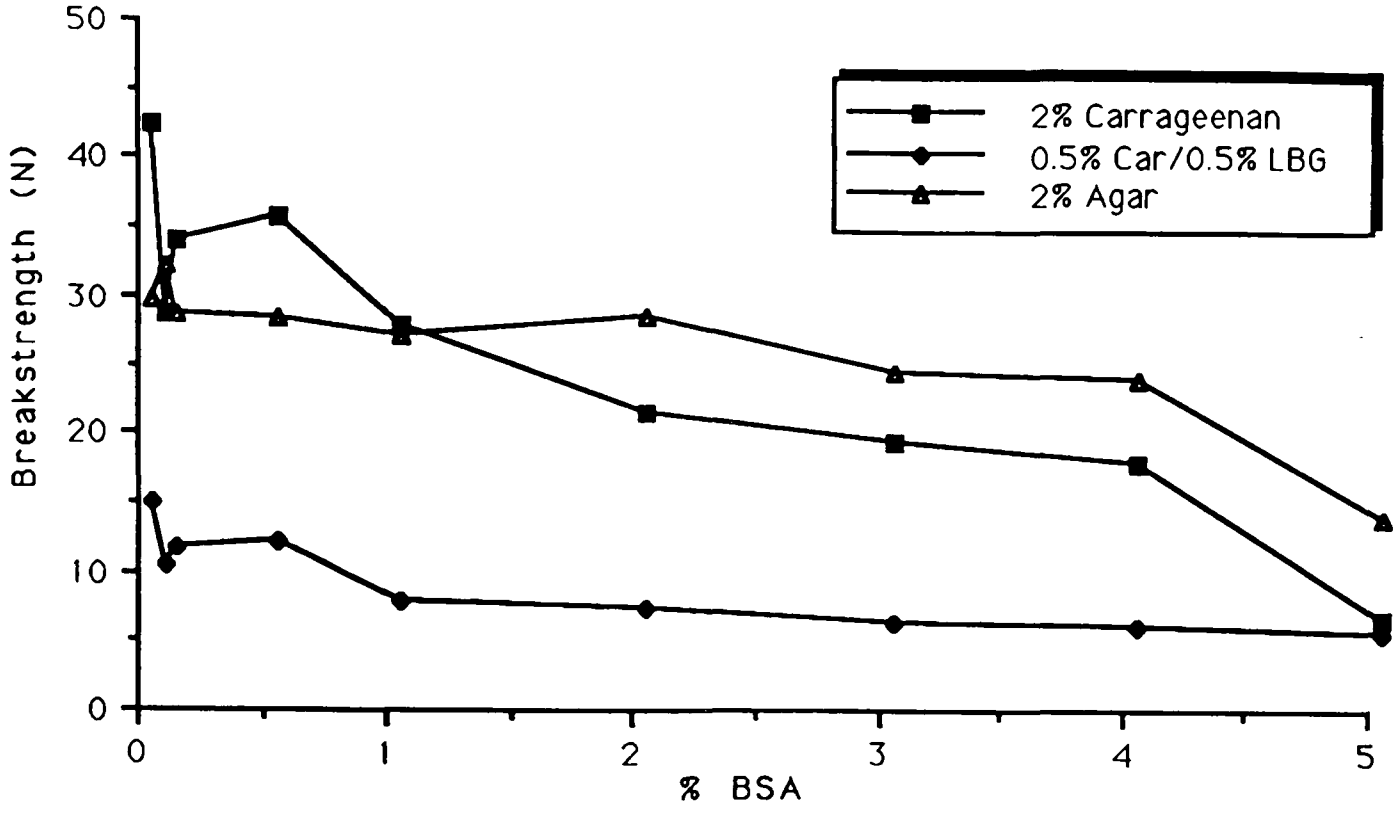


Figure 4.2.3. Effect of BSA on breakstrength of carrageenan, carrageenan/LBG and agar gels

The k_1 values displayed in Figure 4.2.4 show rather similar BSA dependance to that seen for the breakstrength and F_0 data. All show an overall decrease in k_1 but the extents and onset of this decrease vary. The k_1 value for the carrageenan gel decreases more rapidly than is found for the other systems and the final value is about a third of the control value. There is some evidence for a slight initial increase in k1 on BSA addition for the agar and mixed carrageenan/LBG gels.

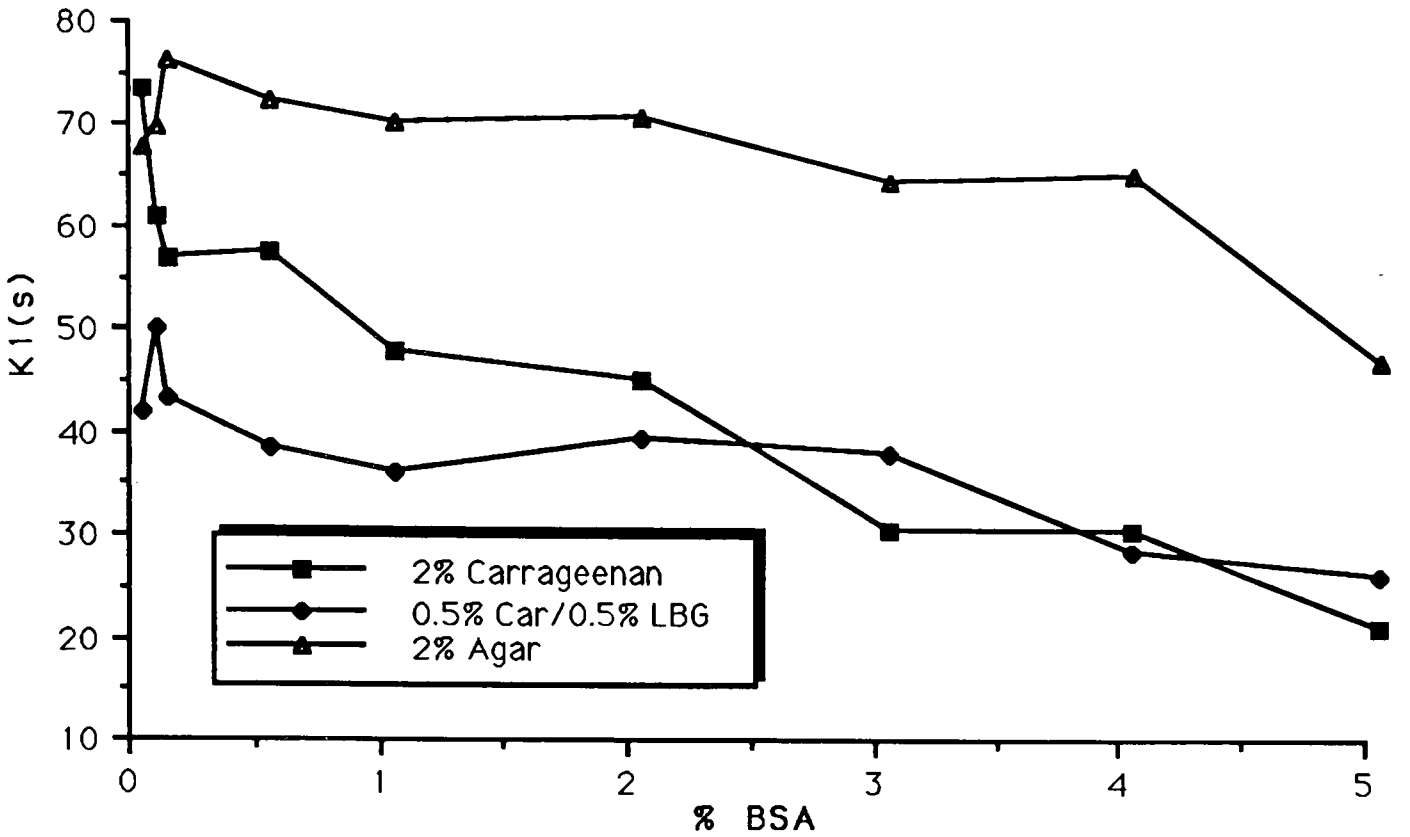


Figure 4.2.4 Effect of BSA on the k_1 value of carrageenan, carrageenan/LBG and agar gels

The k_2 data shown in Figure 4.2.5 shows no real effect overall on addition of BSA. All the systems occupy a very narrow range of values for the whole BSA range. Apart from one data point the agar gel increases slightly on BSA addition unlike the carrageenan-containing gels which merely drift about a mid-point.

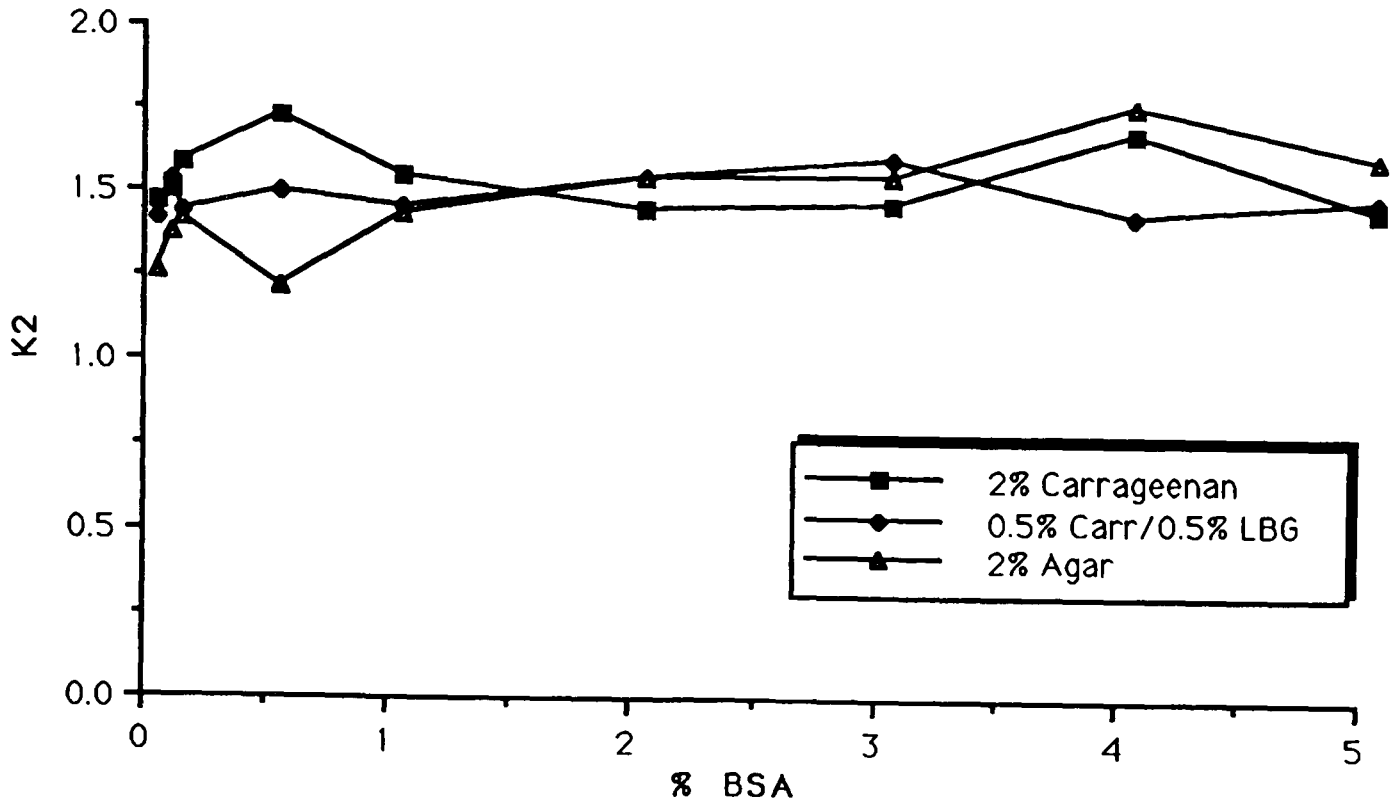


Figure 4.2.5. Effect of BSA on k_2 values of carrageenan, carrageenan/LBG and agar gels

Considering the last parameter derived from the stress relaxation test now the asymptotic modulus, E_a, it is apparent that very marked differences exist between the three systems. Agar increases, carrageenan decreases whilst the carrageenan/LBG gel stays almost exactly the same after the initial very small decrease. The asymptotic modulus is derived from k_1 and k_2. The increase found for agar is a consequence of the increase in k_2 and the weak dependence of F_0 on the BSA concentration. Explaining the responses shown is based partly on the results of Figure 4.2.6 but also on the assumptions made from the previous Figures. Agar is perhaps the best polysaccharide geller known to Man requiring temperatures of ~80-90°C for dissolution of the powder before any gel can begin to form on cooling. Since thermogelling proteins such as BSA require high temperatures to denature and consequently gel the agar will presumably be in the dissolved state before this happens and thus having already acquired optimum conditions for gelation. The BSA will either have already gelled, if temperature and time of heating permitted such, or else will get no further chance to once heat is removed. On cooling the agar gel can then form, perhaps around the protein gel or perhaps incorporating it within its own network as a coupled network maybe, and since the agar gel will have already established itself any additions of protein to the system simply add to the overall gel strength by its own discrete contribution. This is very different to the underlying mechanism of the agar-gelatin system whereby the gelation of the protein on *cooling* results in competition for the continuous phase. This was shown to be determined by

the relative concentrations of each component with a phase inversion occurring at 3-5% gelatin.

From Figure 4.2.6 below, in contrast to the response shown by the agar, the carrageenan gel undergoes an overall loss in modulus value to a final value that is less than half that of the control. The isolated increases that appear to occur at 0.5% BSA and 4% BSA are most likely a knock-on effect of the sporadic k_2 data, from which the asymptotic modulus is partly derived. Thus the carrageenan gel is somehow negatively affected by the presence of BSA. The most likely reasoning is based on the disruption of the carrageenan network by the incorporation of the protein gel. In the carrageenan/LBG gel it could perhaps also be expected that such a disruption of the carrageenan network would also arise in the presence of protein but this decrease, if arguably any, is so small as to be extremely different to that of the carrageenan gel. It is possible that the almost horizontal line observed is, in fact, a product of both a negative response (the reduction seen with the carrageenan gels) and a positive response of the carrageenan/LBG mix to the BSA. It is this latter suggestion that is interesting and forms the basis of the last section of this chapter.

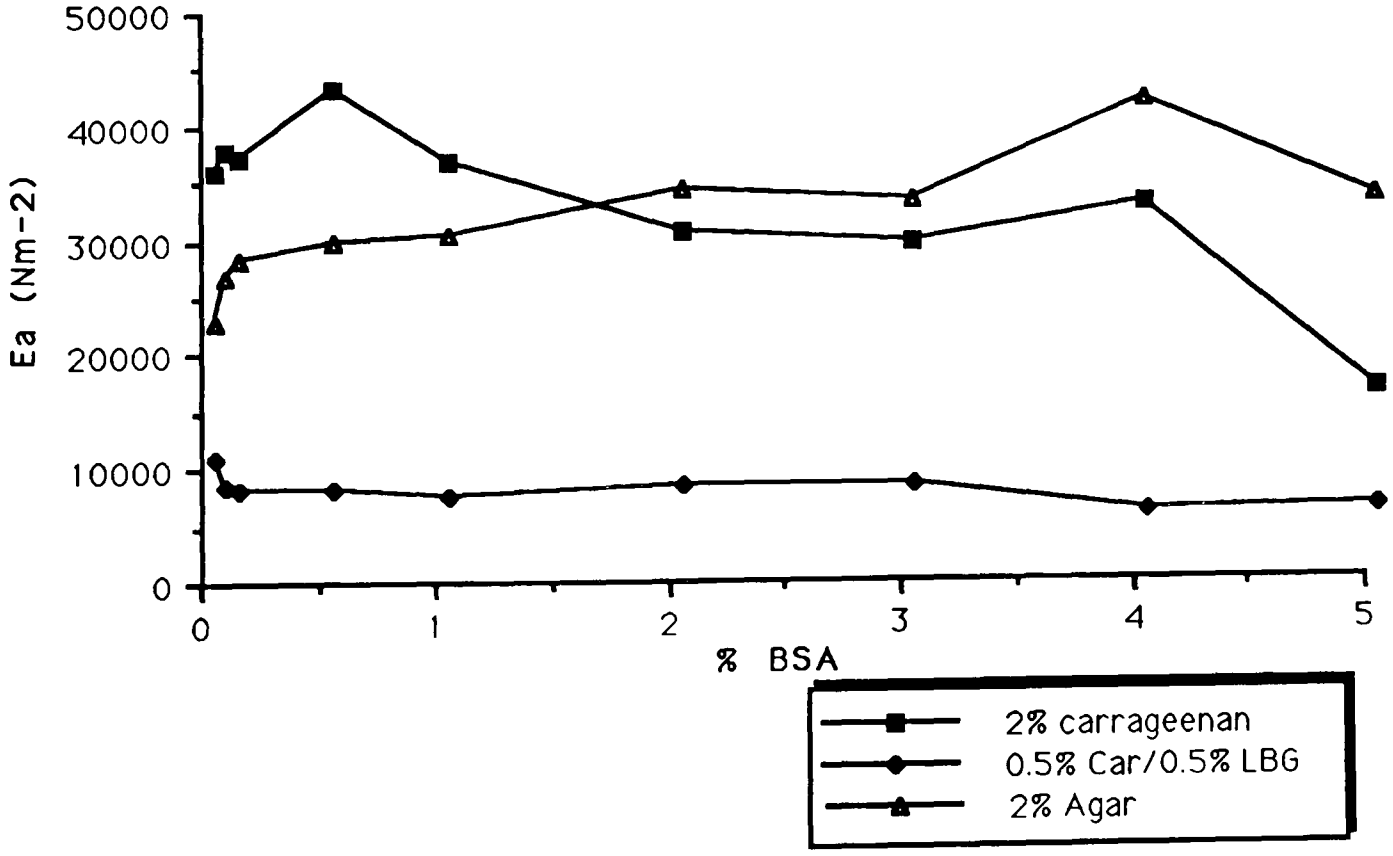


Figure 4.2.6. Effect of BSA on the E_a values of carrageenan, carrageenan/LBG and agar gels

4.3 Section 3: Dried blood plasma-carrageenan/LBG

This section shows photographically the results of the larger scale preparation of carrageenan/LBG-protein gels in small cans undergoing an autoclaving step. The protein used in this case was a dried blood plasma (commercial name of Vepro® 75) which was considered to be an acceptable substitute for BSA in larger scale production and representing more closely the food industry usage.

The photographs of Figures 4.3.2 and 4.3.3 show the effect of DBP inclusion and autoclaving on a 0.3% carrageenan/0.3% LBG gel in pH 7.0 phosphate buffer with 0.1M KCL. Both static and rotating autoclaves (the cans in the latter rotate at a rate of approximately 4 revolutions per minute) were employed for the same time and temperature (121°C, 1hour). Figure 4.3.2 shows that in the statically autoclaved mixed gels of 0.5% DBP and upwards an area of opaque, spongy, material lies at the base of the gel (the photographs show the inverted gels and thus this area is seen at the top), and as with the rotary autoclaved gels, increases in volume with increasing DBP to the point where at 5% protein it accounts for almost all of the gel. Comparison to Figure 4.3.3a shows both non-autoclaved and rotary autoclaved gels before removal from the can. The main feature of the non-autoclaved gels on the photograph is the change in colour (brown → white) and increasing opacity with increasing levels of DBP. However on rotary autoclaving it becomes apparent that at 0.5% DBP a distinct "spongy" phase exists in the centre of the surrounding translucent gel. The triplicate tests shown indicate the reproducibility of this phenomenon. As the DBP level increases to 1% the volume of this central phase becomes greater. At 5% this opaque protein gel accounts for almost all of the gel volume. So what is actually being observed?

At such high temperatures and at sufficient concentration thermo-gelling proteins such as are contained in blood plasma will gel whilst the polysaccharide mixed gel loses considerable viscosity. Hence the heavy protein gel will sink out of the solution formed. This is observed in the normal sedimentation to the base in the statically autoclaved gels and in the central positioning of the rotary autoclaved gels. But should DBP at just 0.5% concentration form the gel mass that is observed?

This is not the end of the story. An additional feature of the autoclaved gels is the location of the LBG crude husk material, observable as coarse "grits" within the gel. Although not clear from the photographs, the rotary autoclaved gel containing 0.1% DBP has these LBG grits distributed fairly evenly throughout the body of the gel.

From 0.5% DBP however the central protein gel region also contains within it all the LBG husk material. Slicing the protein gel indicates that the LBG husk is distributed evenly throughout the whole region. The surrounding supporting gel phase loses much of its dark colour and becomes paler yellow (similar to that of a carrageenan gel alone) with increasing level of DBP. No LBG husk material is to be found in this surrounding gel at all.

Therefore it would appear that an interaction may be occurring between the husk component of the LBG and the DBP. The LBG sample actually contains significant amounts of protein, which is to be found as the largest component of the husk (source: supplier information). In fact the low grade refinement of this product gives an LBG with a 12.0% (by weight) protein content (supplier information). Since the precise nature of the protein in the LBG is not known, the details of the interaction will not be considered in any more depth. At DBP levels of only 0.5% we would not expect a protein to gel alone, as it is indeed seen to do so in the mixed carrageenan/LBG-DBP gel. A control protein sample at this level (not shown) did not form the gel shown in the mixed system. Thus it must be getting help from something else: the additional protein of the LBG perhaps. In order to obtain the resultant gel seen it would suggest that any mechanism would have to be synergistic since overall protein levels are low: 0.5% (DBP) + 0.036% (LBG).

However in postulating this idea it was also considered that the location of the LBG "grits" could be simply due to the gelled protein phase physically supporting the heavy grits as they settle out of the polysaccharide solution and hence provide an alternative reason for the co-existence of the LBG grits and the DBP gel. This can be argued against though by: (1) the LBG husk remains in the whole body of the gel in the rotary autoclaved sample in the absence of DBP rather than just in the centre, i.e. as would occur if it was merely settling out of solution and (2) the LBG husk is evenly distributed throughout the height and width of the protein gel rather than having a predominance at the periphery of the protein gel (i.e. for physical entrapment) or at the centre (i.e. for sedimentation). To obtain such an even distribution this would also suggest that the protein and LBG material gel together; the DBP interacting with the husk material, taking it out of bulk solution and gelling. However since the protein-protein interaction is tentative this alternative idea should not be ruled out at this stage.

Support for the idea can be perhaps gained through the measured gel strengths of the gels as given in Figure 4.3.1. Compressed within the can to a distance of 10mm (sufficient to rupture the gel) the added DBP reduces gel strength in the absence of

autoclaving with increasing protein inclusions. This agrees well with the results of section 2 (Figure 4.2.3) for BSA. A reduction is again seen, at lower absolute levels, on autoclaving up to 1% DBP with a large increase at 5%. Reference to the photographs of Figure 4.3.1 will show however that at this distance of penetration the probe is actually measuring the gel strength of the protein gel at 5% DBP. Between 0.5% and 1% DBP the gel strength of the surrounding yellow gel is measured, and assuming the reduction in gel strength seen between these levels is a real effect and not an artefact, then the loss of colour may correlate to the loss of gel strength (i.e. possible removal of LBG from the carrageenan phase into the protein phase). The gel strength of the 5% DBP gel cannot be accounted for solely through DBP gelation as the value for a blood plasma gel (1.75N) shows.

For autoclaved gels:

gel strength reduced as DBP added ↓

at 1%, weakened surrounding gel ↓

at 5% probe measures protein gel strength ↓

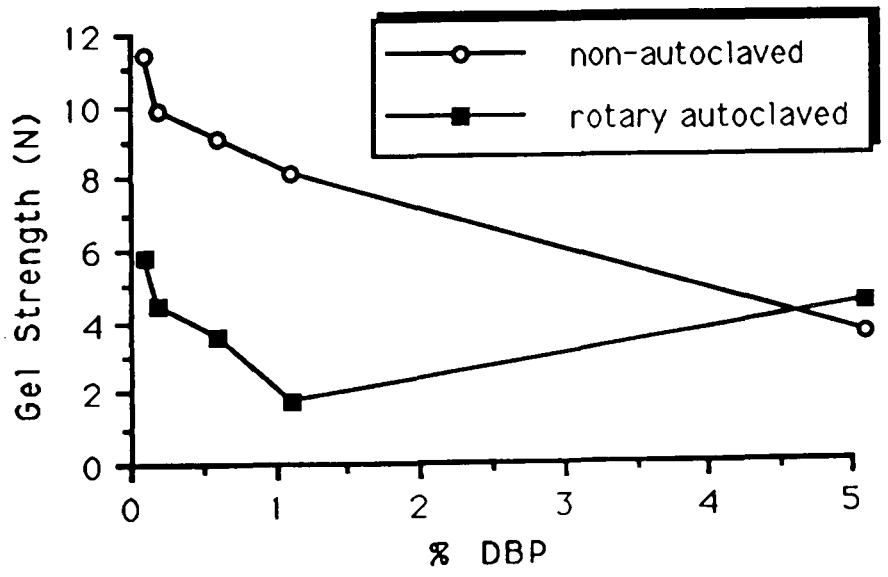
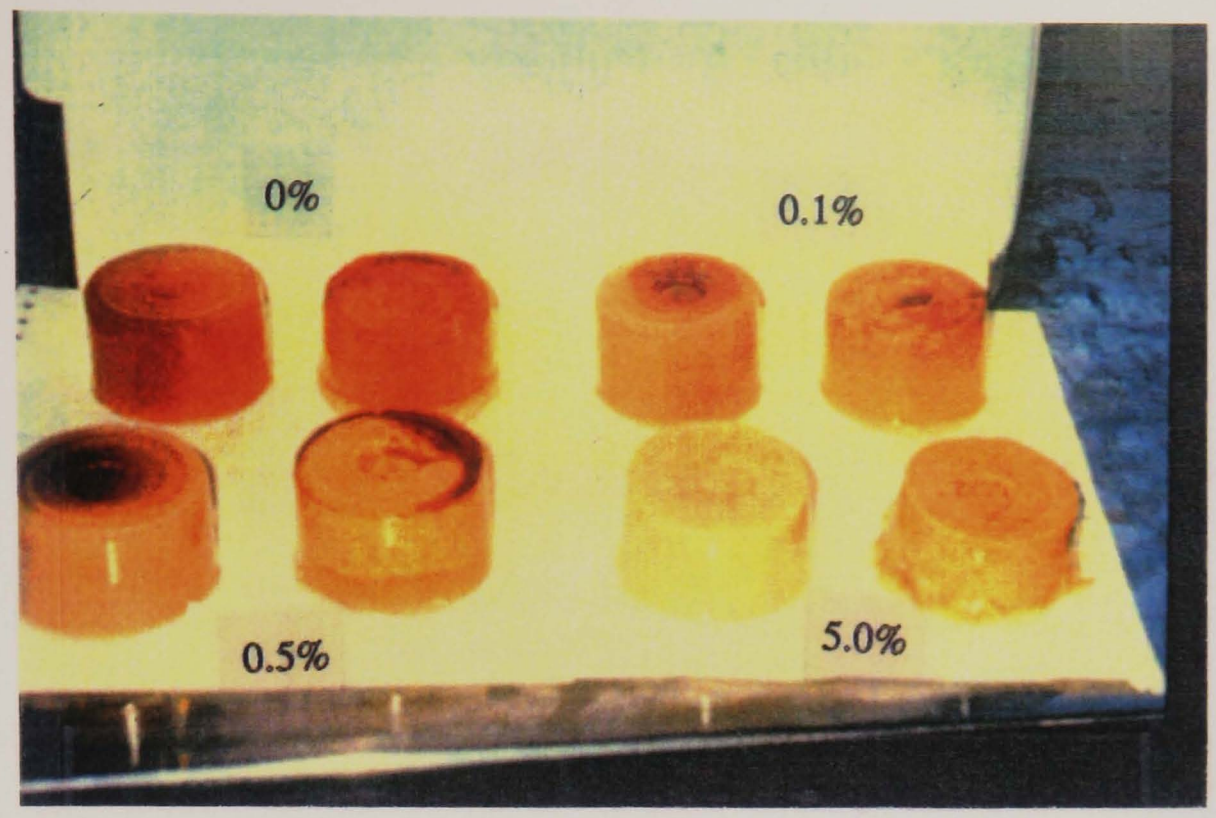


Figure 4.3.1 Effect of DBP on the gel strength of mixed 0.3% carrageenan/0.3% LBG as a function of rotary autoclaving. NB: a 5% autoclaved DBP gel (no polysaccharide) has a gel strength of 1.75N.

Additionally, on autoclaving in the absence of DBP the gel becomes darker red-brown (possibly a Maillard reaction from the protein in the LBG?) and translucent and the grits themselves also become darker in colour (same reasoning?). The question that arises next if the idea of some sort of interaction between the protein component of the LBG and the DBP is believed is that: how is this protein located on

the galactomannan chain and does the binding of the protein take the active galactomannan out of available use for the carrageenan?



L R

(a) non-autoclaved (= left of each pair) and statically autoclaved (= right of each pair) gels at 0%, 0.1%, 0.5% and 5% DBP inclusion levels. Gels shown inverted.

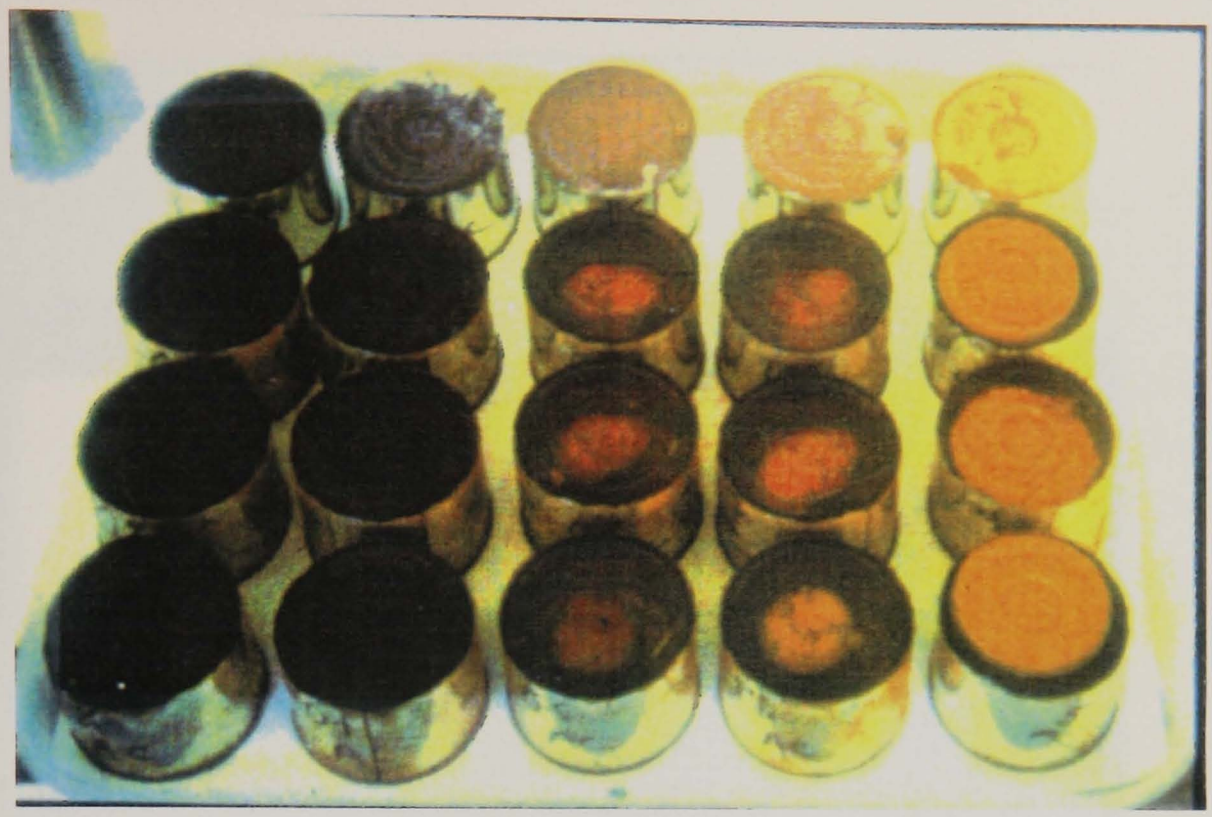
L R

(b) Comparison of effects of autoclaving on 0.5% DBP gel (in the foreground of photograph). Gels shown inverted. Left = non-autoclaved; Right = autoclaved.

Figure 4.3.2: Effect of DBP and static autoclaving (121°C, 1h) on 0.3% carrageenan/0.3% LBG gels in pH 7.0 phosphate buffer (0.1MKCL)

(c) Highlighting the two phases of the 0.5% DBP autoclaved gel (correct way up)

Figure 4.3.2 : Effect of DBP and static autoclaving (121°C, 1h) on 0.3% carrageenan/0.3% LBG gels in pH 7.0 phosphate buffer (0.1MKCL)



L R

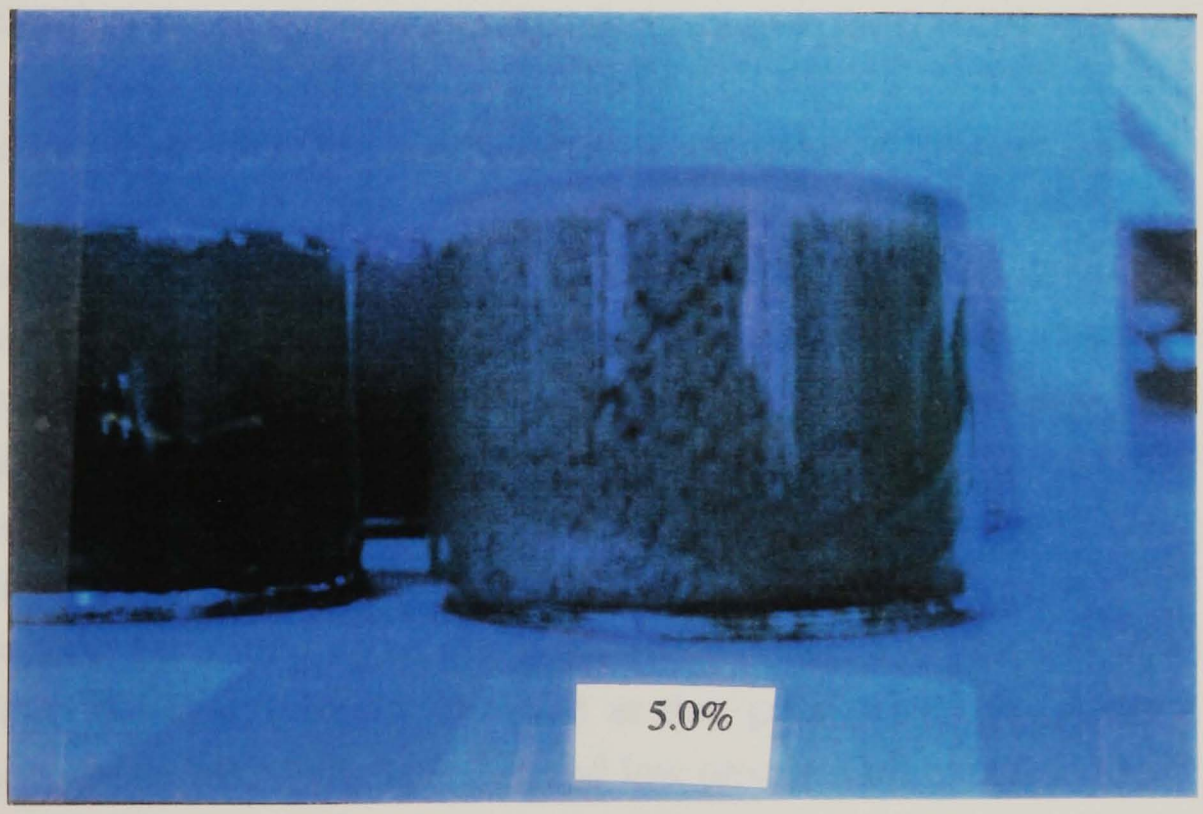
(a) Non-autoclaved (back row) and rotary autoclaved replicate gels before removing from cans. Levels of DBP inclusion from Left → Right = 0%, 0.1%, 0.5%, 1.0%, 5.0%.

L R

(b) Replicates of autoclaved gels removed from cans. Levels of DBP inclusion from Left → Right = 0%, 0.1%, 0.5%, 1.0%, 5.0%

Figure 4.3.3: Effect of DBP and rotary autoclaving (121°C, 1h) on 0.3% carrageenan/0.3% LBG gels in pH 7.0 phosphate buffer (0.1MKCL)

(c) rotary autoclaved at the 0.5% DBP inclusion level



(d) rotary autoclaved at the 5% DBP inclusion level

Figure 4.3.3: Effect of DBP and rotary autoclaving (121°C, 1h) on 0.3% carrageenan/0.3% LBG gels in pH 7.0 phosphate buffer (0.1MKCL)

4.4 Section 4: Elucidation of the BSA-carrageenan/LBG interaction - Replacement of the galactomannan

From the interesting results yielded from the BSA-polysaccharide system of section 2 coupled with the photographic evidence of DBP-polysaccharide gels of Section 3 a further study investigating the possibility of an LBG-protein interaction was made. The increase in melting point seen on the addition of BSA to the carrageenan/LBG (Figure 4.2.1) along with the apparent "clearing" of LBG from the carrageenan phase in a 5% DBP gel (Figure 4.3.1) stimulated the search for the underlying mechanism. The differences between the effect of BSA on the melting points of carrageenan compared with the mixed carrageenan/LBG gels make it reasonable to assume that the inclusion of LBG in the gel promotes an interaction with the protein.

If the LBG is responsible for an interaction with the protein - BSA or DBP - it is most likely influenced by one or both of two factors: (i) the galactomannan ratio (G/M) of the material, (which is the key factor in determining the synergistic gelation of carrageenan with LBG), also influences protein gelation, perhaps through a similar mechanism to that of carrageenan-LBG or (ii) an intrinsic component of the LBG other than the galatomannan is directly responsible for the LBG-BSA interaction.

To investigate both of these possibilities two types of LBG replacer were used. The first focuses on the galactomannan ratio in order to ascertain the effect of this. LBG has a G/M ratio of ~20% (see Chapter 1) and it is this low ratio which allows the interaction with carrageenan at the "smooth" regions of the chain lacking in any mannose residues. This extent of interaction with carrageenan will be reduced as the G/M ratio is increased. Guar gum and tara gum with G/M ratios of ~33% and ~25% respectively were selected. The second type of replacer used in this study are LBG's with stated varying levels of non-galactomannan material (protein and husk). The standard LBG used up to this point contains 12.0% protein by weight contained within the husk (visually observed as the "grits" within the powder, solution or carrageenan-LBG gel). The high and low protein LBG samples contain 16.9% and 8.1% protein by weight respectively. The gels were prepared exactly as before but replacing the standard LBG with the galactomannans in the dry mixed powders before addition to the buffer in the manner described in Chapter 2.

The objective of this particular study was to assess the effect on the protein of replacing the standard LBG with another galactomannan and thus only the two extreme concentrations of BSA were used, namely 0% and 5%. In order to provide

complete comparison the same melting behaviour and stress relaxation tests were performed on these gels as for the first and second sections of this chapter. In presenting this data, whilst fully appreciating that a line graph may not be the most correct or reliable way of relating just two points, it proved to be the best method here. Following the format of previous sections the first result to follow is the melting point data of Figure 4.4.1 below.

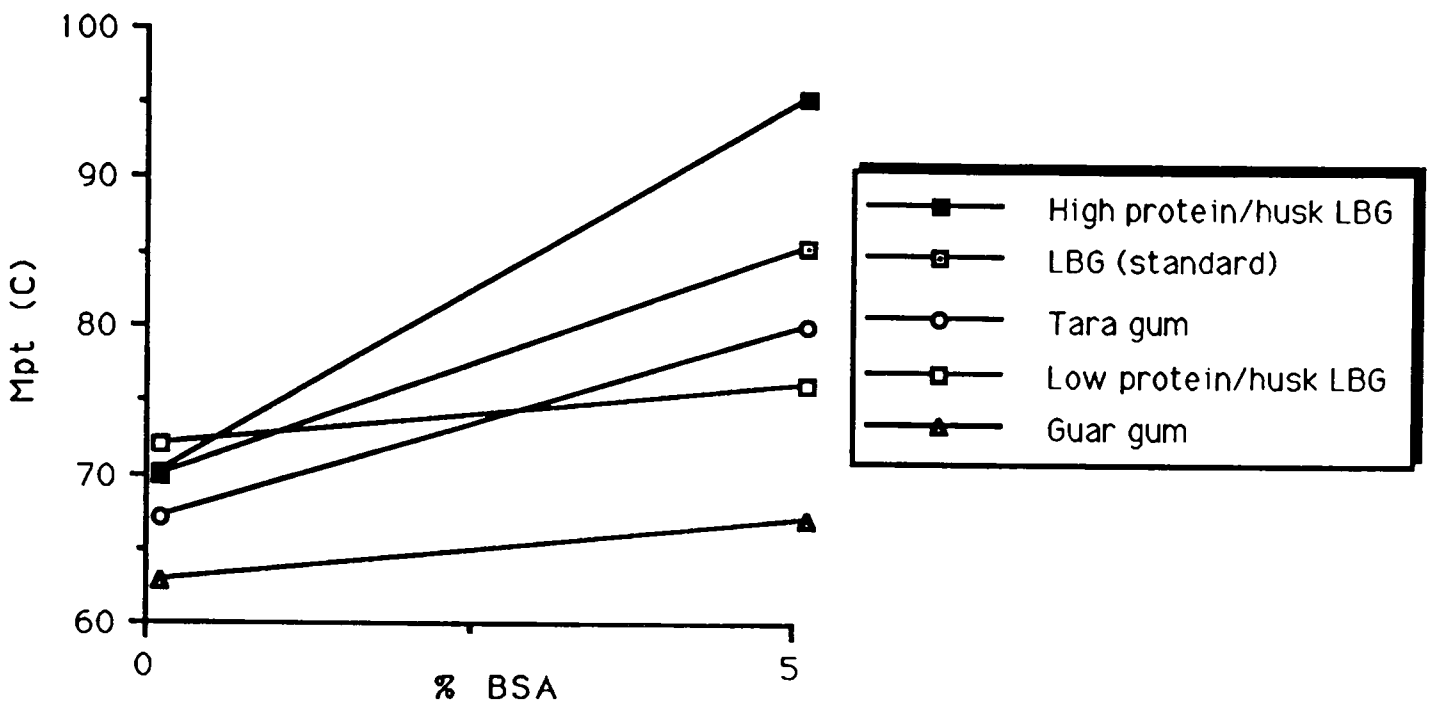


Figure 4.4.1. Effect of Galactomannan in the 0.5% carrageenan/0.5% galactomannan gel in the absence and presence of BSA - melting point

The LBG/carrageenan gels in the absence of BSA all have higher melting points than either the guar/carrageenan or tara/carrageenan gels as would be expected if the degree of interaction is related to the G/M ratio. In fact the only result obtained for the guar is the melting point data. Preparation within a glass tube allowed the containment of a structure and subsequent measurement of a melting temperature. Although this combination could support the 4mm steel ball-bearing without problems it was not possible to remove a sufficiently "solid" gel from the dialysis tubing.

The melting point order in the absence of BSA (all LBG's > tara > guar) reflects the G/M ratio of the three galactomannans. The low protein LBG actually shows a slightly higher melting point than the other LBG samples. This may be attributable to the higher proportion of galactomannan (i.e. the "active" ingredient) in the material.

The results for the gels containing 5% BSA are interesting. Excluding guar, which as already stated, does not show a synergistic interaction with carrageenan the increase

in melting point on 5% BSA addition is related to the protein content of the galactomannan preparation. The low protein LBG which showed the highest melting temperature as a control gel now occupies the penultimate position from the bottom. The highest melting temperature gel is the high protein LBG at values close to the boiling point of water. The standard LBG lies almost exactly intermediately between the high and low protein LBG's and this correlates well with the intermediate protein content of the standard LBG. Tara gum showed a similar increase in melting point to that found for the standard LBG. The colour of both the powder and the subsequent gel solution in this study were found to be indicative of protein content. The deep red-brown colour of the tara gum and the mixed gel formed from this material confirm the level of protein stated by the manufacturer to be ~10.5%.

Thus it appears that in the absence of any BSA the extent of interaction with carrageenan is governed by the G/M ratio of the galactomannan and follows the pattern of gelation cited else where (Fernandes *et al.*, 1993). However in the presence of BSA the resistance to melting is dictated by the protein/husk content of the galactomannan. This then suggests that in the presence of BSA an interaction with the non-galactomannan component is involved.

From the F_O values shown in Figure 4.4.2 this same order is again apparent. The strongest gel (i.e. those that resist compression most) in the presence of BSA is the LBG with the highest protein content. The highest value in the absence of BSA is shown by the low protein LBG. This sample has the higher purity in terms of galactomannan content due to the low husk/protein content and thus has a greater tendency to gel in combination with carrageenan.

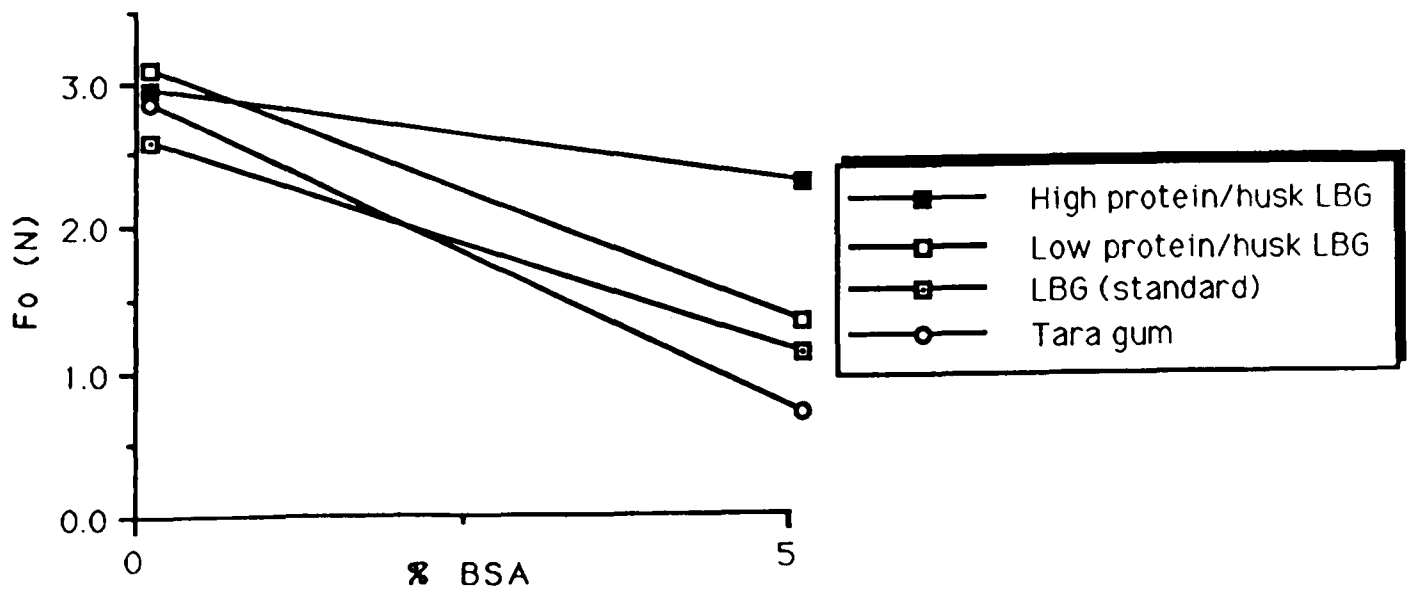


Figure 4.4.2. Effect of Galactomannan in the 0.5% carrageenan/0.5% galactomannan gel in the absence and presence of BSA - F_O values

The results of the other parameters of the large deformation stress relaxation studies are now presented.

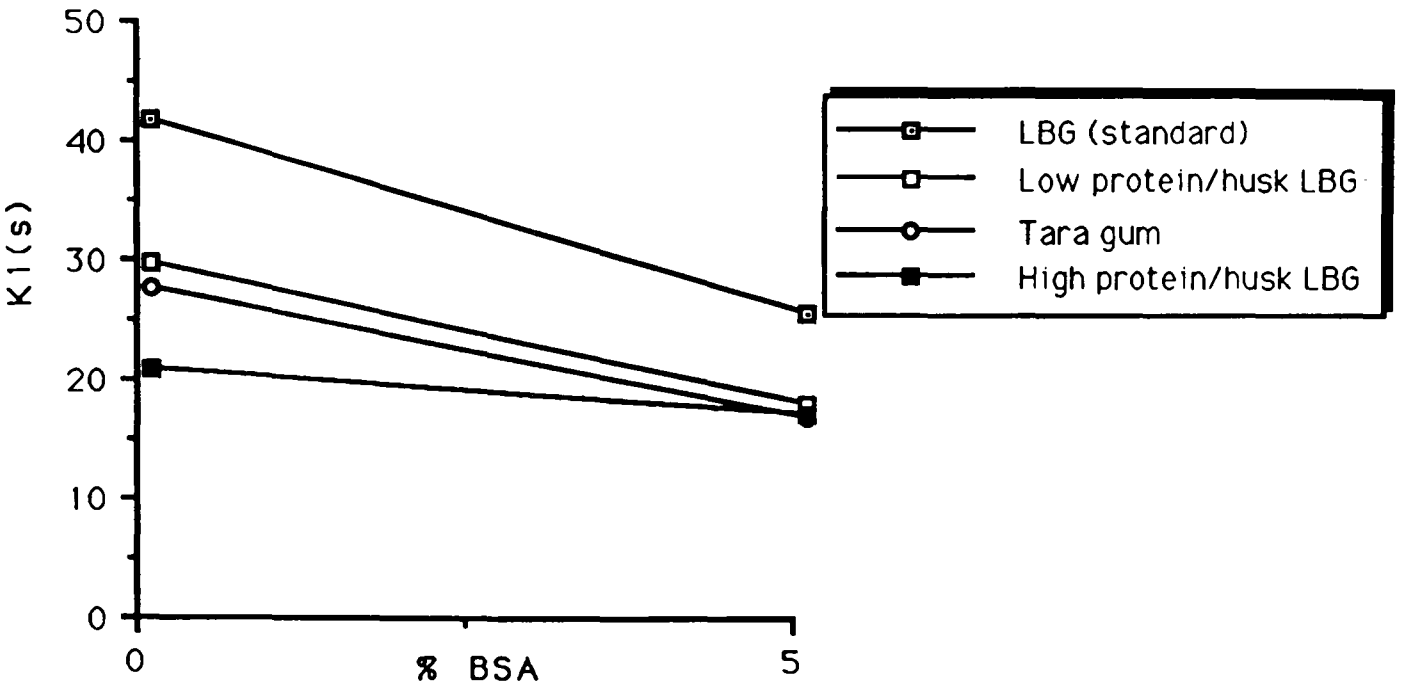


Figure 4.4.3. Effect of Galactomannan in the 0.5% carrageenan/0.5% galactomannan gel in absence and presence of BSA - k_1 values

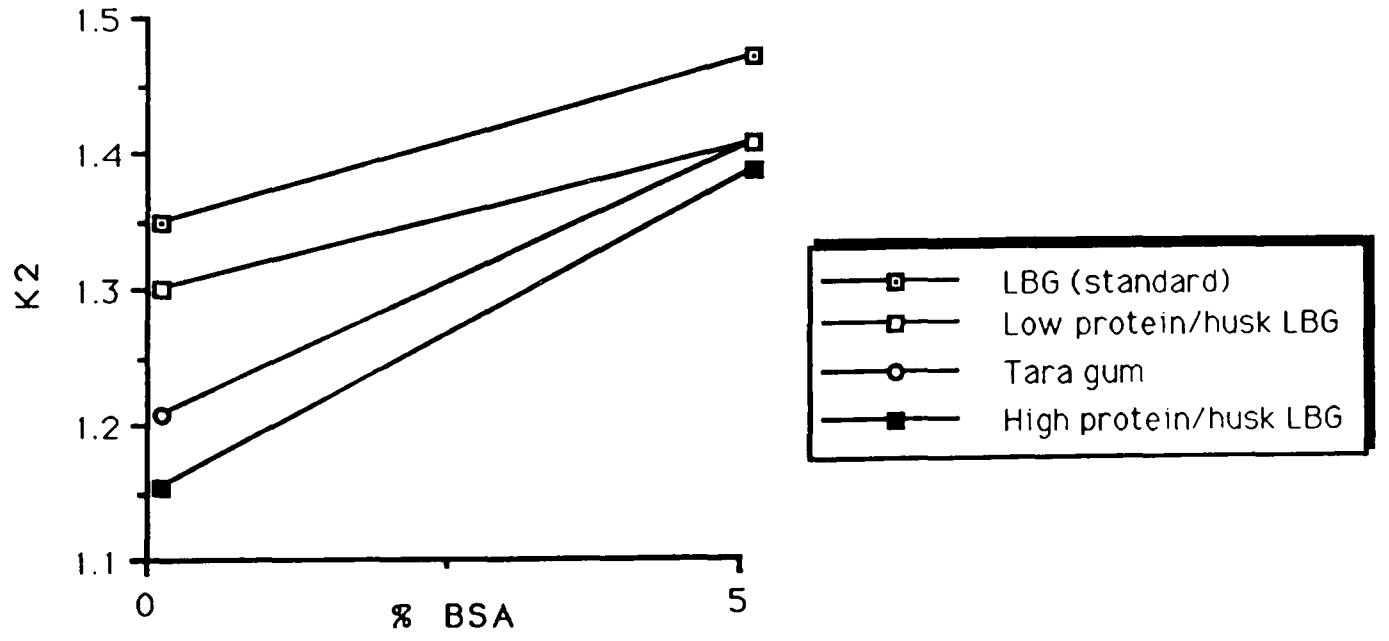


Figure 4.4.4. Effect of Galactomannan in the 0.5% carrageenan/0.5% galactomannan gel in absence and presence of BSA - k_2 values

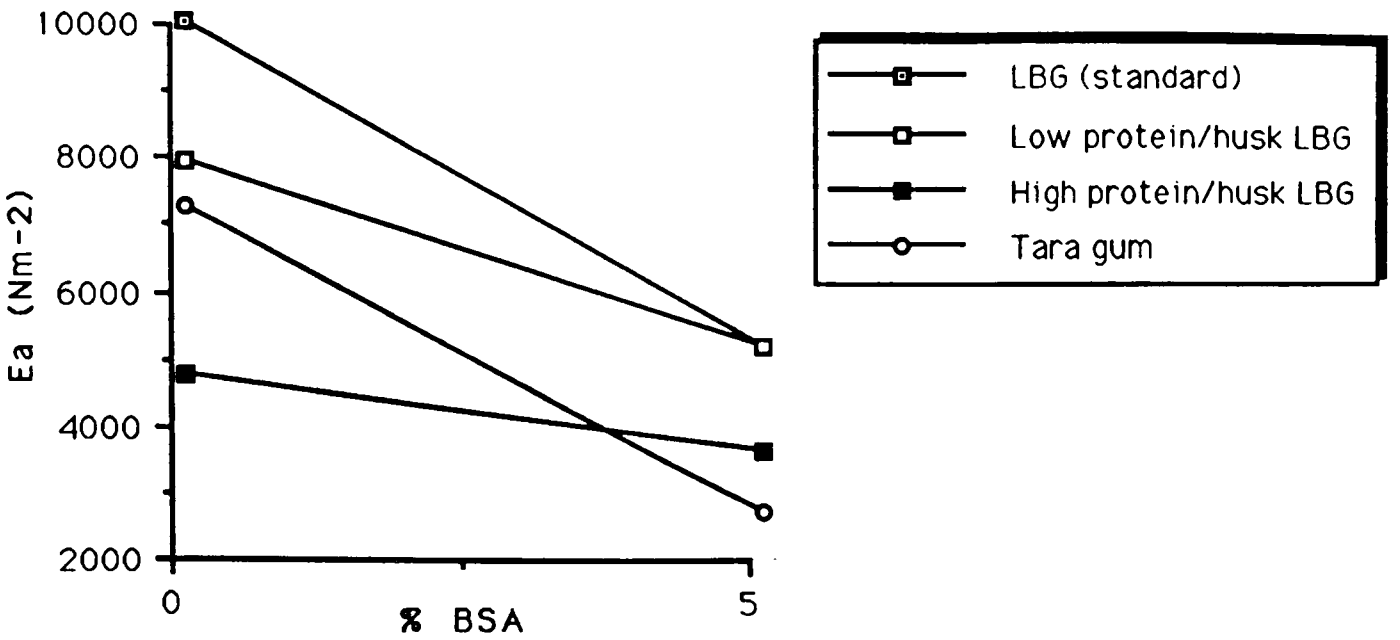


Figure 4.4.5. Effect of Galactomannan in the 0.5% carrageenan/0.5% galactomannan gel in absence and presence of BSA - E_a values

4.5 Discussion of Chapter 4

4.5.1 Phase Behaviour of Polysaccharide-Gelatin Gels

One very striking observation is made from the gelatin-polysaccharide studies - agar and gelatin gels phase separate and phase inversion occurs over a very limited range of gelatin concentration. The data for the melting points shows this best and indicates that this transition occurs over the 3-7% gelatin range. Over this region the gel changes from an agar-continuous network to a gelatin-continuous network with a final melting point characteristic of a gelatin gel. The same concentration range is also highlighted by the viscoelastic parameters, k_1 and k_2. The k_1 data indicates a sharp discontinuity in the short range order of the gel over the 3-3.5% gelatin range. The F_o data suggests a weakening of the gel structure immediately on first additions of gelatin with a continual decrease until the 5% gelatin level. After 10% gelatin addition, the overall strength of the gel picks up but never recovers to the level in the absence of gelatin.

This observation of phase inversion is not new. A number of workers have reported similar findings in the literature. The work of Clark and co-workers (1983) used microscopy and mechanical measurements to clearly show that agar and gelatin show phase inversion over a specific concentration range. At 1% agar, inversion of the continuous phase was seen using microscopy at gelatin concentrations of 2.5-3%. These workers also varied the concentration used for the agar component. At agar concentrations of 2%, phase inversion occurred between 5 and 6% gelatin which is consistent with the results of my studies. It was also noted that both the supporting and supported gel phases, irrespective of which occupies which role, were inhomogeneous. Microscopically small areas of gelatin network can be seen randomly distributed throughout the agar inclusions and *vice versa*. Also in agreement with this study is the work of Shiinoki and Yano (1986) who investigated agar-gelatin mixed gels with respect to viscoelastic behaviour as a function of composition. In addition, Moritaka *et al.*, (1980) reported a melting point transition for 0.5% agar at gelatin inclusion levels of 6.25-7.5%.

In the light of the above studies I believe the mechanism responsible for the results obtained for the agar/gelatin gel in this thesis are as follows. At zero gelatin addition an agar continuous network exists, producing the characteristic strong agar gel (as shown by high F_o values) of high melting point. On addition of gelatin, small, discrete regions of gelatin become dispersed throughout the agar phase and this has

the effect of weakening the agar network and reducing F_0. As the size of the discrete gelatin dispersions increase the disruptions to the agar network become greater and this continues to reduce the gel strength. The melting point at this level however is unaffected as the agar network still effectively dominates the gel; the inhomogenous nature - as suggested by Clark and co-workers - of the gelatin regions will probably also elevate the overall melting point of the dispersed phase. The melting point shows no significant effect until a gelatin concentration of ~3.5% is reached and this is followed by a more significant drop in F_0 value. The area occupied by the gelatin phase becomes more critical now and starts to exert an influence on the melting regime. The highest concentration of gelatin sustainable in the discontinuous phase, as indicated by melting point, is 5% which also correlates to the minimum in F_0 value. The mid-point of the (melting) phase transition is found to be 6% gelatin with the gelatin continuous phase attained at 7% gelatin. The discrete agar inclusions now impart no influence on the gel point which resembles that of a gelatin gel; the slight increase in melting point from 7% to 20% merely reflects an increase in concentration in the continuous gelatin phase. In terms of the F_0 value this reinforcement is shown through the increase to levels close to that of a 20% gelatin gel alone.

The carrageenan gels, whilst not undergoing any phase change as determined by the melting point, show minima in their F_0 values at 3% gelatin whereafter the gel strength increases to finally exceed that of carrageenan alone. Perhaps on first addition, the gelatin simply serves to affect the charge balance of the carrageenan without contributing any gel strength of its own but at higher levels the gelatin does add to the gel strength; the value of F_0 at 20% gelatin may in this case be an "additive product" of the two components. Even at the high levels of gelatin there must be a continuous carrageenan or carrageenan/LBG network to explain the melting point data.

So having established that agar and gelatin phase separate but carrageenan-gelatin and carrageenan/LBG-gelatin either do not phase separate or the polysaccharide network remains the continuous one at high gelatin levels, why should this be the case? Two explanations can be suggested:

(i) The entropically unfavourable counter-ion distribution that would result when a mixture of a polyelectrolyte and a non-ionic polymer phase separate prevents carrageenan/gelatin phase separation
(ii) the close vicinity of the setting temperatures of agar and gelatin compared with carrageenan and gelatin

The first idea is based on the fact that whereas agar has little polyelectrolyte character (i.e. few sulphate groups) in solution carrageenan behaves as a polyelectrolyte due to its higher sulphate content. For electrical neutrality to be preserved counter-ions must be present. If such a system phase separated in the mixed gel the counter-ions would remain in the carrageenan domain. Since gelatin possess little charge there would be an uneven distribution of counter-ions which is entropically unfavourable. The much less polyelectrolyte character of agar would result in a much lower counter-ion concentration difference between the two phases. If this mechanism was responsible for the different behaviour it might be expected that high levels of electrolyte would swamp this counter-ion effect for carrageenan promoting phase separation. (This was the reason suggested for the different behaviour found for the maize starch-caseinate system in buffer compared with water). Additional experiments by C. Le Bon investigating the effects of NaCl on the carrageenan/gelatin gel would shed doubt on this mechanism as being responsible since the addition of up to 0.2M NaCl did not promote phase separation as monitored by melting point.

The second idea focuses on the very different gel setting temperatures of carrageenan and agar. Whereas carrageenan gels form at around 60°C, agar gels form at much lower temperatures, around ~30°C (Ross-Murphy & Shatwell, 1983) -which is much closer to the gelatin-setting temperature of just below 30°C (Djabourov *et al*, 1993). It is this similarity of setting temperature that is crucial. As a carrageenan-gelatin mixed gel is cooled the carrageenan phase will have fully gelled before the gelatin starts to gel and therefore will have already established its own network. The gelation of the gelatin may then result in an interpenetrating network but in which the carrageenan remains as the dominant phase or alternatively, discrete regions of gelatin are dispersed throughout the continuous carrageenan phase. However on gelation of agar-gelatin mixed gels both components will be establishing their networks at roughly the same temperature and thus a phase separated system could result. At low levels of gelatin the agar can form the continuous phase slightly earlier than the gelatin which then forms the discontinuous phase, but as the gelatin concentration increases the situation is reversed. The microscopic evidence of Clark *et al.* (1983) for inhomogeneity of each phase would perhaps support this idea - if both components set at roughly the same temperature (and therefore time) it is possible that "contamination" of each phase with the other could arise.

4.5.2 Rheology of Polysaccharide-Gelatin Gels

It is quite clear that agar and gelatin phase separate but it is not quite so clear as to the situation for the gelatin-carrageenan mixed systems. Two possible extreme models can be identified: (1) phase separation involving gelatin inclusions in a continuous carrageenan network or (2) a carrageenan/gelatin network at all gelatin concentrations with complete mixing resulting in interpenetrating networks, as shown below in Figure 4.5.1

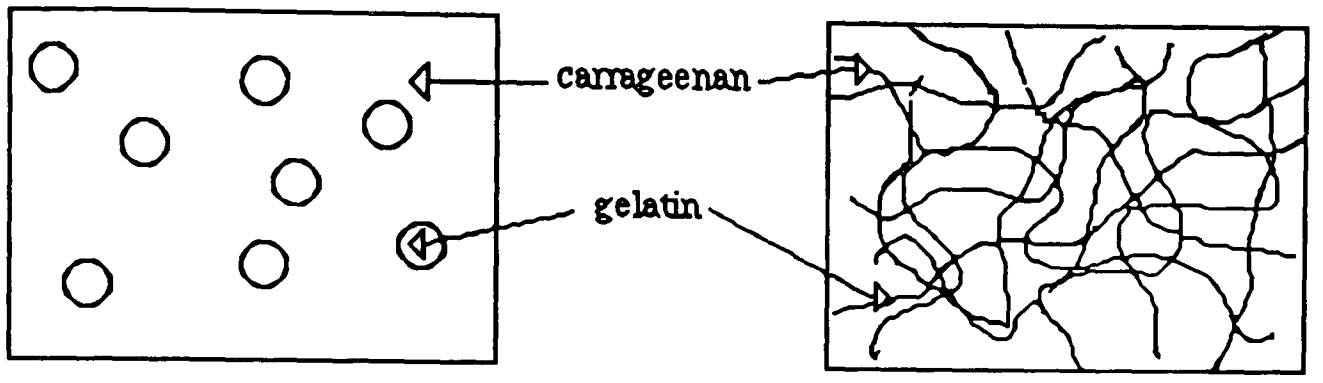


Figure 4.5.1 Schematic representation of the two possible outcomes of gelatin-carrageenan mixing: (a) phase separation (b) interpenetrated network formation.

Both would be expected to give the higher melting point of a carrageenan continuous gel. The difference between these two models is of practical importance because the presence of a large domain of melt in the mouth (gelatin gel) would be expected to have implications for both mouthfeel and flavour release. It would have been very useful to have used microscopy to distinguish between these two models. It should however be possible to come to some conclusions based on the rheological measurements. To do this we need to consider how the rheological properties of these two systems differ.

Since, over the whole of the concentration range, gelatin would be expected to give a weaker gel than carrageenan it would seem probable that isostrain behaviour predominates at all levels of gelatin inclusion - if the phase separated model holds. If the interpenetrating network model holds, however, and there is no interaction between carrageenan and gelatin then the modulus would be expected to be proportional to the number of network domains in the system. This would imply additivity between the two modulii. Indeed the Fo values would appear to be more consistent with this second theory.

If it is the case that phase separation occurs (and it is assumed that the phase volume for the two phases are equal) then the stronger carrageenan gel must remain the continuous phase; isostrain behaviour would then be observed over the whole of the gelatin range, assuming the concentration behaviour of the modulus is similar in both cases. This simple idea would give $\phi_{car} = \phi_{gel}$ and for a 2% carrageenan and 20% gelatin gel, result in effective concentrations of 4% and 40% for carrageenan and gelatin in their separate domains respectively. Since carrageenan gels first and the gelatin concentration is higher than the carrageenan concentration in almost all situations, it seems unlikely water would move from the gelatin to the carrageenan phase. This would result in a weaker gelatin gel than would be expected for the $\phi_{car} = \phi_{gel}$ situation and still give isostrain behaviour.

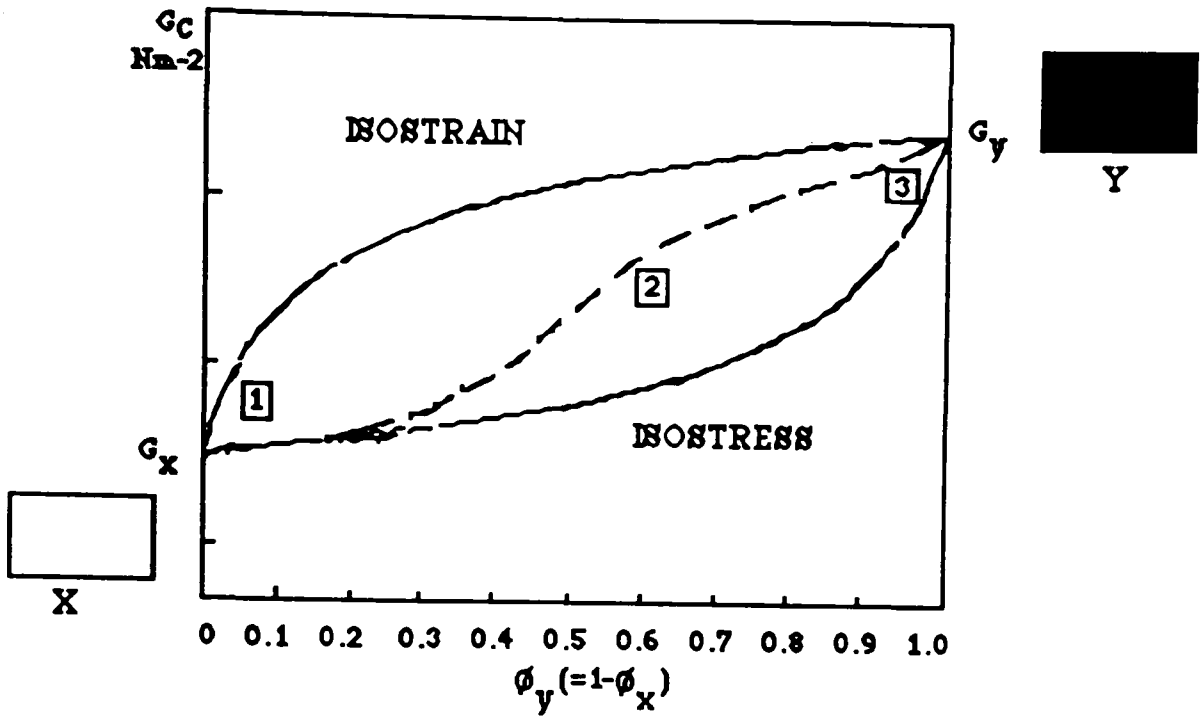
To quantitatively apply these ideas it is essential to perform small deformation dynamic rheological investigations on these systems, ideally also confirming the structure with microscopy. This is suggested in the further work of chapter 6.

In many investigations into phase separated mixed gels (Clark *et al.*, 1983; Walkenström & Hermansson, 1994; Clark *et al.*, 1982) the Takayanagi (1963) models are applied. These consider the mechanical behaviour of phase-separated mixtures of polymers of known volume fraction. This initially dealt with phases that showed perfectly elastic behaviour but progressed through to viscoelastic and plastic-phase and rubber-phase composite models. Considering the core ideas first: If X and Y represent the two components in a mixed gel, with each then having individual modulii of G_X and G_Y respectively, the extreme states of isostress and isostrain are outlined by:

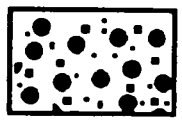
$$G_C = \phi_X G_X + \phi_Y G_Y \qquad \text{isostrain}$$

$$1/G_C = \phi_X/G_X + \phi_Y/G_Y \qquad \text{isostress}$$

where ϕ_X and ϕ_Y are the volume fractions of each respective phase. The isostrain, corresponding obviously to a homogeneous strain in the gel, is depicted by the parallel model and denotes the upper bound. The converse then follows for the isostress model; this gives a homogeneous stress, depicted by the series model and denotes the lower bound on the behaviour of the composite. Therefore if the weaker component occupied the continuous phase then the experimental curve would follow the lower bound. A transition between the lower and upper bounds is seen at phase inversion. This idea is shown schematically in Figure 4. 5.2.

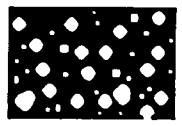


Microscopic detail from(for example) Clark et al., 1983



1. High concentrations of X

2. Intermediate (pre-phase inversion) behaviour



3. High concentrations of Y

Figure 4.5.2 Schematic isostress and isostrain bounds for a component X/component Y biphasic composite system (for simplicity no solvent is included). (From Morris, 1990) and the microscopic evidence of Clark et al, 1983 for such a phase inversion system

However since the largest component of a gel is the solvent, usually water, this should form part of the consideration also. Indeed the volume fraction and effective concentration of X and Y are determined by the way in which the solvent partitions itself between X and Y. To account for this, adaptations of the Takayanagi approach are made assuming that: (1) complete demixing of X and Y occur as a result of phase separation and (2) a fraction of solvent, denoted α, associates with X and $(1-\alpha)$ with Y. The solvent fraction depends on both the composition of the system and the relative affinity of each component for solvent, ρ, the latter variable given as:

$$\alpha = \frac{\rho x}{(\rho x + y)}$$

and the effective concentrations (that concentration of each polymer in their corresponding phases after gelation) becomes:

$$C_X^{eff} = \frac{100\,(\rho x + y)}{(100\,\rho + (1-\rho)\,y)}$$

$$C_Y^{eff} = \frac{100\,(\rho x + y)}{(100\ + (1-\rho)\,x)}$$

where x and y are the nominal concentrations of the polymers in solution and ρ and α are as described above. The second aspect of the volume fraction may now be defined as:

$$\phi_x = \frac{x\,(100\,\rho + (1-\rho)\,y)}{100\,(\rho x + y)}$$

$$\phi_y = \frac{y\,(100\,\rho + (1-\rho)\,x)}{100\,(\rho x + y)}$$

In this thesis although no small deformation rheology was carried out, viscoelasticity measurements at larger deformations were made. The stress relaxation experiments were analysed in terms of the viscoelastic parameters, k_1 and k_2. Before considering how these depend on gel structure it will be helpful to outline their meaning. The difference between the k_1 and k_2 parameters can be understood most easily in terms of the equation used to define them:

$$\frac{F(o)t}{F(o) - F(t)} = k_1 + k_2 t$$

At short times $k_1 \gg k_2$ and the equation can be written as:

$$\frac{F(o)t}{F(o) - F(t)} = k_1$$

which can be rearranged to:

$$\frac{F(o)t}{k_1} = F(o) - F(t)$$

and:

$$F(t) = F(o) - \frac{F(o)t}{k_1}$$

and ultimately to:

$$\frac{F(t)}{F(o)} = 1 - \frac{t}{k_1}$$

Hence the initial slope of a F(t)/F(o) plot will have a gradient of $-(1/k_1)$. Thus the more rapidly the force decays at short times the smaller the value of k_1 will be.

Conversely at long times $k_2 \gg k_1$ and the equation is written as:

$$\frac{F(o)t}{F(o) - F(t)} = k_2 t$$

and:

$$k_2 = \frac{F(o)}{F(o) - F(\infty)}$$

where $F(\infty)$ is the value of F(t) at long times. Following this through:

$$k_2 [F(o) - F(\infty)] = F(o)$$

and removing the brackets:

$$k_2 . F(o) - k_2 . F(\infty) = F(o)$$

and:

$$k_2 . F(\infty) = k_2 . F(o) - F(o)$$

and ultimately to:

$$F(\infty) = F(o) . [1 - (1/k_2)]$$

Thus the lower the k_2 value the smaller the final value of the force. So how do these two k parameters affect the shape of the stress relaxation curve? Below is shown the four model outcomes of each k_1 and k_2 parameter. The height of the first peak obviously here gives the value for F_0.

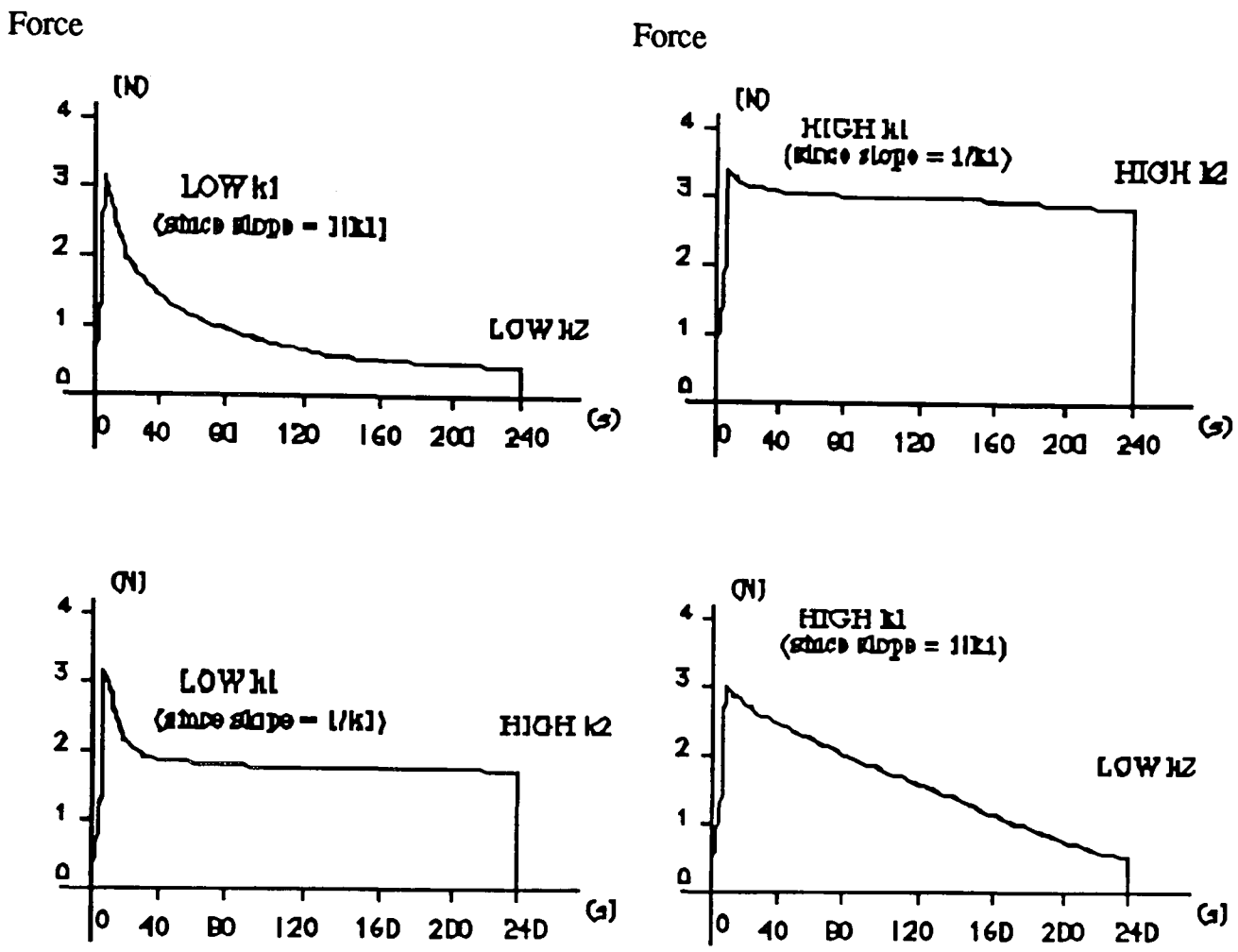


Figure 4.5.3 Model stress relaxation curves for materials with different values of k_1 and k_2

So how do my data relate to these ideas? Using the curves above gelatin represents the HIGH k_1/HIGH k_2 plot whereas agar is best represented by the LOW k_1/LOW k_2 curve. Composites of these two biopolymers at concentrations greatly removed from the phase inversion concentration will also follow the curve of the dominant biopolymer, since the continuous phase dictates the gel properties (see Figure 4.5.2). However at gelatin concentrations in the vicinity of the phase inversion concentration the resultant stress relaxation curve could be a compromise between the extreme curves and be represented by the remaining plots. Even within a biopolymer species, however, very different gel parameters can be obtained. This was seen clearly by the large discrepancy between the k_1 values of the control (zero protein) gels of section 1 and section 2 of this chapter. Further on, the critical nature of the agar purity is given as the most likely reason for this observation. Such variety of values are also quoted in the literature and a few examples are given below, to demonstrate the dependence

of k_1 on agar purity, concentration, preparation conditions (solvent) and testing conditions (deformation).

Table 4.5.1 Comparison of k_1 and k_2 data for agar gels @ 20% compression (unless otherwise stated)

Source of data	k_1(s)	k_2	Reason?
section 1 agar (2%)	5.5	1.20	low grade, "plate-count" quality ; prepared in water
section 2 agar (2%)	65	1.25	high grade, analytical quality; prepared in buffer
Ref. 1 (1.25%)	8.0	1.03	lower concentration; lower grade?
Ref. 2 (2%)*	67	1.54	"food grade"; prepared in water
Ref. 2 (1%)*	55	1.25	"food grade"; lower concentration; water
Ref. 3 (2%)	50	1.13	food grade; prepared in water
Ref. 3* (2%)	120	1.84	food grade; prepared in water

References:

1. Winwood *et al.*, (1986)
2. Nussinovitch *et al.*, (1989)
3. Nussinovitch *et al.*, (1990)

* tested at 10% deformation

The low values of the k_1 are probably indicative of fracture within the gel on a microscopic scale. This may reflect differences in the molecular weight of the samples and the presence of non-agar contaminants, in addition to actual concentration. In the light of these ideas we can perhaps consider which of the carrageenan/gelatin models (as discussed earlier) is most appropriate. For agar and gelatin a very distinct k_1 behaviour (Fig. 4.1.3) is observed. It would appear that the presence of small amounts of gelatin results in a sharp increase in k_1, suggesting that the microscopic fracture within the agar continuous phase is reduced. This may reflect the greater rupture strength of agar as a result of its effective concentration within its own domain. At the onset of the phase inversion region there is a sharp drop in k_1, probably indicating the weaknesses within regions of gelatin at low concentrations which start to influence the gel as it becomes continuous. The longer time structure reflected by the k_2 parameter is unaffected until the start of the phase inversion region where it shows a large increase. This may suggest that the final level of contact points within the agar phase is not affected by the low levels of gelatin inclusion but the rate

of breakage and reformation is. At high gelatin levels the long time k_2 parameter reaches that of gelatin alone as a result of domination by the continuous gelatin phase whereas the k1 value approaches that for gelatin alone but not attaining it.

For carrageenan and carrageenan/LBG we observe a very different pattern - a gradual increase in both k_1 and k_2 parameters with increasing gelatin concentration. This increase is more pronounced in the lower concentration carrageenan(/LBG) gel. Both parameters, even at the high gelatin inclusions, fall short of the value expected for gelatin alone and lower than that seen for the agar/gelatin system. It would therefore seem that at all concentrations the network is affected by gelatin and carrageenan. At low gelatin concentrations it is the longer time, k_2, parameter which is affected by gelatin rather than the short time, k_1, value. This is consistent with the idea that the shorter, stiffer carrageenan network chains in an interpenetrating network dominate k_1. This is easily understandable since it is only when the junction zones move or break that the influence of gelatin is seen. It would therefore be easier to interpret this data on the basis of the interpenetrating network model.

4.5.3 BSA/Blood Plasma-Polysaccharide Gels

Unlike the gelatin-polysaccharide systems where distinct conclusions can be made the interactions of BSA with the polysaccharides used here are not as clear. BSA presumably aggregates on heating forming inclusions within the polysaccharide gel. The results of the mixed carrageenan/LBG-BSA gel indicate that this is not the whole story.

The addition of BSA to a 2% carrageenan or 2% agar gel has no effect on the melting point. This would therefore suggest that no interaction occurs between the protein and the polysaccharide, the denatured aggregated protein merely existing as a small phase in the established polysaccharide gel. An interesting study by Clark and co-workers (1982) investigated the mechanical properties of BSA/agar co-gels. By changing the temperature regime of the mixture these workers found characteristic differences between mixed gels, based on which component gelled first. Allowing the agar to gel first produced a co-gel these workers termed agar(BSA) and allowing the protein to gel first produced a BSA(agar) co-gel. Microscopic studies showed the discrete domains formed by both components in the mixed gel and indicated the differences arising depending on which component gelled first and the protein concentration. At low (0.5%) BSA concentrations the appearance of the protein aggregates is similar in both types of gel; the only distinguishing feature being the

slightly larger size of aggregate in the BSA(agar) gel. This is the structure envisaged in my work. This latter observation was reasoned to be due to the lower constraints imposed by the agar sol than by the agar gel. However at higher protein concentrations (10%) microstructural differences become greater. The phase boundaries are less obvious in the agar(BSA) gel, perhaps due to the greater degree of intimacy of the microphases, whereas in the BSA(agar) gel a distinct agar phase is seen surrounded by the BSA gel phase. However having said this it was also concluded that the microstructure was heterogeneous - one phase also contained the other. But before a direct comparison is made with my results it should be noted that the Clark study employed mixed predissolved solutions of components, whereas I added dry mixtures of powders to the buffer. Since the solubility of BSA in limited in agar solutions (Clark *et al.*, 1982) this could be important.

In the case of the carrageenan/LBG gel an elevation of melting point occurs on addition of BSA particularly at BSA concentrations higher than 2%. At 5% BSA the melting point enhancement is 15°C. Why this should happen at first glance is not obvious especially when the large deformation stress relaxation data is considered. Although BSA causes a significant increase in melting point of carrageenan/LBG gels this is not translated into an increase in gel strength of the system although the reduction on protein addition is less than found for carrageenan alone gels. Since the carrageenan-BSA gel does not give the same resistance to melting it would appear that the LBG somehow interacts with the protein. The interaction could occur with (i) the galactomannan in the material *or* (ii) an intrinsic component of the LBG other than the galatomannan. If the interaction is with the galactomannan then it would be expected to be dependant on the G/M ratio.

By replacement of standard LBG by galactomannans of varying (a) G/M ratio and (b) protein/husk content the two ideas proposed above are investigated. Whilst the melting point in the absence of BSA is as would be expected given the G/M ratio this is not the case at 5% BSA; instead the protein content of LBG is important here. This is seen through the LBG>tara>guar melting point order in the absence of BSA. Of the LBG's the sample with the lowest protein content shows the highest melting temperature and this merely serves to reiterate this idea; a greater degree of refinement yields a higher proportion of galactomannan for interaction with carrageenan. At 5% BSA inclusions the highest melting temperature gel is that containing the high protein LBG. The melting point of the mixed gel in the presence of BSA decreases with decreasing LBG protein content. In addition the melting point observed with tara gum for the mixed system is consistent with its 10.5% protein content. Thus in the absence of any BSA the extent of interaction with carrageenan is

governed by the G/M ratio of the galactomannan as already known (see for example, Fernandes *et al.*, 1993) but with BSA included the melting temperature is dictated by the protein/husk content of the galactomannan. This would then strongly suggest that in the presence of BSA an interaction with the non-galactomannan component is involved.

The F_O values, which represent the peak force on the compression cycle, indicate that addition of BSA to all gels produces a similar trend of reduction in gel strength. Whereas the initial gel strength reduction, seen in the previous system on the addition of gelatin, reverses at the point where the gelatin contributes gel strength of its own this is not the case with BSA at sub-gelling concentrations for the protein. The extent of reduction is greatest on the carrageenan gel, whereby the "gel strength" halves at maximum BSA addition. Both agar and carrageenan/LBG gels show a smaller overall reduction in F_O but all three gels undergo the most significant decrease on first additions of BSA. It is interesting that the gel strength drop seen with the carrageenan gel at the higher BSA levels is prevented when LBG forms part of this polysaccharide blend. A good correlation exists between the F_O and breakstrength data. Whereas BSA reduces the breakstrength values of the carrageenan/LBG and agar gels to 50%, for the carrageenan gel this value is only 20% of the control value.

Although the evidence presented indicates that whilst any protein in the LBG contributes significantly to the melting behaviour of the carrageenan/LBG gel in the presence of protein it does not impart a particular improvement on gel strength. It can therefore be suggested that insoluble aggregates are formed as a result of a interaction between the BSA and some protein element of the LBG. The reason why this causes a melting point enhancement could be due to the increased volume occupied by the high-melting point phase.

Such an idea is further supported by the photographic results of section 3 where the inclusions of dried blood plasma (termed DBP here) in the carrageenan/LBG gel produces a "clearing" of the gritty material, representative of the insoluble material (husk/protein) in the LBG, from the surrounding carrageenan gel into the central protein gel. If this is believed to be a real effect (i.e. as opposed to being due to the supporting, higher viscosity phase of the gelled blood plasma) then it goes some way to providing evidence for the blood protein/LBG(protein) interaction. Whether the removal of the protein element of the LBG drags any galactomannan with it is unknown but if this was so than this could account for the loss of gel strength seen on addition of BSA and DBP.

Therefore the governing factor in the control (zero BSA) gels is the M/G ratio of the galactomannan (a polysaccharide-polysaccharide interaction) but in the presence of BSA the protein/husk content of the galactomannan becomes important (and possibly giving rise to a protein-protein interaction).

CHAPTER 5. RESULTS: BSA-ALGINATE

This short chapter presents the results of studies into the mixed system of bovine serum albumin (BSA) with sodium alginate in dilute solution using the techniques of microelectrophoresis and ultracentrifugation to study potential complex formation between these two macromolecules. The objective of the work was to characterize the interaction between heat or surface denatured BSA and an ionic polysaccharide. The results would be expected to have some relevance to the BSA-carrageenan mixed gels; because of the tendency for carrageenan to self-associate it was considered that a study of dilute solution carrageenan-protein mixtures would be too complicated. The microlelectrophoresis work is relevant to emulsion stability. Both techniques demand the concentrations of each component be low, as compared to the concentrations regimes found elsewhere in this thesis. The results of the two experimental techniques will be considered separately.

5.1 Section 1: Microelectrophoresis

Microelectrophoresis is the study of the movement of charged particles suspended in a liquid under the influence of an applied electric field. Figure 5.1.1 below compares the electrophoretic mobility's of BSA alone and in combination with alginate. Clearly at pH's just above the isoelectric point of the protein (pI = 4.9) the droplets are much more negatively charged in the presence of the alginate. This can be explained by the fact that proteins partially unfold on adsorbing to a surface and would suggest that at pH's above the isoelectric point such unfolded proteins can complex with anionic polysaccharides such as sodium alginate or carrageenan.

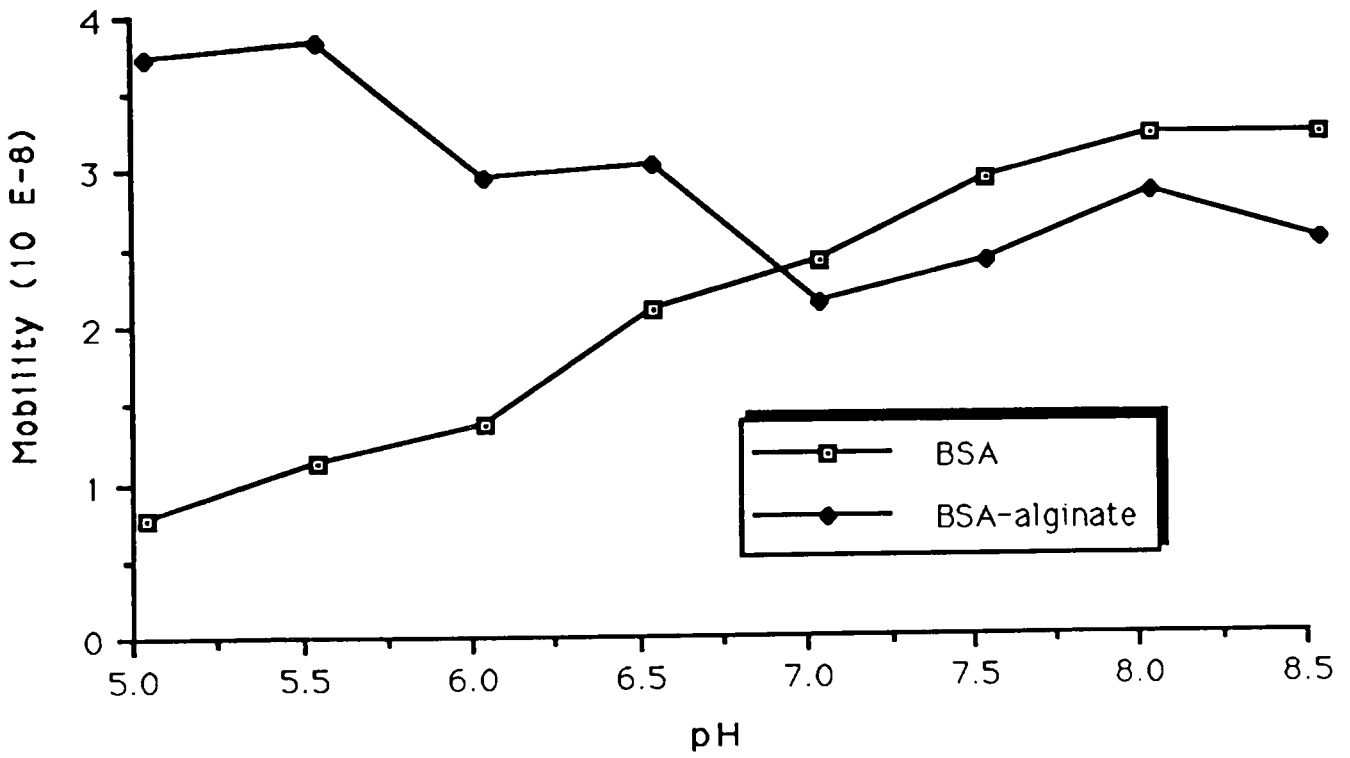


Figure 5.1.1. Electrophoretic Mobility of BSA both alone and complexed with sodium alginate

In order to see the net effect of electrophoretic mobility the difference between the two cases is illustrated in Figure 5.1.2. This data would suggest that alginate only interacts with BSA at the oil droplet surface at pH's up to about 6.5 to 7.0

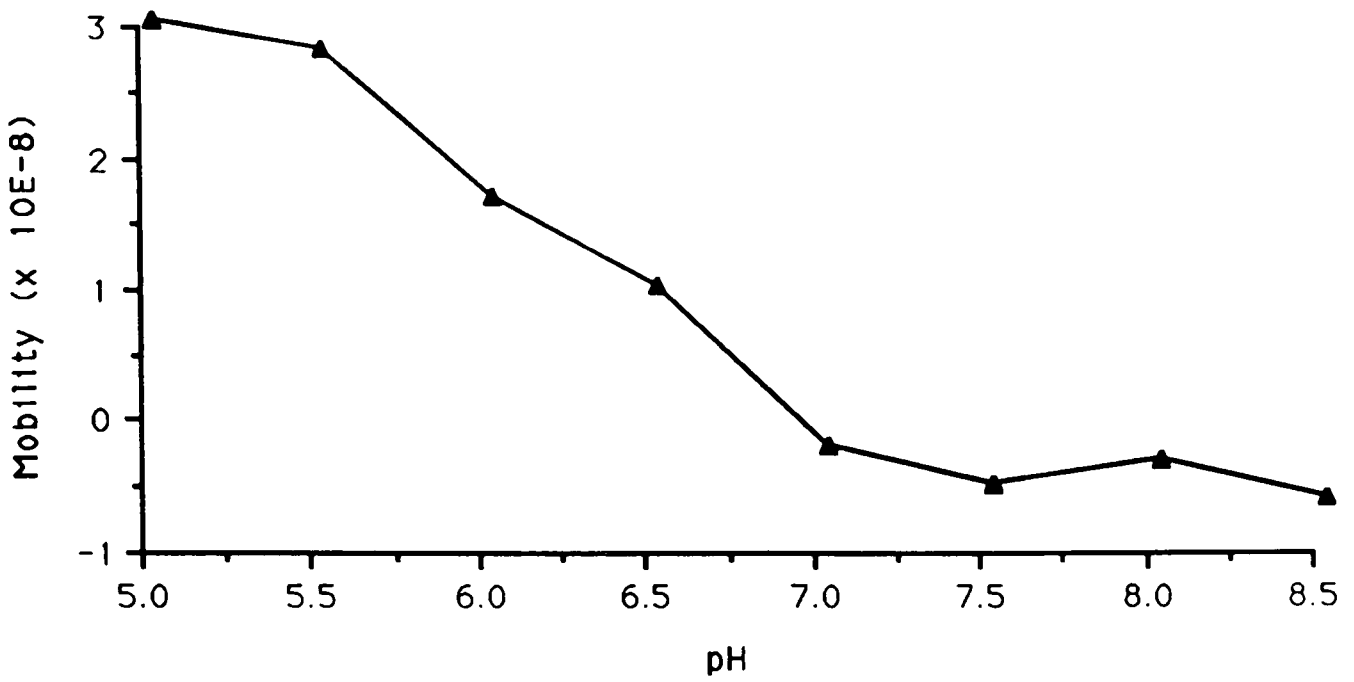
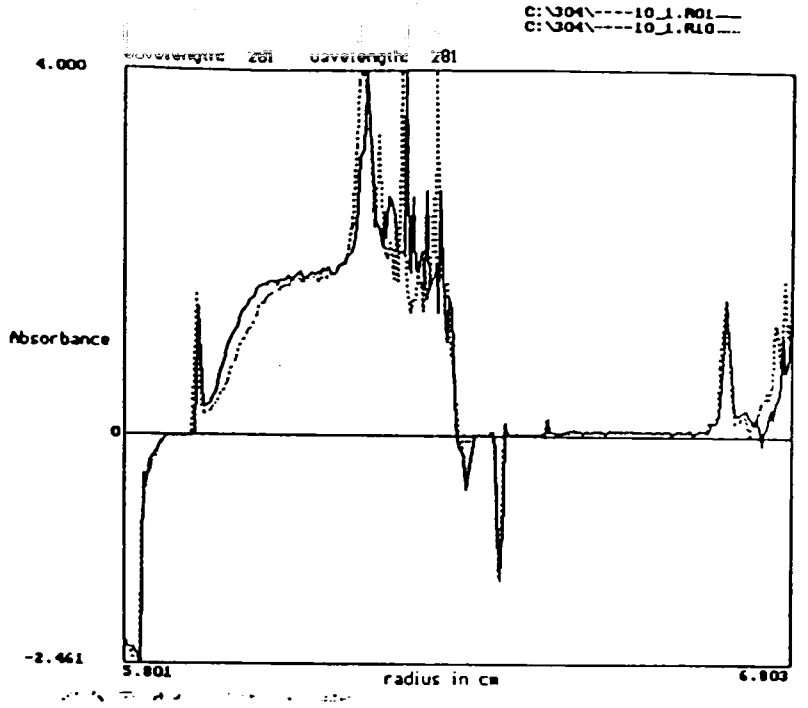


Figure 5.1.2. Difference in electrophoretic mobility between the BSA-alginate complex and BSA alone.

5.2 Section 2: Analytical Ultracentrifugation

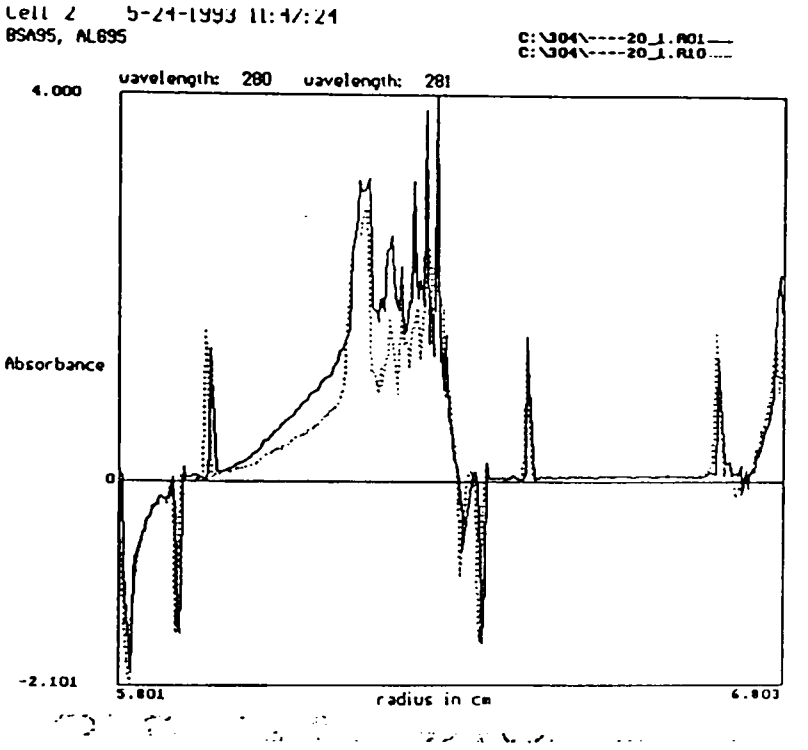
The presence of large white aggregated material , presumably an interaction product, in the mixture after 95°C heating was not present in the 85°C treatment. Figures 5.2.1a-c indicate the temperature sensitivity of the system. The s_{25} values calculated from the 1st and 10th scans of the sedimenting boundaries show that at 85°C the value obtained for the mixture corresponds almost exactly to that of BSA alone. However under 95°C heating, the sedimentation of the interaction products was too fast for a record of the sedimenting boundary to be established; this would strongly suggest the existence of very large aggregates.



(a) BSA - LHS

S value = 66S

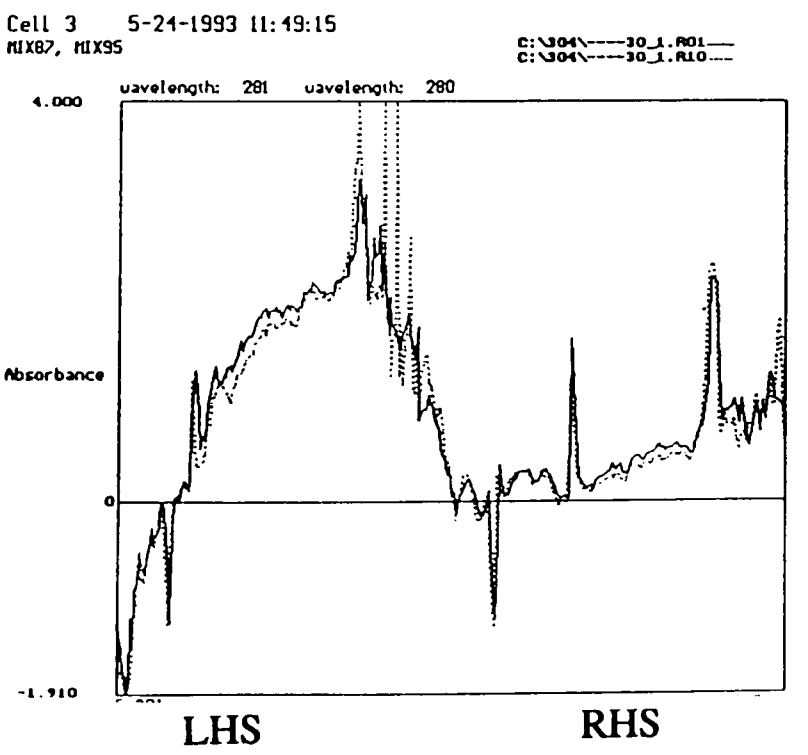
Alginate - RHS



(b) BSA - LHS

S value = 122S

Alginate - RHS



(c) BSA/Alginate @ 85°C - LHS

S value = 68S

BSA/Alginate @ 95°C - RHS

S value = not determinable

Figure 5.2.1 (a) Single components at 85°C heating; (b) Single components at 95°C heating; (c) Mixed components at both heating temperatures

5.3 Discussion of Chapter 5

Through this study it has been shown that BSA, whilst a macromolecule with a net negative charge, can complex with sodium alginate, an anionic polysaccharide. This is achieved by the unfolding of the native globular protein structure to reveal local sites of positive charge. Such unfolding of a protein molecule occurs on denaturation and this can be induced by the two different methods used in this study, namely thermal denaturation or that brought about by adsorbing to a surface.

BSA has been shown to interact with alginate on heat denaturation before (Kelly *et al.*, 1994) but this study also highlighted the temperature sensitivity of the interaction; whereas the complex formed at 95°C was accounted for in terms of an electrostatic interaction between the two components at 85°C the resultant species was believed to be simply due to a weight average of free alginate and aggregated BSA. Independent microelectrophoretic studies by Ward-Smith and co-workers (1994) on oil droplets in the presence of BSA/haemoglobin mixtures suggest that the alginate is adsorbed onto the protein-water surface.

The results of Figure 5.2.1 indicate the temperature sensitivity of the system. At 85°C heat treatment the sedimentation coefficient of the mixture is 68S and this value simply presents that of the individual components heated to the same temperature; the s_{25} value of the BSA in isolation is 66S. However at the higher heating temperature of 95°C the s_{25} value of the mixture cannot be determined due to the rapid sedimentation of the (presumably electrostatic) complex. The significant stages of BSA denaturation and aggregation can be seen by the doubling of the s_{25} value at the higher heating temperature. Recent work by La Rosa and co-workers (1994) also found this temperature dependence at high temperatures. Using DSC and scanning dilatometry (DS) they showed new phenomena in the range 80-97°C on the thermal denaturation of BSA, and in particular that in the range 85-93°C aggregation is the major force but at >93°C the system undergoes a spatial rearrangement accompanied by a sudden reduction in specific volume. At the pH ~7.0 used in this study BSA will be in its monomeric form prior to denaturation (Kinsella & Whitehead, 1989).

Thus the exposure of positive sites of the unfolded protein allows an electrostatic interaction with the negative charged alginate. These positively charged binding sites of BSA are the arginyl and lysyl side chains which project more from the surface than the negatively charged glutamyl and aspartyl side chains (Nozaki, *et al.*, 1974). In addition BSA has a hydrophobic pocket (Tanford, 1970), located in the second

domain, or more precisely, at amino acids 198-389 (Peters, 1985). Whilst being important in the binding of other species (i.e. one BSA molecule retains one enantiomer molecule assuming no steric hindrance etc. (Jacobson *et al.*, 1993)) this would not provide a potential binding-site in the case of charged polysaccharides. BSA contains one free thiol group which is inaccessible to solvent at or below neutral pH (Papiz *et al.*, 1986; Kinsella & Whitehead, 1989). The overall hydrophobicity of BSA is 995 kcal/residue; a value lower than other globular proteins such as α-lactoglobulin and β-lactalbumin (Eigel *et al.*, 1984).

In terms of thermal denaturation Takeda and co-workers (1993) showed that a reduction in helicity of BSA depended on the fragment and the temperature; for the Asp^{1} -Asp^{306} fragment helicity was reduced from 59% helicity before heating to 46% at 65°C (Takeda *et al.*, 1993). Thus a substantial proportion of "native state" still exists at temperatures close to the denaturation temperature of BSA and the temperature dependence is again demonstrated.

In the microelectrophoretic study the underlying feature is that proteins will adsorb at air-water and oil-water interfaces because of the presence of both polar and non-polar groups (Mitchell, 1988). At pH's close to that of the pI of BSA (pI = 4.7-4.9 (Eigel *et al.*, 1984)) the protein molecule is electrically neutral and therefore will not migrate in an electric field. This is demonstrated by the minimum in electrophoretic mobility shown in Figure 5.1.1. As pH is increased the overall charge on the protein increases. The mobility of the molecule steadily increases at this point. This is detected by the movement of oil droplets, sufficiently large to be observable under the microscope. Although at high pH the net charge on a protein will be negative, local regions of positive charge will still be apparent. This may become more accessible if the molecule reorientates itself in the process of becoming more charged. BSA is different to other proteins in that it can "swell" or expand in acid or alkaline media, and thereby behave like a polyelectrolyte, unlike most proteins which do not do this even at several pH units away from their pI (Yang, 1961). The apparent absence of an interaction above pH 6.5-7.0 suggests that decrease in the extent of the positively charged patches on the protein or alternatively the magnitude of the overall repulsion between negative charges is sufficient to prevent the interaction.

In order to be a good emulsifier the protein must diffuse and adsorb relatively rapidly at the interface and for this to happen the molecule must possess some flexibility. The tertiary structure of BSA is arranged in three domains (Peters, 1985) to form a prolate ellipsoid (Squire *et al.*, 1968) with strong association between two of the domains. The latter statement is based on the accepted good homology with human serum

albumin (HSA) (Carter *et al.*, 1989). Although BSA is a big molecule and has 17 disulphide bridges it is still relatively flexible due to its high helix content and this multi-domain nature (Suttiprasit *et al.*, 1992). Indeed it is the multi-domain structure which is responsible for its anomalous behaviour under denaturation conditions as well as its interfacial activity (see Sutisprasit *et al.*, 1992).

The binding properties of BSA are well known: for example this protein binds many drugs or low molecular weight bioactive substances (Peters, 1985) and has many applications in pharmaceuticals. Blood adsorption onto artificial solid surfaces in drug delivery systems for example (Peula & Nieves, 1993) necessitates an understanding of the mechanisms involved. More relevant to this thesis however is the interactions that mixtures of BSA-alginate undergo in food systems. The surface activity of proteins is important in many food applications. The accumulation of a protein at an air-water or air-oil interface produces an interfacial layer that results in stabilization of a foam or emulsion by reducing interfacial tension and preventing collapse and coalescence (Halling, 1981). In many food products, either because of the intrinsic ingredients or through the addition for other functional properties such as thickening, polysaccharides are often also present and thus how these two components interact is important. In food processing too, information on surface properties should also be known in order to reduce fouling of membrane and heat exchange surfaces (Kim & Lund, 1989).

CHAPTER 6. OVERALL CONCLUSIONS AND SUGGESTIONS FOR FURTHER WORK

6.1 Conclusions

- The action of caseinate on potato starch is through a non-specific ionic strength effect. The caseinate exerts a marginally stronger effect on the viscosity of the potato starch when hydrated before or at the same time as the starch. However additions of caseinate to the starch in a dry form and at very advanced stages of pasting still produce almost the same massive loss in viscosity.

- The response of maize starch is very dependant upon the solvent used. This is attributed to different mechanisms of interaction: the high salt content of the buffer- pasted system encourages phase separation through the swamping of the unfavourable entropy decrease that would otherwise result. Thus the amylose is maintained within the granular phase and, as a consequence of this retrogradation, is also prevented at high caseinate levels. In water-pasted systems the mechanism is thought to consist of interpenetrating caseinate, swollen starch and released amylose allowing retrogradation to occur as normal.

- Some preliminary experiments to determine if caseinate prevented "cook-out" of soluble starch showed little difference from the control in the absence of caseinate.

- Gelatin and agar mixed gels undergo phase separation with the region of phase inversion occurring at 3-5% gelatin when the agar concentration is 2%. This is not seen with carrageenan-gelatin or carrageenan/locust bean gum (LBG)-gelatin.

- BSA at levels up to 5% gives a marked increase in the melting point of mixed carrageenan/LBG gels. This is not seen when the protein is added to carrageenan alone or agar gels. BSA addition results in some decrease in the breaking strength.

- Results obtained on the replacement of standard LBG in the carrageenan/LBG mix with a range of other galactomannans suggests that an interaction between the protein/husk content of the LBG and the BSA is possibly responsible for the elevation of melting point seen.

- Visual observation of the mixed gels containing dried blood plasma and carrageenan/LBG suggests that the insoluble material in LBG (presumably protein/husk) is "cleared" from the carrageenan gel when the protein is incorporated into the gel. Such an interaction between the protein of the LBG and the blood protein lends further support for the previous conclusion.

- At pH's above its isoelectric point and whereby it is a negatively charged macromolecule, BSA can complex with anionic sodium alginate. Microelectrophoresis shows that this occurs at the oil-water interface and ultra centrifugation demonstrates that this also occurs in bulk solution provided the protein is denatured.

6.2 Suggestions for Further Work

As with any research project, time is a limiting factor. In the case of this PhD, the interest of the industrial companies supporting the work meant that a broad range of topics had to be investigated. A number of interesting follow-up investigations then become apparent based on the results of this study. These include:

- Confirm the reversible swelling/de-swelling on the gelatinised potato starch granule. Since Table 3.1.1 and Figure 3.1.10 showed that addition of unhydrated caseinate powder to the already swollen potato starch produced a similar massive drop in viscosity to those systems where caseinate is included in solution initially. It would be interesting to dialyse ions out of the system and see if the viscosity recovers.

- Modelling of the effect of ionic strength on potato starch using theories developed for the swelling of polyelectrolyte gels.

- Gain further evidence for the idea postulated that the structure of the maize starch-caseinate system depends on ionic strength. Techniques which would be appropriate include light and electron microscopy and dynamic rheology to follow retrogtradation kinetics. These would be followed over a range of salt concentrations.

- Extend the maize starch/caseinate investigations to milk systems and examine the potential for freeze-thaw stability in such food products. If retrogradation is prevented by caseinate inclusion then freeze-thaw stability might be expected to be enhanced.

- As discussed in Chapter 3 it would have been better to have performed a carbohydrate analysis rather than a total solids measurement on the centrifuged starch supernatants in order to clarify the solubility data obtained. This could be then be extended to the high caseinate levels of the maize starch system.

- Microscopy of the carrageenan-BSA/gelatin gels to complement the data of Clark *et al.* (1983; 1984) showing micrographs of the agar-BSA/gelatin gels respectively. Can what I suggest from the melting point and stress relaxation data be confirmed by microscopy studies?

- Microscopy of the blood protein/carrageenan-LBG gel. Investigations into whether the galactomannan or any other element of the LBG (i.e. not the protein/husk component) is responsible for the interaction postulated with blood proteins. Attempts could be made to remove the protein element of the LBG and thereby eliminate any possibility of protein involvement in the interaction. Use of LBG fractions of differing solubility's to ascertain if the component reacting with the blood proteins is contained within the higher temperature solubility fractions. Either way, aim to elucidate the mechanism of interaction (i.e. what is the nature of the protein-protein interaction, if indeed there is one?)

- Further studies on the large deformation viscoelasticity of mixed gels: effect of actual deformation. All stress relaxation tests were performed at 20% deformation in this study but perhaps more information could be gained by deforming to different levels. Table 4.5.1 shows that the degree of deformation does effect the stress response of agar gels.

- An investigation of the small deformation rheology of the mixed carrageenan-gelatin system. In particular, is it possible to ascertain a gelatin melting within this system? Modulus measurements at different temperatures should allow the contribution of each component to the overall modulus to be established.

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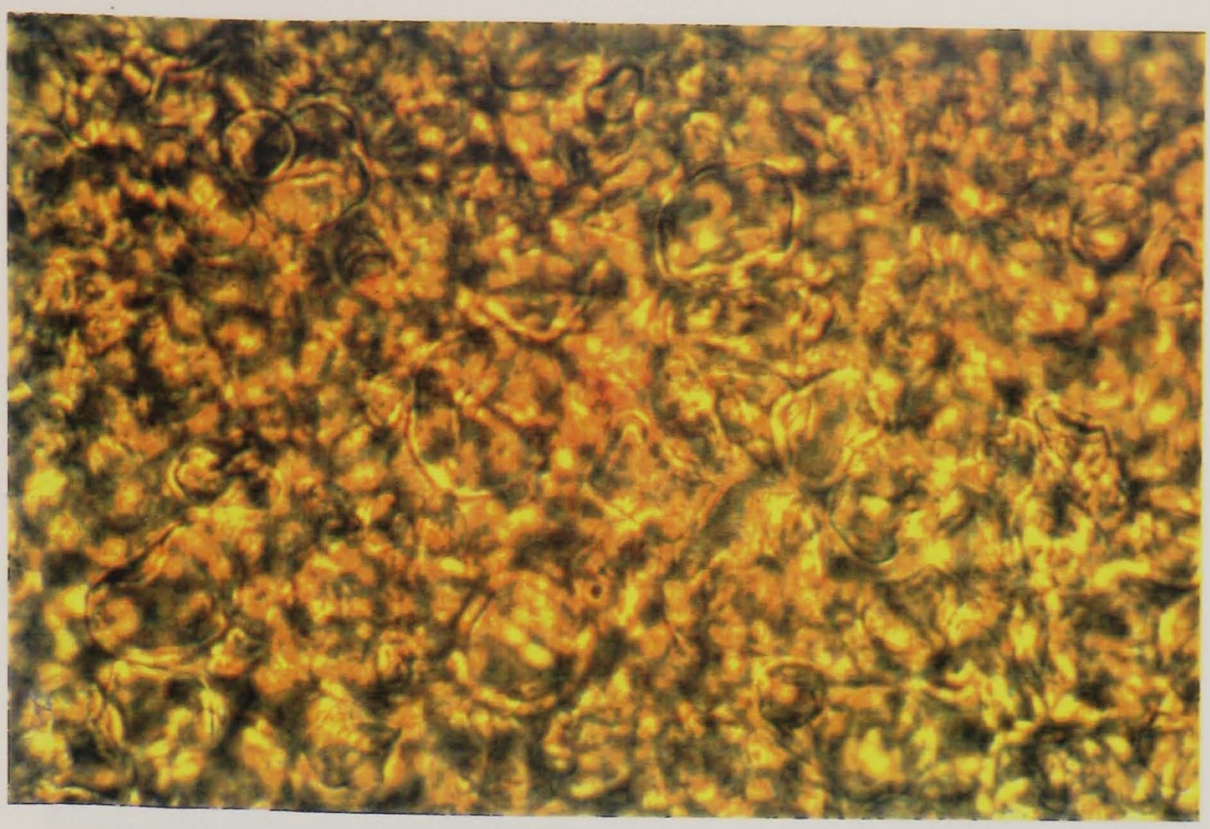
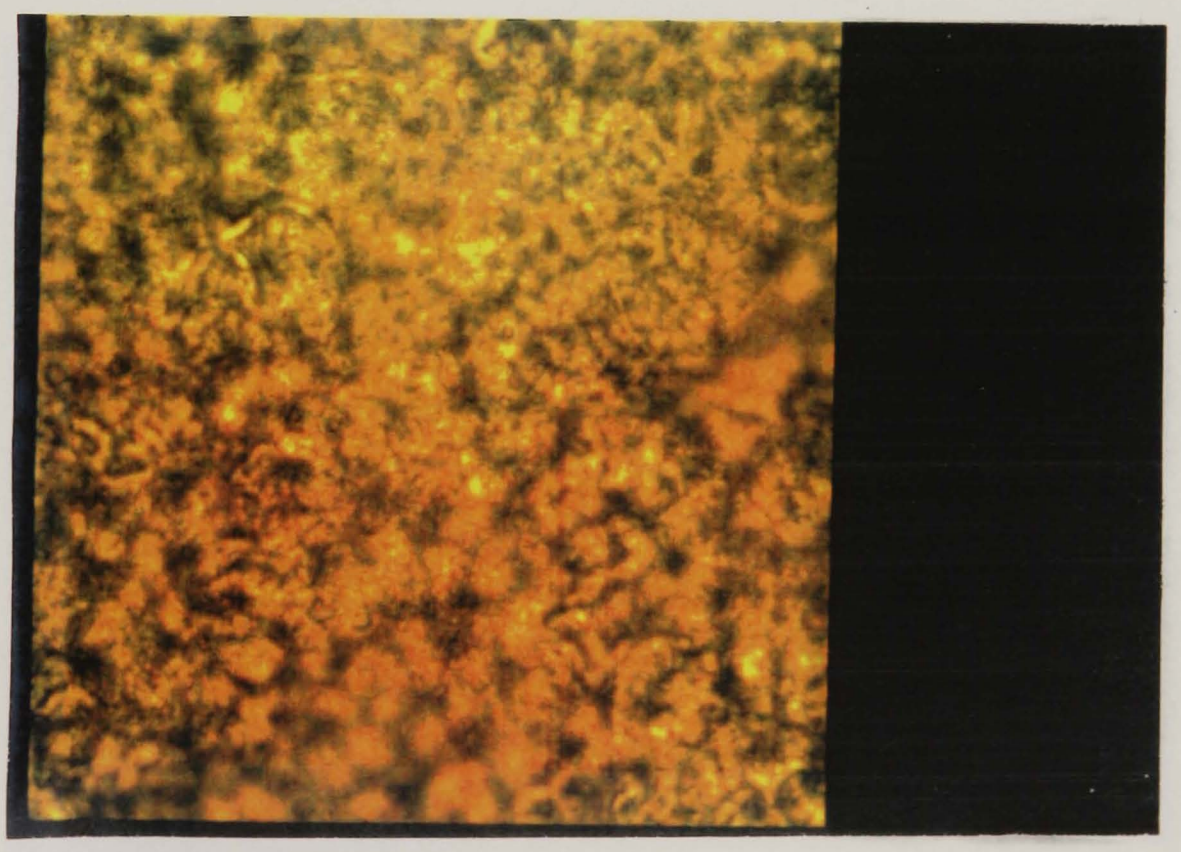
APPENDICES



(a)

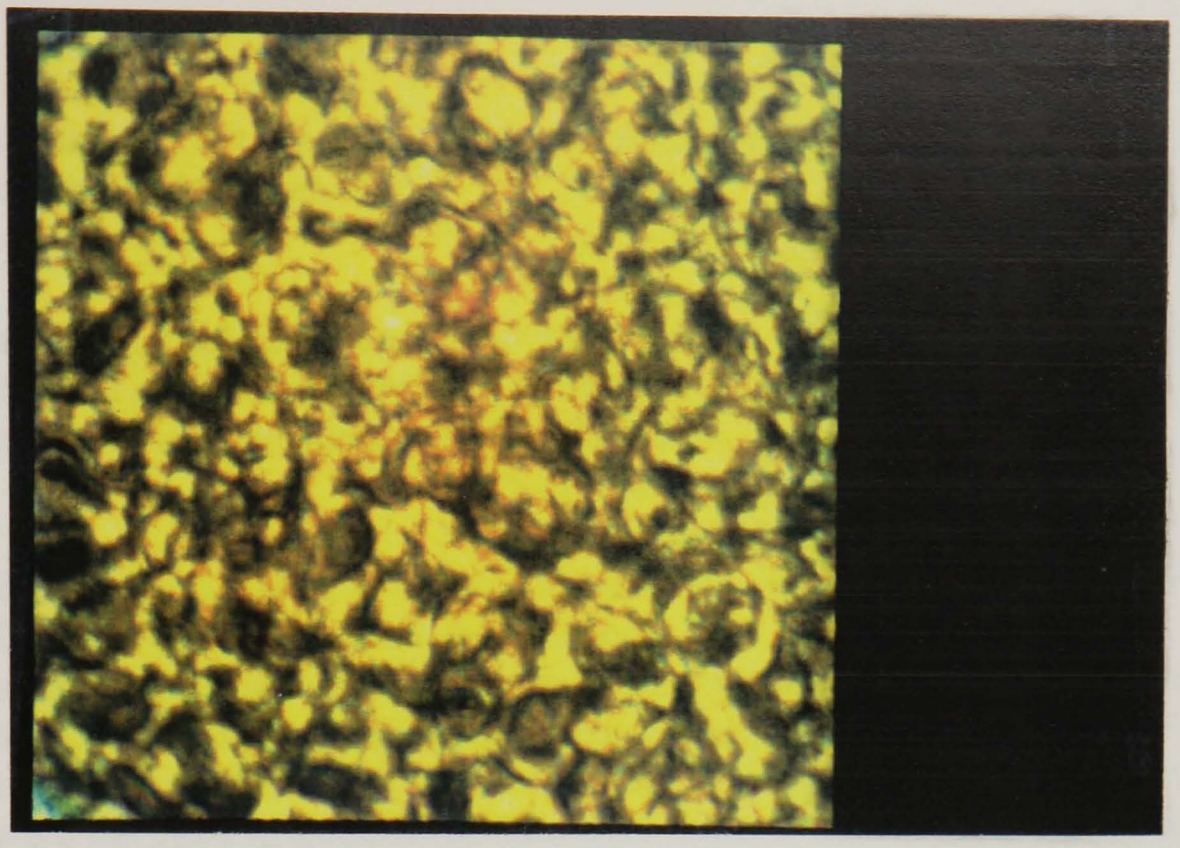
50μm

I————I



(b)

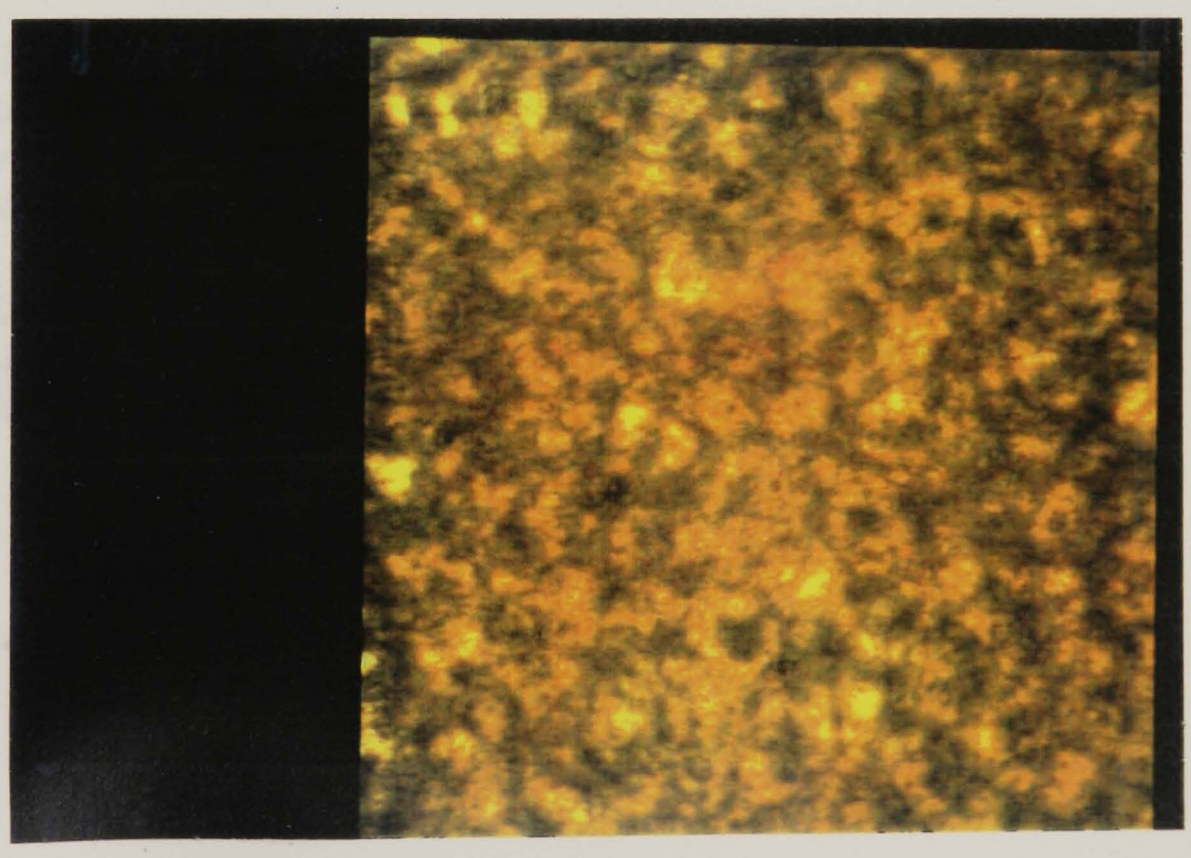
Appendix 1: Figure A.1. Micrographs of 4% maize starch pasted in (a) distilled, deionized water and (b) 5% caseinate in distilled, deionized water



(a)

50μm

I————I



(b)

Appendix 2: Figure A.2. Micrographs of 4% wheat starch pasted in (a) distilled, deionized water and (b) 5% caseinate in distilled, deionized water

The Addition of Cereal and Caseinate in Meat Products

Aim

To determine if a synergy exists between caseinate and starch by using cookout, chunk strength and rheological methods.

Method

A standard meat emulsion (30kg) was prepared. The entire batch was frozen, so it could then be defrosted in three separate 10kg batches as required. The three separate products made consisted of 85% meat emulsion and (1) 15% Potato Starch or
(2) 15% Wheat Starch or
(3) 15% Wheat Flour
To this sodium caseinate (0-5%) was added on the basis of the flour or starch (15%) so the amount of meat emulsion used remained constant.

The starch/flour and caseinate were blended together and then mixed with the meat emulsion using a large Hobart mixer for approximately five minutes. The emulsion was canned and the weight of each can noted, this was in order for the cookout to be calculated after processing. The cans were sterilised in a bench top autoclave under a standard process. A sample of each of the raw emulsions was collected and run on the Carri-med rheometer using a temperature sweep from 20°C-80°C (for Carri- med procedure see *Appendix II*).

The post-process emulsion was used to calculate percentage cook-out (for procedure see *Appendix IV*) and the break strength of the emulsion was determined using the Instron texture analyser (for procedure see *Appendix III*).

Results

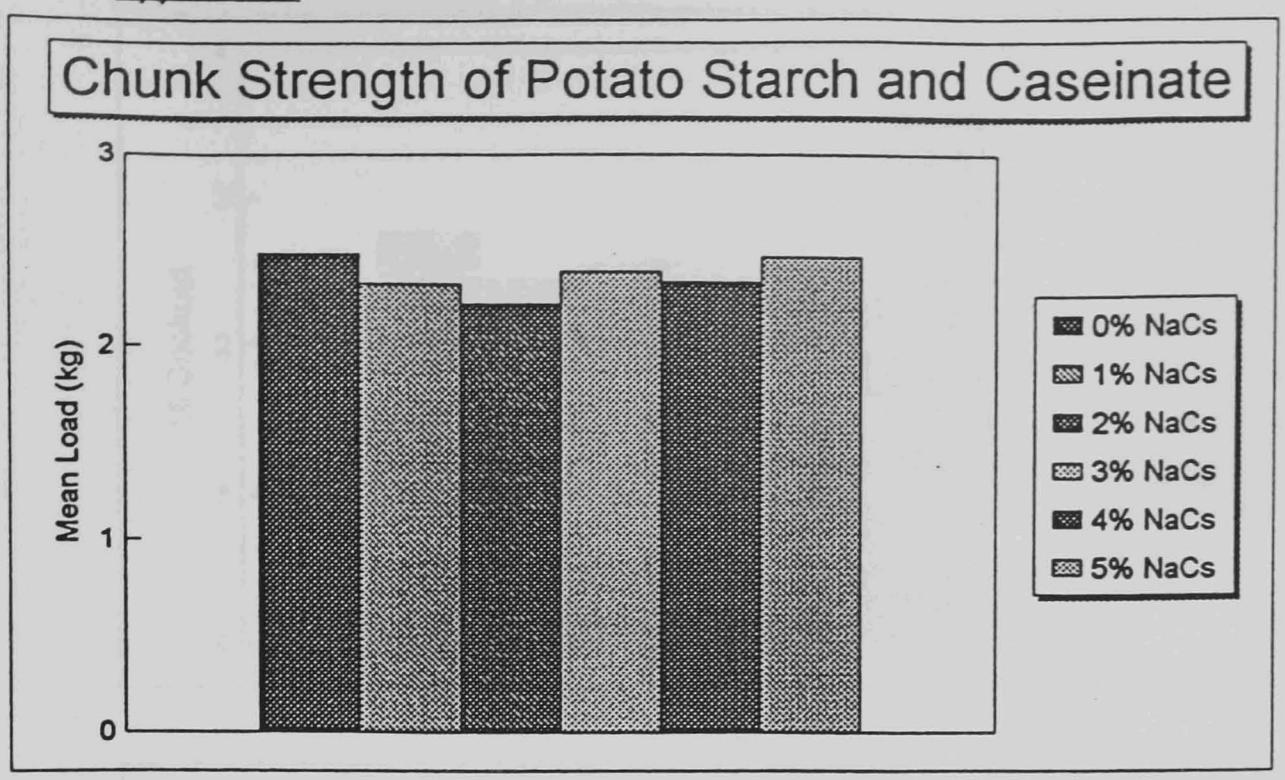
The ability of caseinate and starch/flour to synergise with one another was evaluated. Comparing the viscosity profiles of the different emulsions, cookout and instron values (for graphs see *Appendix I*) there were small variations in the samples but these were not significant or consistent enough to suggest that a synergy exists.

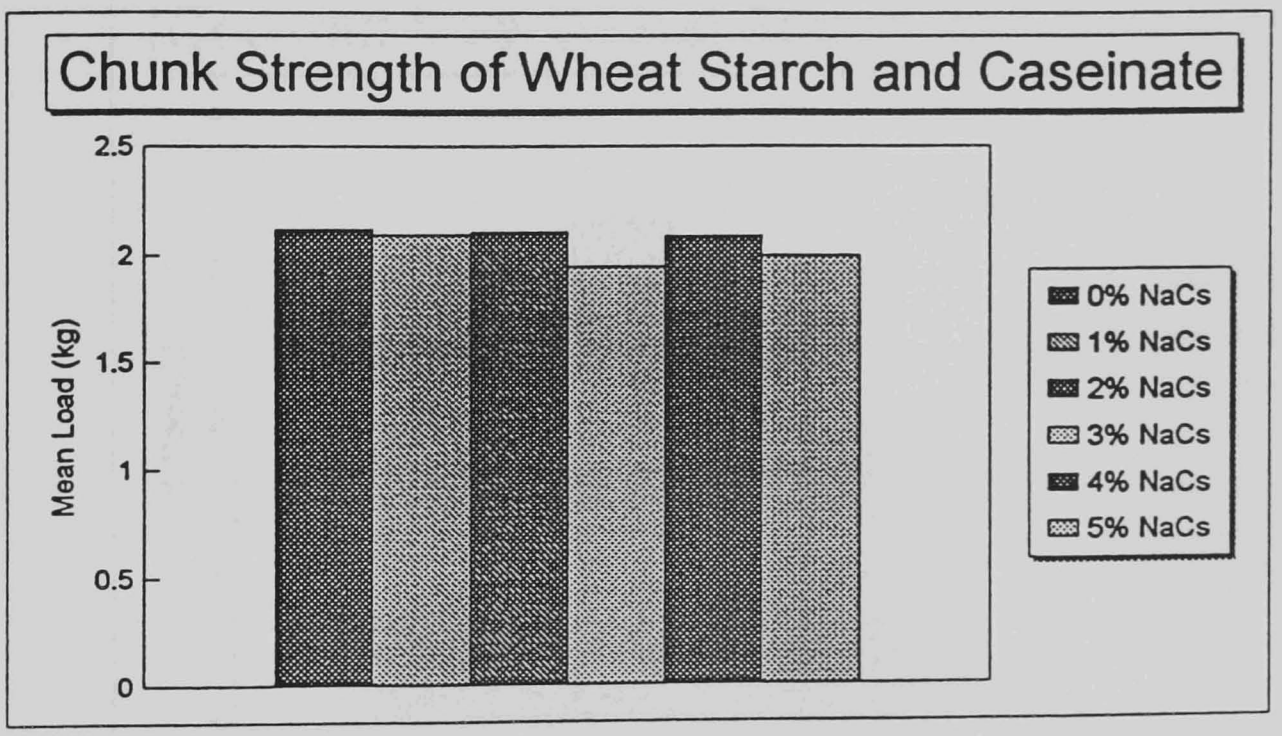
Conclusion and Recommendations

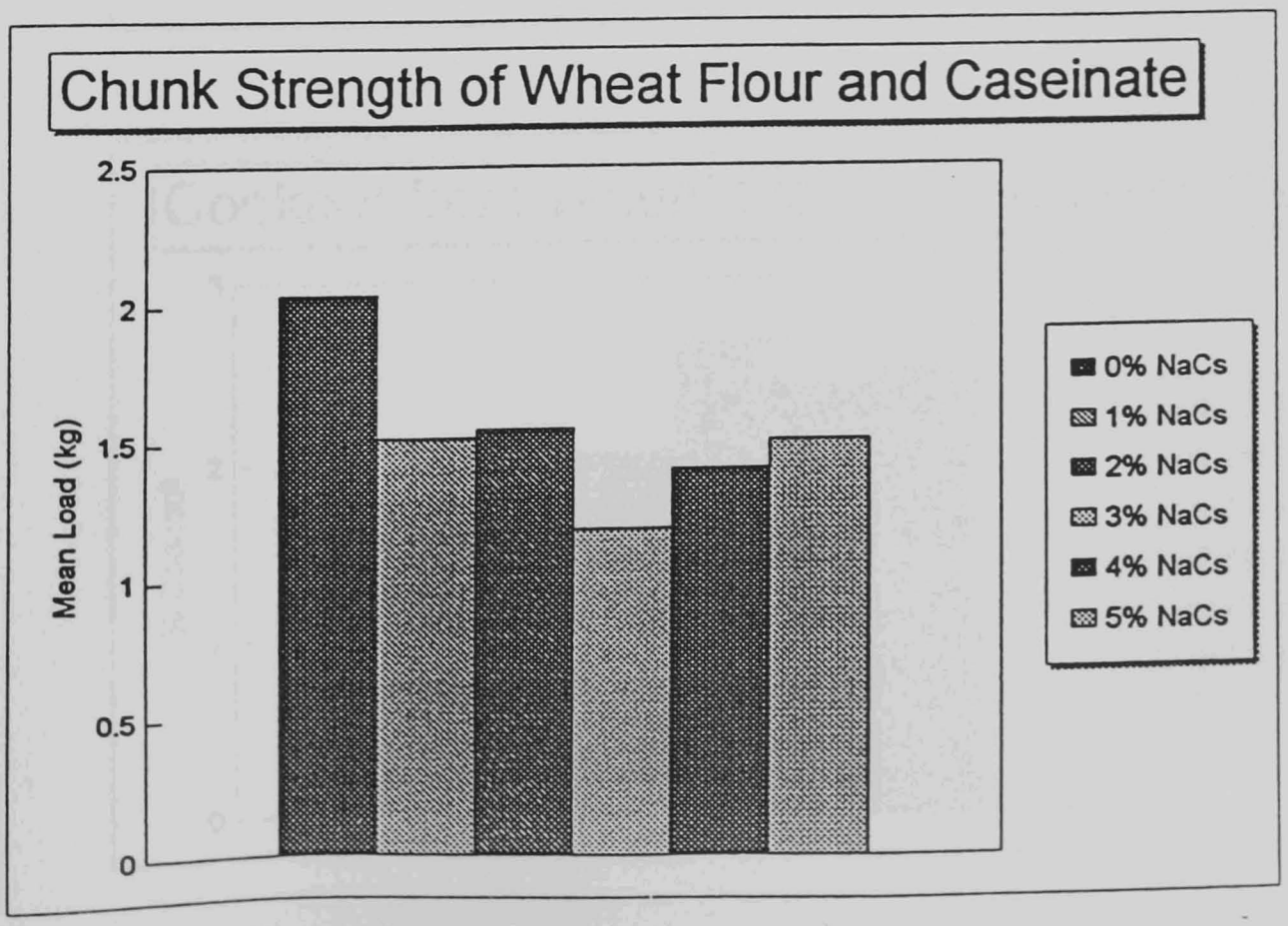
At the level of caseinate and starch/flour used in this experiment there is no synergistic relationship between the two.

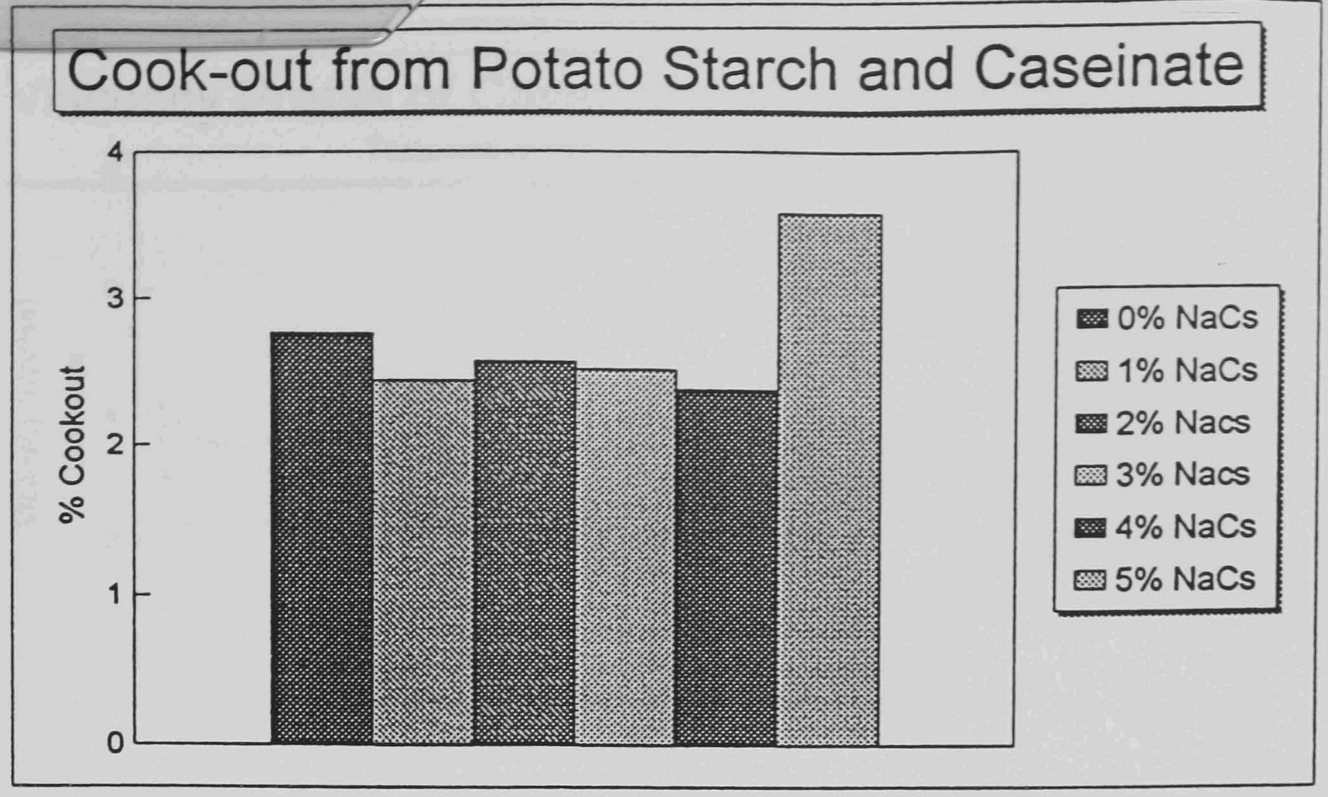
Appendix:

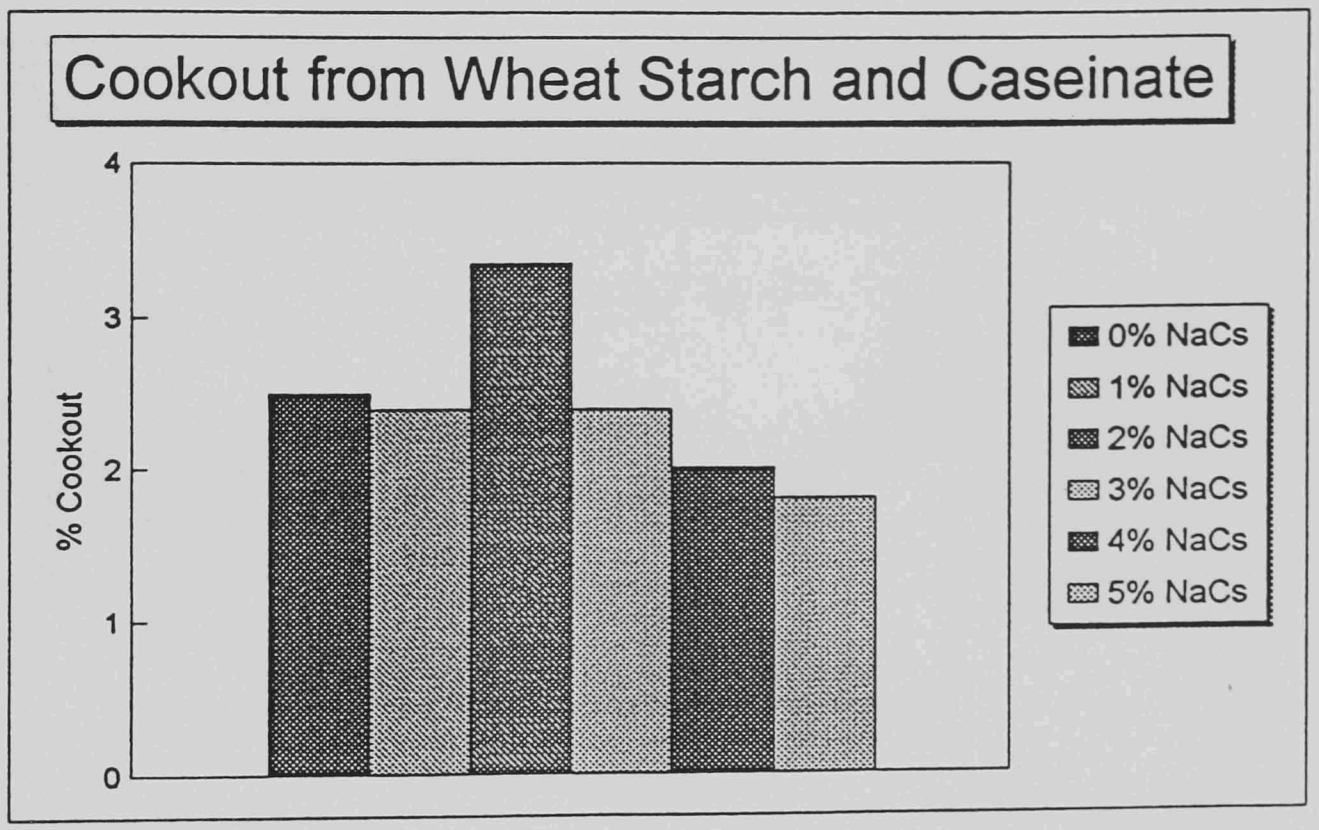
Appendix I:

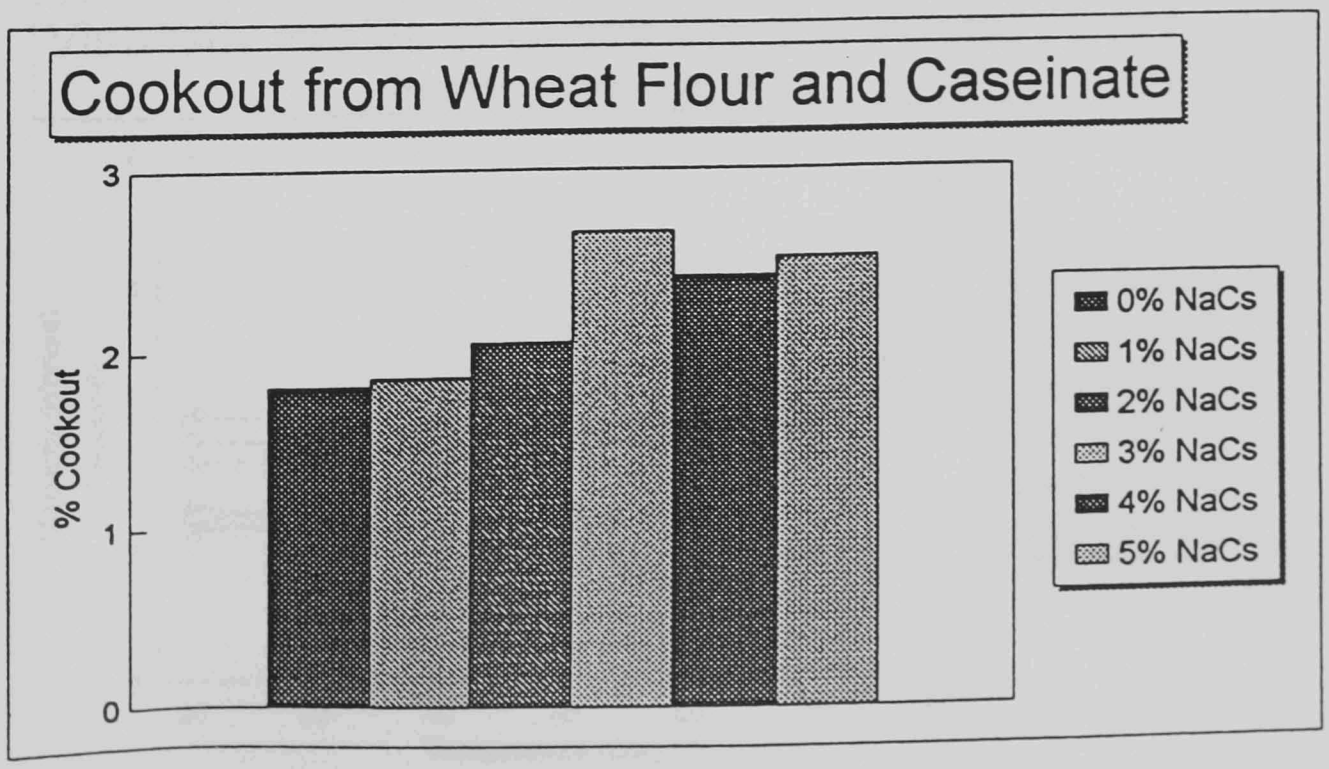


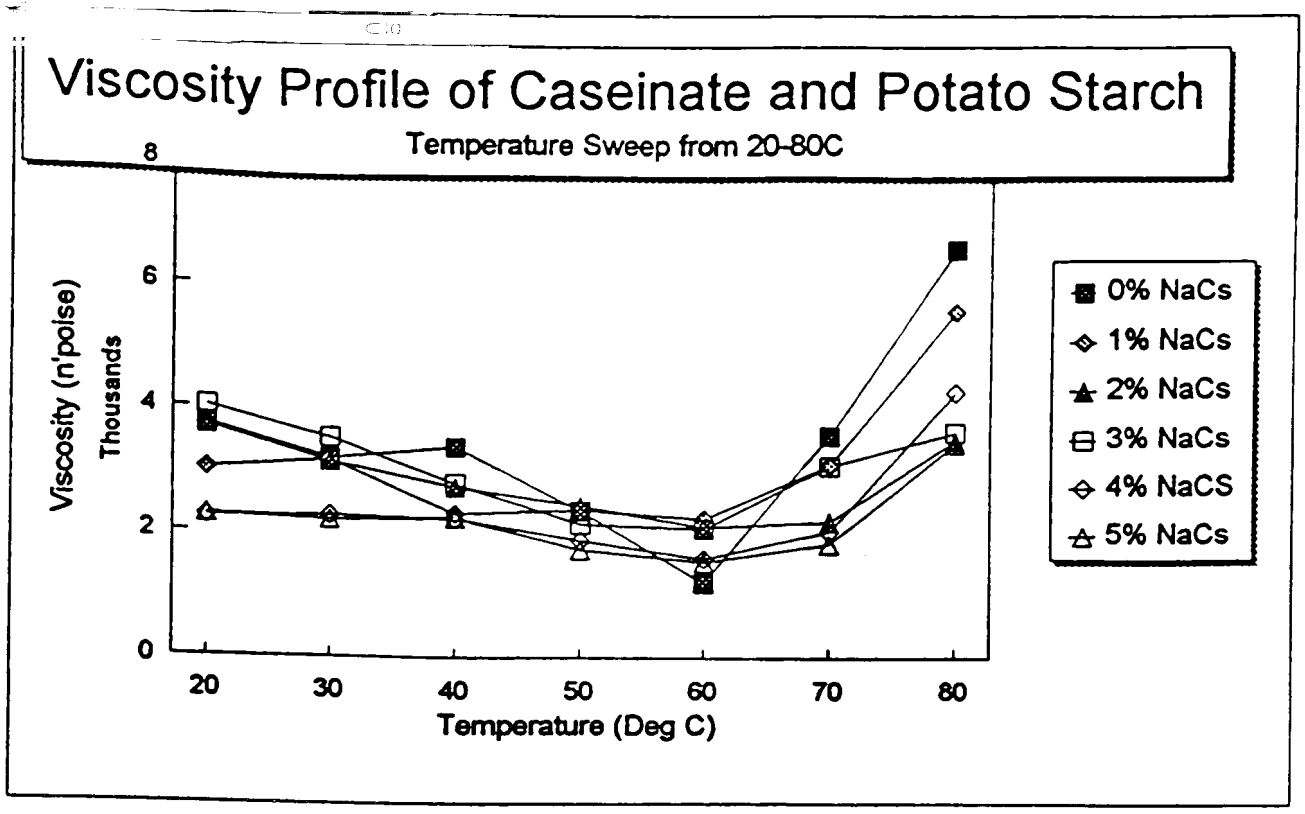


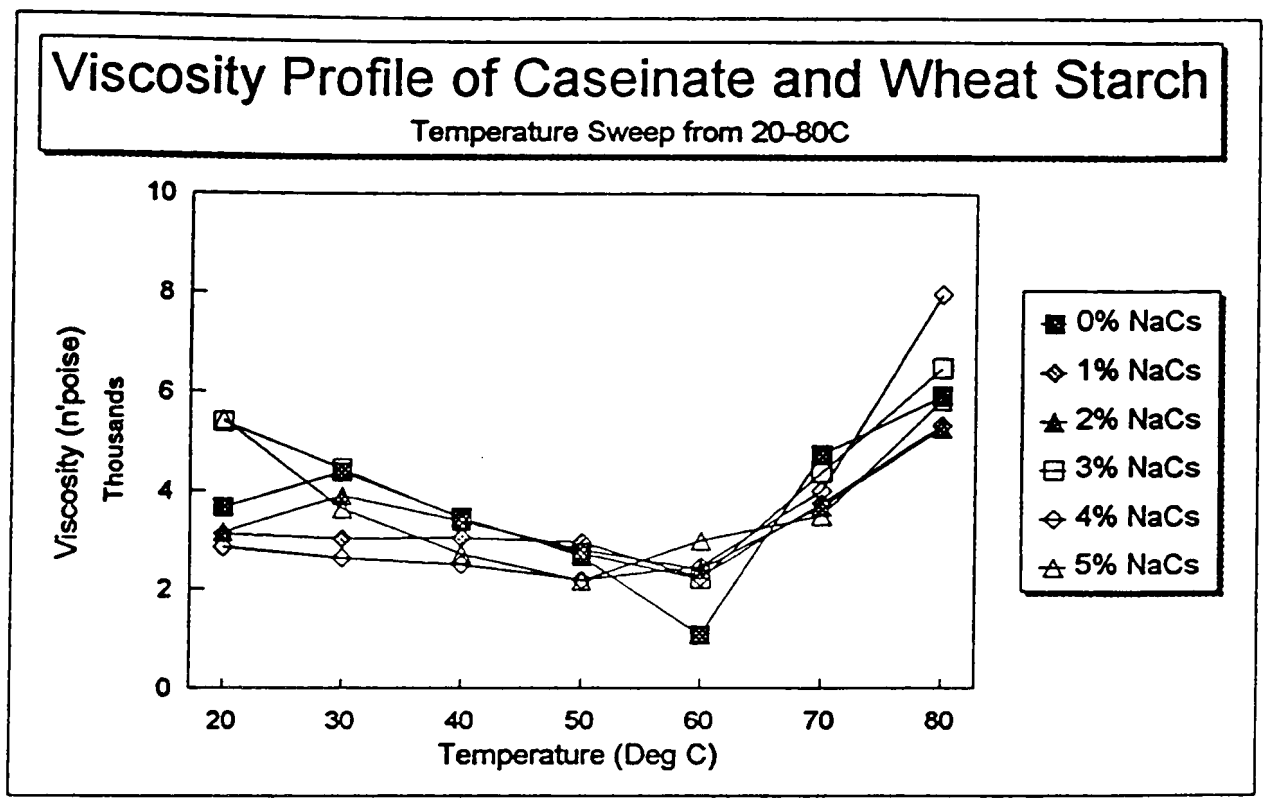


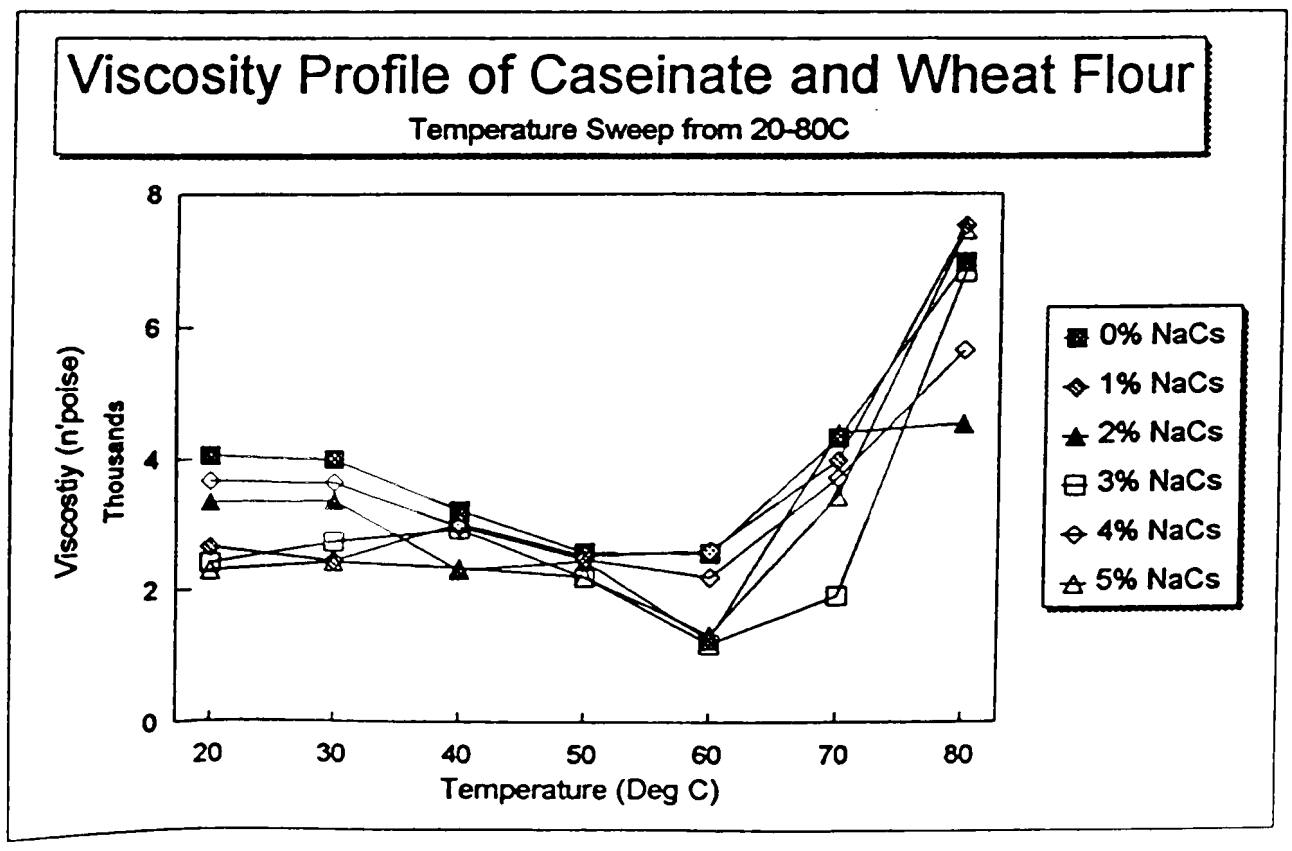

Cook-out from Potato Starch and Caseinate
% Cookout
0
1
2
3
4
0% NaCs
1% NaCs
2% Nacs
3% Nacs
4% NaCs
5% NaCs


Cookout from Wheat Starch and Caseinate
% Cookout
0
1
2
3
4
0% NaCs
1% NaCs
2% NaCs
3% NaCs
4% NaCs
5% NaCs


Cookout from Wheat Flour and Caseinate
% Cookout
0
1
2
3
0% NaCs
1% NaCs
2% NaCs
3% NaCs
4% NaCs
5% NaCs


Viscosity Profile of Caseinate and Potato Starch
Temperature Sweep from 20-80C
Viscosity (n'poise)
Thousands
0
2
4
6
8
20
30
40
50
60
70
80
Temperature (Deg C)
0% NaCs
1% NaCs
2% NaCs
3% NaCs
4% NaCS
5% NaCs


Viscosity Profile of Caseinate and Wheat Starch
Temperature Sweep from 20-80C
Viscosity (n'poise)
Thousands
0
2
4
6
8
10
20
30
40
50
60
70
80
Temperature (Deg C)
0% NaCs
1% NaCs
2% NaCs
3% NaCs
4% NaCs
5% NaCs


Viscosity Profile of Caseinate and Wheat Flour
Temperature Sweep from 20-80C
Viscostiy (n'poise)
Thousands
0
2
4
6
8
20
30
40
50
60
70
80
Temperature (Deg C)
0% NaCs
1% NaCs
2% NaCs
3% NaCs
4% NaCs
5% NaCs

Standard Procedure to Measure Meat Emulsion Viscosity Using a Carri-med Rheometer

(1) Go through the Carri-med start up procedure.

(2) Select the Oscillation package. The oscillation technique is used because it will measure heat gelation without damaging the structure of the sample during the gelation.

(3) Choose the 4cm parallel plate and set the gap to 5000 microns, then calibrate the interia.

(4) Take a sample of meat emulsion at room temperature and place it on the peltier plate.

(5) Select the auto torque procedure. This is run at 20°C over a torque range of 20 to 100 dyne cm A torque and displacement value at the lower range of the viscoelastic region of the sample is chosen.

(6) Change the procedure to a temperature sweep over a range of 20°C-80°C and set the torque and displacement values (obtained from above).

(7) Place a fresh sample of meat emulsion on the peltier plate and ensure sample details are correct before starting the test.

(8) At the end of the temperature sweep print off the data and cool the peltier plate to 20°C before testing the next meat emulsion sample.

(9) Repeat the temperature sweep three times for each meat emulsion, to ensure a consistent result.

Standard Procedure for Chunk Compression using an Instron Texture Analyser

(1) Equilibrate all products at laboratory temperature (20°C) overnight. This is crucial to standardising as product temperature affects texture.

(2) Calibrate the instron.

(3) Open the cans and reweigh the meat loaf.

(4) Remove both ends of the loaf and cut the remainder into chunks (approx dimension 2x2x2).

(5) Place a meat chunk under the instron probe so that it is central. Press [enter] on the keyboard.

(6) The crosshead will move down, pushing the probe into the chunk and giving a moving readout on the graph on the screen.

(7) Once the probe reaches 20 mm it stops and returns to the zero position. By using the arrow keys the cursor in the graph can be placed at the point where the chunk yielded. This will be recorded in the table as a displacement and a load.

(8) Remove the old chunk and replace with a fresh chunk. Repeat the procedure for ten chunks that are representative of the sample.

(9) Repeat the whole procedure three times for each sample.

Appendix IV:

Cookout Procedure

(1) Weigh the unprocessed can of emulsion (minus the weight of the can).

(2) Process the cans (use a standard process).

(3) Drain off cookout at room temperature.

(4) Reweigh the meat loaf.

(5) % Cookout = $\frac{\text{Weight before process - Weight after process}}{\text{Weight before process}} \times 100$

Publications

(1) Harding, S., Jumel, K., **Kelly, R.**, Gudo, E., Horton, J. & Mitchell, J. 1993."The structure and nature of protein-polysaccharide complexes" In "Food Proteins - Structure and Functionality". Edited by Schwenke, K.D. & Mothes, R. Published by VCH. From the 4th Symposium on Food Proteins "Structure-Function Relationships" held at Reinhardsbrunn, Germany, 5th-8th October 1992.

(2) **Kelly, R.**, Gudo, E., Mitchell, J., & Harding, S. 1994. "Some observations on the nature of heated mixtures of bovine serum albumin with an alginate and a pectin" In Carbohydrate Polymers 23, Elsevier Science Limited. pp. 115-120.

(3) **Kelly, R.,** van Wagenberg, M., Latham, J. and Mitchell, J. "A rheological comparison between the effects of sodium caseinate on potato and corn starches". *In press* - accepted for publication by Carbohydrate Polymers.

(4) *In preparation* **Kelly, R.,** Le Bon, C., Pinat, F. and Mitchell, J." The Effects of Gelatin on Melting Point and Large Deformation Stress Relaxation of Agar, Carrageenan and Carrageenan/LBG Gels".

Communications

Poster presentations:

(1) "The Effects of Sodium Caseinate on Corn and Potato Starches" **Kelly, R.**, van Wagenberg, M., Latham, J. and Mitchell, J. Biopolymer Mixtures, Nottingham. September 1994.

(2) "Large Deformation Stress Relaxation Studies on Gelatin-Agar and Gelatin-Carrageenan/LBG Gels" Le Bon, C., Pinat, F., **Kelly, R.** and Mitchell, J. Biopolymers Mixtures, Nottingham, September 1994.

(3) "The Influence of Low Levels of Electrolytes on the Swelling and Viscosity of Potato Starches" **Kelly, R.**, Paterson, L., Hill, S. and Mitchell, J.Gums and Stabilizers for the Food Industry - 8, Wrexham, July 1995.

(4) "The Influence of Gelatin Inclusion on Melting Points and Large Deformation Stress Relaxation Behaviour of Carrageenan and Agar Gels" **Kelly, R.**, Le Bon, C. and Mitchell, J., Gums and Stabilizers for the Food Industry - 8, Wrexham, July 1995.

(5) "The Effects of Gelatin and Bovine Serum Albumin on the Melting Points and Large Deformation Stress Relaxation Behaviour of Agar and Carrageenan Gels" **Kelly, R.**, Le Bon, C. and Mitchell, J. At The 9th World Congress of Food Science and Technology, Budapest, August 1995.

Oral Presentations:

(1) "The Influence of Low Levels of Electrolytes on the Swelling and Viscosity of Potato Starches" **Kelly, R.**, Paterson, L., Hill, S. and Mitchell, J. Gums and Stabilizers for the Food Industry - 8, Wrexham, July 1995.

(2) "The Influence of Gelatin Inclusion on Melting Points and Large Deformation Stress Relaxation Behaviour of Carrageenan and Agar Gels" **Kelly, R.**, Le Bon, C. and Mitchell, J., Gums and Stabilizers for the Food Industry - 8, Wrexham, July 1995.