GLUCONO-δ-LACTONE - INDUCED GELATION OF SOME MEAT COMPONENTS AT CHILLED TEMPERATURES

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ABSTRACT

In this study, 1,5-glucono-δ-lactone, was used to achieve acid-induced gelation of meat, myofibrillar protein and myosin at 4°C. The mechanisms of gelation of myofibrillar protein and myosin were investigated. The effects of addition of sodium chloride and tetrasodium pyrophosphate to myosin and myofibrillar protein, with and without 1,5-glucono-δ-lactone, were also studied. In addition, the presence of other phosphates, orthophosphate, tripolyphosphate and hexametaphosphate, in a myosin system were studied to aid in an understanding of the the observed effects of tetrasodium pyrophosphate on myosin.

At about pH 4.5, it was observed that extraction of the A-band of myofibrillar protein occurred. It was suggested that an impregnated composite system of myosin reinforcing the myofibrillar structure had formed. At about pH 4.0, complete extraction of the A-band occurred. Dissolution of the myofibrillar structure was suggested to result in myosin network formation of weaker Young's Modulus than the impregnated composite system.

Addition of 1,5-glucono-δ-lactone to myosin resulted in the exposure of hydrophobic sites as the pH decreased and it was suggested that acid-induced denaturation had occurred. Gel formation occurred parallel to denaturation. At pH 4.0, the gel became liquid-like and was suggested to be a result of excess repulsive electrostatic interactions. Hydrogen bonding and hydrophobic interactions were shown to be involved in gel formation, whereas sulfhydryl bonding appeared not to be involved in gelation. Sodium chloride was postulated to enhance gel rigidity through its effects on the isoelectric point of myosin. The inclusion of tetrasodium pyrophosphate resulted in network formation prior to acid-induced denaturation and was suggested to enhance hydrogen bonding.

The acid-induced gels appeared to revert to myosin or myofibrillar protein when immersed in quiescent water, a condition where unimpeded diffusion of ions was obtained. However, a slow rate of ion diffusion resulted in the formation of a 'strong', translucent gel which was dense to the point of being effectively impermeable to ion migration. These gels were hypothesized to have formed through the displacement of sodium and potassium ions with protons, enhancing hydrogen bonding. Myosin was observed to have a stronger affinity for sodium than for potassium.

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ABBREVIATIONS

Abbreviations of units

A	amps
C	coulombs
°C	degrees Celsius
Da	daltons
g	gram(s)
h	hour(s)
Hz	hertz
J	joules
l	litre(s)
m	metre
M	molar
min	minute(s)
mol	mole(s)
N	Newtons
Pa	pascals
rad	radians
rpm	revolutions per minute
s	second(s)
v	volume
V	volts
w	weight
W	watts

Other Abbreviations

A1	alkali light chain one
A2	alkali light chain two
ADP	adenosine diphosphate
ANS	8-anilino-1-naphthalenesulfonic acid
AR	analytical reagent grade
ATP	adenosine triphosphate
bis	N,N'-methylene-bis-acrylamide
BSA	bovine serum albumin
CD	circular dichroism
CPA	*cis*-parinaric acid
DSC	differential scanning calorimetry
DTNB	5,5'-dithiobis(2-nitrobenzoic acid)
DTT	dithiothreitol
e.d.	external diameter
EDTA	ethylenediaminetetraacetic acid

EGTA	ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid
G'	storage moulus
G"	loss modulus
G^*	complex modulus
GdL	glucono-δ-lactone
HMM	heavy meromyosin
HMP	hexametaphosphate
i	imaginary number = $(-1)^{1/2}$
i.d.	internal diameter
ICP	inductively coupled plasma
IEP	isoelectric point
LMM	light meromyosin
MHC	myosin heavy chain(s)
MW	molecular weight
N	total number of monomers
NDIC	Nomarski differential interference contrast
OP	sodium dihydrogen orthophosphate
OR	optical rotary dispersion
Pc	number density of crosslinkers at the point of incipient gelation
PCMB	p-hydroxy-mercuribenzoic acid
PD	denatured protein
PN	native protein
PPase	pyrophosphatase
PVC	polyvinyl chloride
PVDC	polyvinylidene chloride
q	charge
r	distance separating q
RFI	relative fluorescence intensity
S-1	fragment of HMM containing the head portion
S-2	fragment of HMM between the head and LMM
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate - polyacrylamide gel electrophoresis
SEM	scanning electron microscopy
t	time
TEM	transmission electron microscopy
TEMED	N,N,N',N'-tetramethylethylenediamine
T_m	transition temperature
TPP	tripolyphosphate
Tris	tris(hydroxymethyl)aminomethane
TSPP	tetrasodium pyrophosphate
UV	ultra-violet
Vc	number of crosslinked or branched monomers
WHC	water holding capacity
x	number of protein molecules
Xn	weight average degrees of polymerisation

δ	phase angle
γ	shear strain
γ_o	strain amplitude
$\dot{\gamma}$	strain rate
τ	shear stress
τ_o	stress amplitude
ω	angular frequency

CHAPTER 1

INTRODUCTION

The Meat Industry is a major contributor to New Zealand's standard of living. Since the 1880's it has made significant contributions to New Zealand's Gross Domestic Product and foreign exchange earnings. Meat and related by-products are the country's largest export earners; in 1991 earning $3.25 billion in export receipts which accounted for 21% of the total receipts from merchandise trade in that year (Enterprise New Zealand, 1992). The Meat Industry also provides employment for around 89 900 people in both the rural communities and cities.

On average meat contributes more than 50% of the gross income of a typical sheep and beef farm (Enterprise New Zealand, 1992). There are approximately 40 000 meat producing farms in New Zealand, with around 58 million sheep, 4.6 million beef cattle and 100 000 deer. These farms supply 52 licensed meat processing plants, local abattoirs and live sheep exports to Saudi Arabia. About 1.1 million tonnes of meat are produced of which much is destined for overseas markets with New Zealand contributing 17% of internationally traded meat.

Over the last 120 years the Meat Industry has undertaken two major diversification trends. The first era of diversification was made possible by the introduction of refrigerated ships in 1882. This gave rise to the rapid growth of agricultural exports destined primarily for Britain. The second era occurred in response to government initiatives in the 1960's. For too long New Zealand had depended on a narrow range of agricultural export products destined to the traditional markets of Britain, Australia and more recently the U.S.A. World economic and political allegiances were changing as was the demand for imported goods.

A trend in the industry today is towards further processing before meat reaches the final point of destination. Meat marketing and processing over the last decade has focused more on the

needs of the end users. The higher prices for further processed meat have driven this change. Carcase prices have remained below the price of cuts and, to a greater extent, below the price of boned cuts. Only boned prices have kept ahead of inflation. This added value to the meat earns higher prices and returns for processors and farmers (Enterprise New Zealand, 1992).

Apart from those who for ethnic, racial or religious reasons do not eat meat (mainly in Asia) most of the world's 5.5 billion population are consumers of meat (Harrington, 1992). What is more, the great majority want to eat meat, have few concerns about its production or consumption and have a strong positive attitude towards this food. But the amount they actually consume is influenced by a whole range of market factors; including income, availability, price, visual appeal, fitness for purpose, the choice of alternative foods available and the eating satisfaction experienced. In contrast to these positive consumers, there is a growing minority in some developed countries whose purchase and consumption patterns are influenced not only by the market and taste factors, but by growing concerns about the healthiness of meat and about modern methods of production and their impact.

Market pressures in developed countries with relatively high levels of consumption (83 kg per head per year on average) derive from changing lifestyles and retailing practices (Harrington, 1992). The simple foods regularly bought in the past are, to a degree, supplanted by a wider choice of prepared or processed foods which may contain less meat than the traditional purchase or even no meat at all. Many developed countries are experiencing fundamental changes in lifestyle as social structures change, as affluence increases, as families fragment and as individuals achieve more leisure time. This leads to major changes in meal patterns and the time available for preparation and consumption so increasing the demand for convenience foods. However, despite relative affluence in developed countries, price remains a key factor for a high proportion of the public.

Retail consumers have demonstrated a preference for fresh chilled meat products, especially primal cuts, as indicated by the large proportion of meat sold in this form (Mandigo, 1982; Schmidt and Sofos, 1988). The cheaper cuts and trimmings, of which some is high quality meat, are generally used for inclusion in small goods and hamburger manufacture forming products with relatively low economic value. As a consequence there has been considerable

activity throughout the world in developing technologies that will enable the upgrading of the value of these cheaper cuts (e.g., Mandigo, 1982; Jolley *et al.*, 1988; Schmidt and Sofos, 1988; Wijngaards and Paardekooper, 1988).

The aim of practically all these technologies has been the development of products that are intermediate in value between the primal cuts and the small goods. Many of the products successfully developed rely on the inclusion of additives and must be precooked and/or frozen (Jolley *et al.*, 1988). These processing steps mean that consumers no longer see the product as fresh meat, but as products competing with all other meat substitutes (Field, 1982; Schmidt and Sofos, 1988; Wijngaards and Paardekooper, 1988).

The most important feature of these restructured meat products is the ability of the protein matrix formed to effectively bind the meat pieces together. Effective binding is essential for the product to retain its structural integrity during subsequent handling and slicing (Schmidt and Trout, 1982). Initial research in this area was directed towards studying the binding involved in meat patties and sausages (Hashimoto *et al.*, 1959; Fukazawa *et al.*, 1961a,b,c; Nakayama and Sato, 1971a,b) and has since developed to include chunked and formed meat products (e.g., Schnell *et al.*, 1970; MacFarlane *et al.*, 1977; Ford *et al.*, 1978; Siegel and Schmidt, 1979a,b; Wijngaards and Paardekooper, 1988). Many researchers have substantiated the findings of Fukazawa *et al.* (1961a,b) and Hashimoto *et al.* (1959) showing that myofibrillar proteins, in particular myosin, are primarily responsible for the binding quality in restructured meats and that bind strength bears a close relationship with the amount and nature of myosin contained in and/or liberated as actomyosin from a meat structure as reviewed by Asghar *et al.* (1985).

If meat, myofibrillar proteins, or myosin alone, could be modified in some consumer acceptable way to enable it to preform restructured meat muscles at low temperatures, a range of fresh chilled meat products could be produced. Jolley *et al.* (1988), Mandigo (1982) and Field (1982) have suggested the probable existence of a substantial market for these products.

Because of the perceived need for a chilled restructured meat product, the aim of this study was to develop an understanding of the mechanism of gelation of meat proteins at low

temperatures since binding is a function of gelation. With the trend in New Zealand towards further processing of meat before sale, the amount of trimmings produced will be greater, and hence the return on goods increased with the introduction of a competitive restructured meat product. Determination of the mechanism of gelation could impact on other protein-based industries, especially fish, but could also be as far reaching as the medical arena.

CHAPTER 2

LITERATURE REVIEW

2.1 MEAT

2.1.1 The composition of muscle

Skeletal muscles are one of three main classes of muscle on the basis of histological appearance. They are red or pink in colour and are commonly recognised as the meat or flesh of animals. The composition of lean muscle can be approximated in a broad sense as 75% water, 20% protein, 3% fat and 2% non-nitrogenous soluble substances (Schut, 1976). The 20% protein, measured on the basis of nitrogen content, includes about 1.0 to 1.5% non-protein nitrogenous material such as amino acids, nucleotides, and creatine. The non-nitrogenous substances consist of carbohydrates such as glycogen, glucose, glucose-6-phosphate and lactic acid, and minerals, such as phosphorus, calcium, magnesium, zinc, iron, potassium, sodium and trace metals. The vitamins also belong to this group.

Muscle proteins can be divided into three main categories based on their relative solubilities at different ionic strengths (Schut, 1976; Pearson and Young, 1989). The first group, the sarcoplasmic proteins, are soluble in water or dilute (less than 50 mM) salt solutions. They comprise approximately 5.5% of typical adult mammalian muscle, less in young immature animals. The second group, myofibrillar proteins are soluble in concentrated salt solutions and make up approximately 11.5% of the total muscle. They are responsible for muscle contraction and therefore, movement. Myosin and actin are the principal myofibrillar proteins, accounting for about 50% and 20% of the total myofibrillar protein respectively (Obinata *et al.*, 1981). In meat, myosin and fibrous actin are present in a more or less complexed form called actomyosin. The final category, the connective tissue proteins are structural

components varying in abundance and density. They contribute approximately 2% of the total muscle and are insoluble in both dilute and concentrated salt solutions.

2.1.2 The gross structure of muscle

Skeletal muscle is made up of a grouping of muscle fibres surrounded and supported by connective tissue. The epimysium comprises the heavy sheath of connective tissue surrounding an entire muscle. A finer connective tissue layer, the perimysium, surrounds the fibre bundles and a still finer connective tissue layer, the endomysium, envelopes each individual fibre. Figure 2.1 illustrates the gross structure of the muscle.

The muscle fibre is the fundamental organisational unit of muscle. Each muscle cell or fibre is long, cylindrical, multinucleate and is surrounded by a double membrane called the sarcolemma (Schut, 1976). Each fibre is an independent structure and varies in length from 1 to 40 mm and from 10 to 60 μm in diameter (Leeson and Leeson, 1981). The muscle fibre is composed of smaller longitudinal ordered subunits, the myofibrils, which are surrounded by the fluid sarcoplasm. Myofibrils are thin threads 1 to 2 μm in diameter (Schut, 1976) which represent a half crystalline, contractile protein gel. They make up 60% of the volume of the muscle fibre, the protein concentration being between 15 and 20%. Each myofibril contains a number of smaller long filaments which comprise 80 to 87% of the muscle fibre volume. These myofilaments are largely composed of myosin and actin usually organised into thick filaments containing 300 to 400 myosin molecules and thin filaments of approximately 400 actin molecules. Thick filaments are approximately 14 to 16 nm in diameter, 1.5 μm in length and are separated laterally by approximately 40 nm; thin filaments are 6 to 8 nm in diameter and approximately 1 μm in length (Schut, 1976; Pearson and Young, 1989). Figure 2.2 gives a sketch of a muscle fibre.

2.1.3. The ultrastructural organisation of muscle

On examination of skeletal muscle under the light microscope, alternating light and dark bands are apparent. The A-bands are so-called because they are anisotropic (birefringent), while the

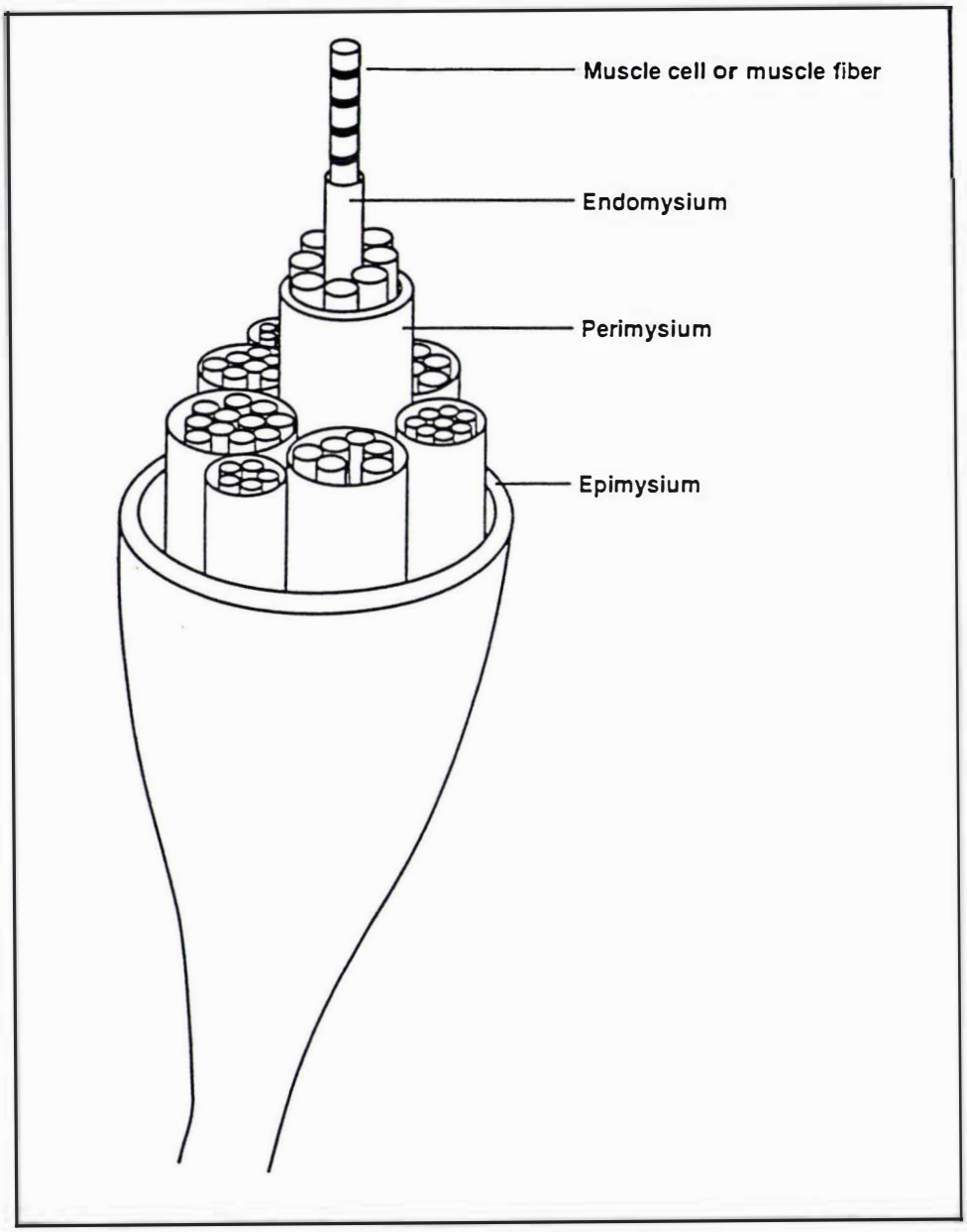
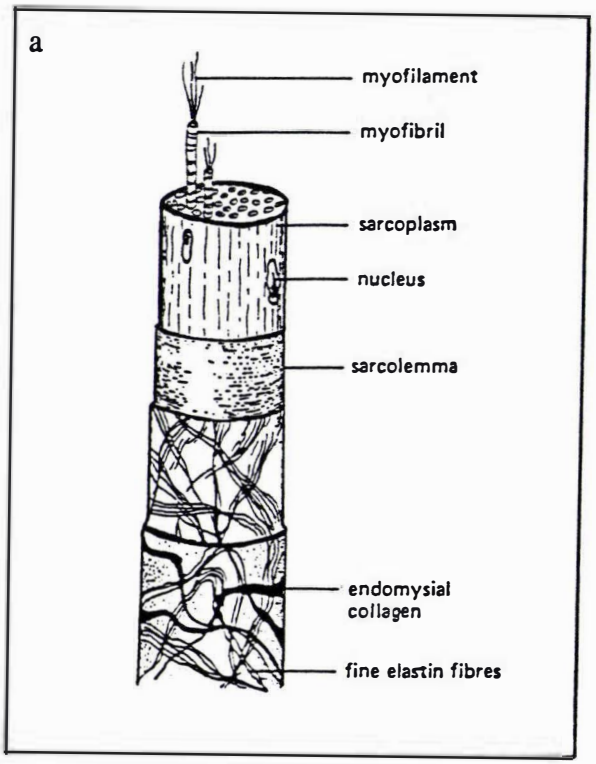


Figure 2.1. Hierarchies of muscle structure. Adapted from Bechtel (1986).



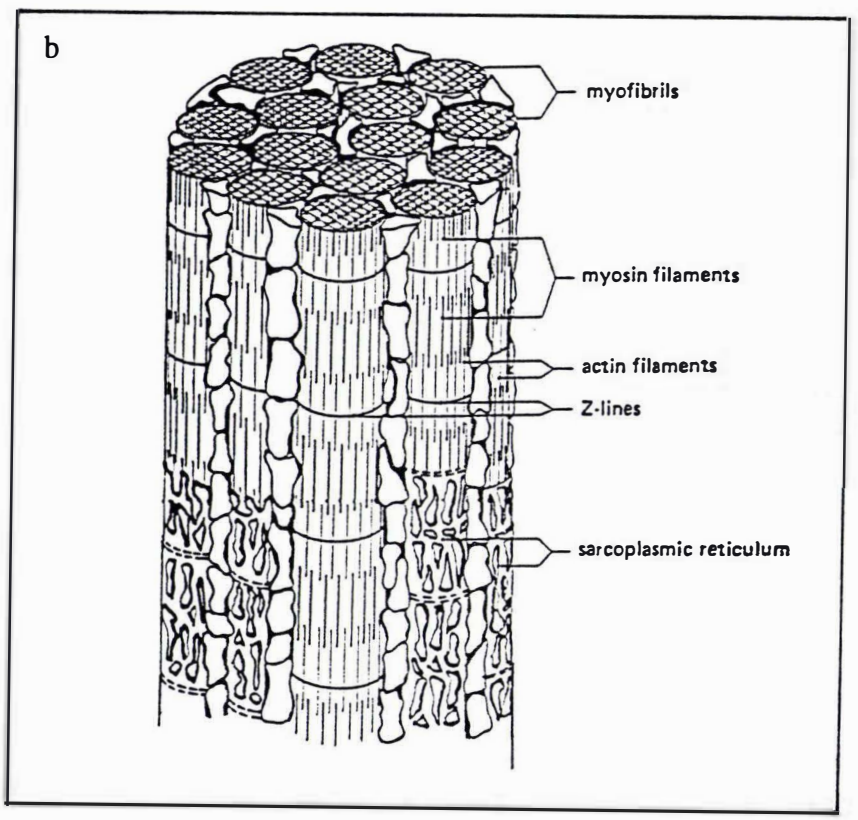


Figure 2.2a and b. A sketch and a section of a muscle fibre. From Schut (1976).

I-bands are isotropic under polarised light (Leeson and Leeson, 1981). The A-bands and I-bands of neighbouring myofibrils lie adjacent to their counterparts giving the entire muscle a cross-banded appearance. Figure 2.3 is a representation of the various components of the sarcomere. The A-bands contain the thick filaments with some overlap of the thin filaments at each end. The H-zone of the A-band contains only thick filaments. The I-bands are made up of the thin filaments on either side of the narrow heavily staining Z-band. Where thick and thin filaments overlap the thick filaments are surrounded by a hexagonal array of thin filaments (Bendall, 1969). The thick filaments are believed to be held in transverse register by a thin structural element that bisects the middle of the H-zone in each sarcomere, known as the M-line. The repeating structural and functional unit in muscle contraction is the sarcomere or the unit lying between two adjoining Z-bands in the same muscle fibre. Each relaxed sarcomere is approximately 2.5 μm in length (Pearson and Young, 1989).

The review by Morrissey *et al.* (1987) recommends several references which discuss the structure of muscle. Only the major myofibrillar proteins will be discussed in more detail in this review.

2.1.4. Myosin

Myosin is a large molecule (approximately 500 kDa; Pearson and Young, 1989). It contains six polypeptide subunits arranged into a native protein molecule with two globular heads attached to a long α-helical rod-like tail (Figure 2.4). The rod portion is responsible for the assembly of myosin into thick filaments, and the two globular heads contain both the enzymatic active site for ATP hydrolysis and the actin binding region. Each head also has two non-covalently bound light chains that appear to be involved in the regulation of contraction. Thus, myosin has the biochemical properties of a globular and a fibrous protein. The myosin molecule is approximately 150 nm in length, with a diameter of approximately 8 nm in the globular region and approximately 1.5 to 2.0 nm in the α-helical region. Myosin has an isoelectric point of 5.4 (Szent-Györgyi, 1951) indicating that it is a negatively charged protein. The amino acid composition (Table 2.1) shows corresponding substantial amounts of aspartic and glutamic acids. No disulphide bonds are present in myosin, however there are

Table 2.1. The amino acid composition of myosin (moles/10^5g).

Amino Acid	Myosin
Cysteine	8.8
Aspartic acid	85
Threonine	44
Serine	39
Glutamic acid	157
Proline	22
Glycine	40
Alanine	78
Valine	43
Methionine	23
Isoleucine	42
Leucine	81
Tyrosine	20
Phenylalanine	29
Histidine	16
Lysine	92
Arginine	43
Total	863

Adapted from Lowey and Cohen (1962) using rabbit myosin.

approximately 42 thiol residues in the myosin molecule of which 12 to 13 are present in each of the two heads (Lowey *et al.*, 1969).

In the muscle, myosin is arranged into thick filaments which are approximately 1.6 μm long (Huxley, 1963). The myosin molecules are packed with the tails in the backbone and the heads at the surface. The packing is anti-parallel near the middle of the filament and parallel for the rest of the length. The myosin heads in vertebrate skeletal muscle thick filaments are arranged approximately helically with an axial repeat of 43 nm and with the myosin heads spaced axially every 14.3 nm (Craig and Knight, 1983).

The myosin molecule contains two identical heavy chains (approximately 220 kDa each) and two sets of light chains that range in size from 14 to 20 kDa depending on species and muscle type. Approximately 50% of each heavy chain starting at the carboxy-terminal end folds together to form a coiled-coil of α-helices that make up the fibrous portion of the molecule. The rod sequence exhibits the heptapeptide repeat (*a-b-c-d-e-f-g*) typical of coiled-coil structures where residues *a* and *d* are hydrophobic and form the interface between

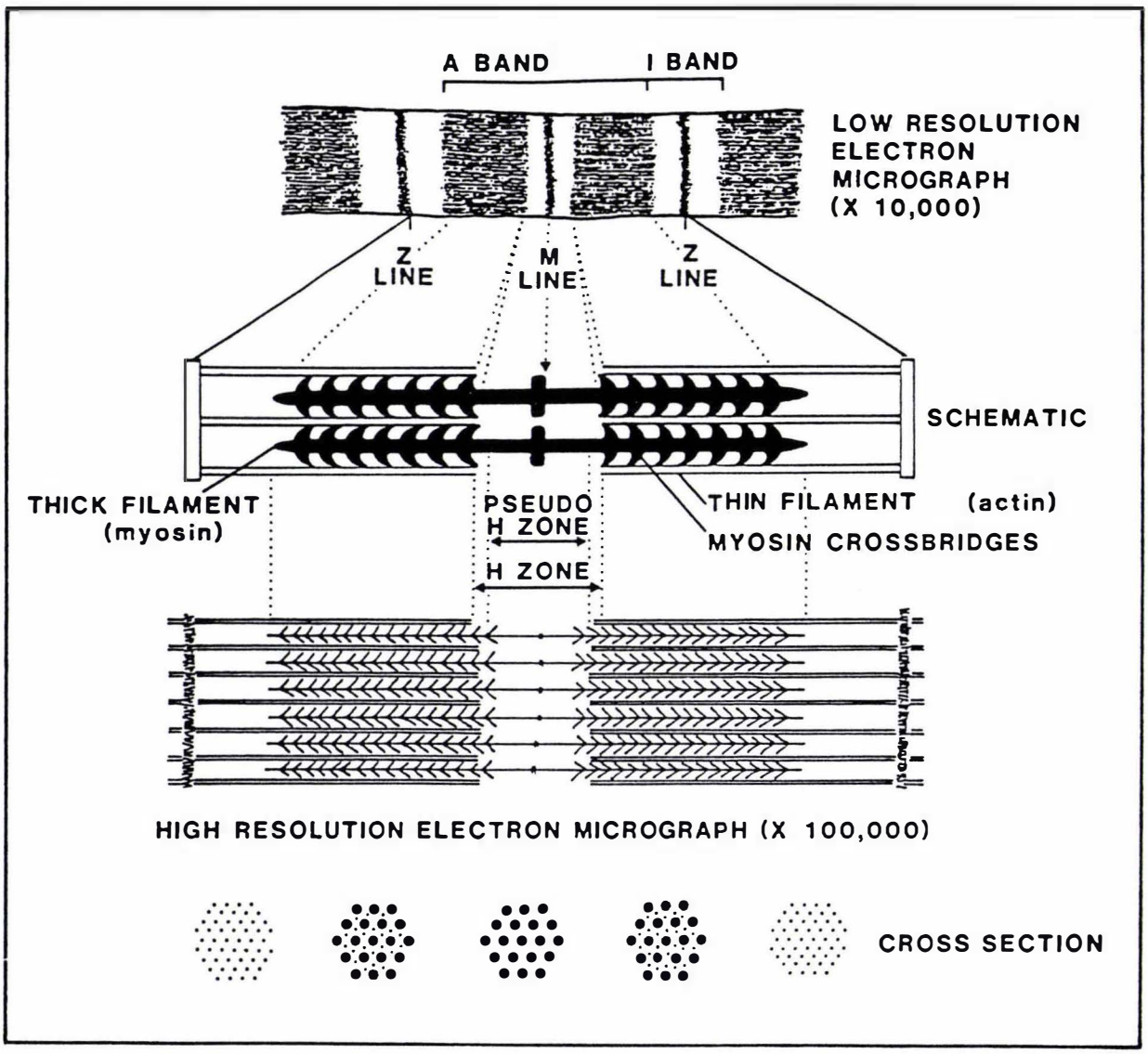


Figure 2.3. The components of the sarcomere. From Pearson and Young (1989).

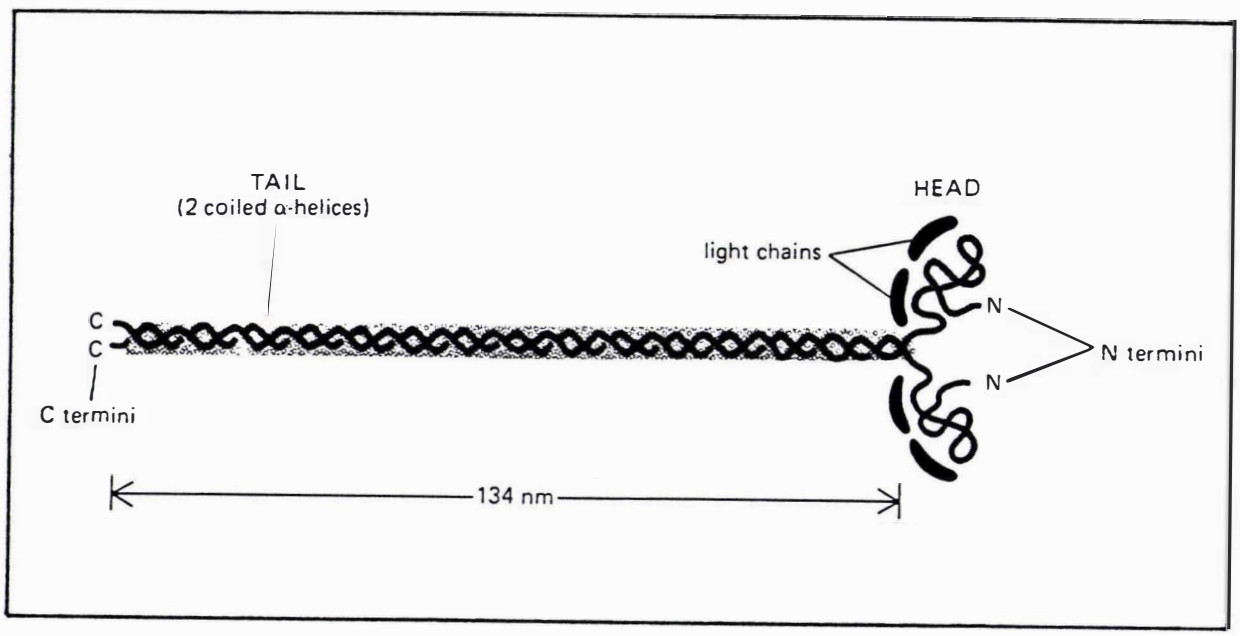


Figure 2.4. The myosin molecule. From Pearson and Young (1989).

the α-helices in the folded protein (Harrington and Rodgers, 1984). Positions *e* and *g* are frequently occupied by acidic and basic residues, respectively (Parry, 1981). The other 50% of the length of the heavy chain is associated with two light chains to form one of the two globular or head regions of the molecule.

Upon electrophoresis three light chain bands are evident rather than two as suggested by Figure 2.4 This discrepancy is explained schematically in Figure 2.5. Adult skeletal myosin may contain two or more populations of myosin molecules that differ in their light chain composition. The myosin light chains in rabbit skeletal muscle that have a molecular mass of approximately 18 kDa are frequently called the "DTNB" light chains, so-called because they are released from the myosin molecule on treatment of native myosin with the sulfhydryl-blocking agent 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB). The alkali light chains (A1 and A2 light chains in Figure 2.5, of 25 and 16 kDa, respectively) of skeletal muscle are so-named because they are released from native myosin following treatment with alkali of pH 11 (Gazith *et al.*, 1970; Weeds and Lowey, 1971). Immunoabsorption experiments have shown that each native myosin molecule contains two A1 light chains or two A2 light chains. No hybrid myosin molecules, containing one A1 light chain and one A2 light chain, are present (Holt and Lowey, 1977).

2.1.5. The proteolytic subunits of myosin

If solutions are treated with either trypsin or chymotrypsin at a ratio of one part enzyme to 300 parts myosin by weight, the viscosity of the solution is reduced sharply within a few minutes. Two new species are generated termed light and heavy meromyosin (Lowey *et al.*, 1969). The heavy meromyosin (HMM) contains all ATPase activity and actin-binding ability; the light meromyosin (LMM) fragment is that portion of the molecule responsible for the packing of myosin into the body of the thick filament. The products of treatment with trypsin, chymotrypsin and papain are illustrated schematically in Figure 2.6

Note that treatment of HMM with papain results in the formation of two additional fragments termed S-1 and S-2. The S-1 fragments (two per native myosin molecule) contain the globular head region of myosin and the S-2 fragment contains the portion of the α-helical

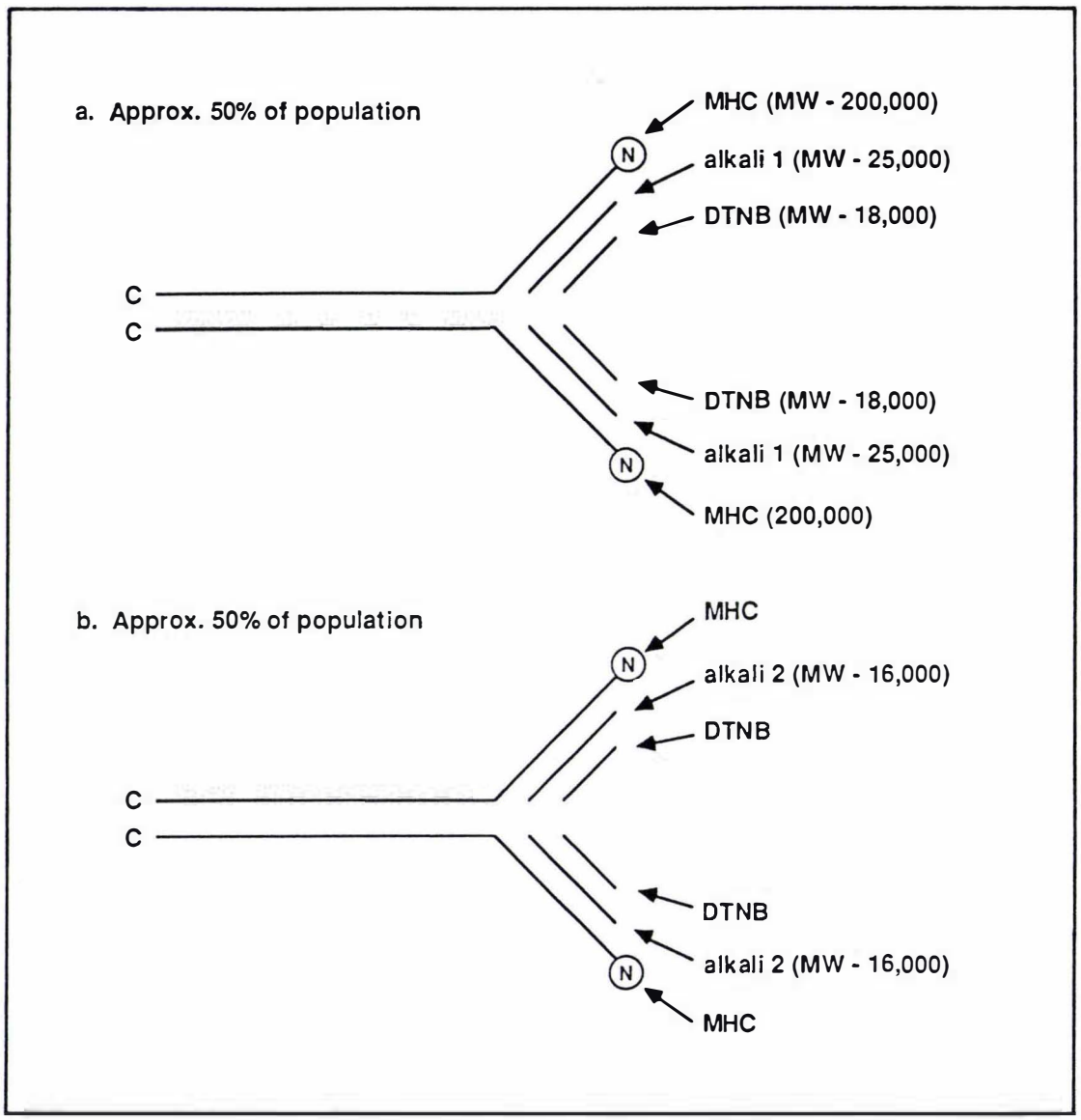


Figure 2.5. Schematic explanation for the appearance of three light chain bands, instead of two, in polyacrylamide gels of myosin purified from adult rabbit skeletal muscle. Approximately half of the population (a) of native myosin consists of two alkali 1 light chains and two DTNB light chains. The other half of the population (b) consists of two alkali 2 light chains and two DTNB light chains. Thus, each native myosin molecule contains either two alkali 1 light chains or two alkali 2 light chains, rather than one of each. From Pearson and Young (1989).

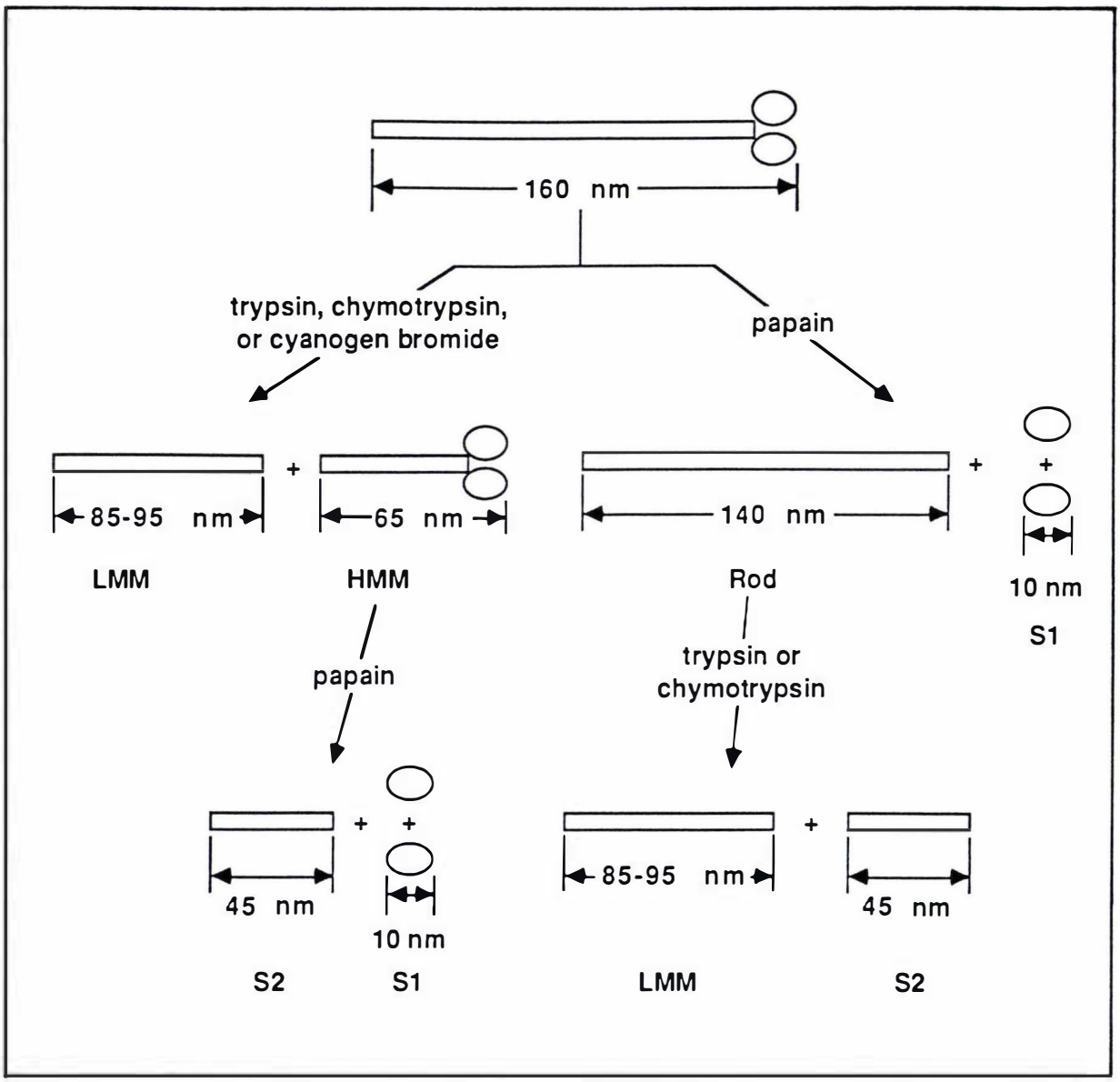


Figure 2.6. The proteolytic subunits of myosin. From Pearson and Young (1989).

region between the head and LMM. In all the proteolytic treatments, the DTNB light chains are dissociated from the HMM or S-1 portion of the myosin molecules. Additional properties of the fragments are summarised in Table 2.2

Table 2.2. Selected properties of the proteolytic subunits of myosin.[a]

Property	Myosin	LMM	HMM	Rod	S-2	S-1
Molecular Mass (kDa)	480	140	310	220	62	115
Percent α-helix	56	95	46	95	90	30
Length (nm)	155	85	70	140	42	10
Diameter (nm)	1.5	1.5	7.0	1.5	1.5	7.0

[a]Each of the values listed is an approximation, since slightly different results have been obtained by individual research laboratories.

Adapted from Pearson and Young (1989).

2.1.6 Other major myofibrillar proteins

Approximately 20% of the total myofibrillar protein is actin (Obinata *et al.*, 1981). The actin monomer is a slightly elongated sphere, so-named globular actin (G-actin), of approximately 42000 molecular weight (Pearson and Young, 1989). The diameter of each molecule is approximately 5.0 nm and slightly less than 30% of the molecule is α-helix. Each actin molecule contains five sulfhydryl groups. Polymerisation of 3 to 400 monomers constitutes fibrous actin (F-actin). There are approximately 400 polymerised G-actin monomers in a thin filament. The classic structural organisation of individual actin monomers in the thin filaments is that of a polymer consisting of two helical cords twisted around each other, with each full turn of the helix involving 28 G-actin monomers (14 in each strand) and requiring approximately 70 nm. The long axis of the actin molecule points radially (side-to-side) rather than longitudinally along the thin filament. When F-actin is formed *in vitro* the length of the polymers is random.

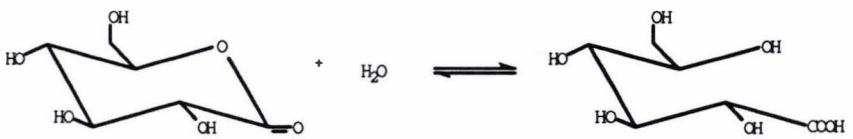
Tropomyosin comprises approximately 7% of the total protein in the myofibril and is a regulatory protein because of its involvement in the regulation of contraction (Pearson and

Young, 1989). Rabbit skeletal muscle tropomyosin consists of two polypeptide chains of approximately 34 kDa each. The two polypeptide chains have the same number of amino acids, but there are 39 differences between the amino acid sequences. Native tropomyosin has a molecular mass of approximately 68 kDa, an overall length of approximately 41 nm, and each native tropomyosin molecule can interact with seven individual actin monomers within a thin filament. Tropomyosin contains virtually 100% α-helix and contains no disulphide bonds.

Troponin is also a regulatory protein and comprises 4.5% of total myofibrillar protein. The native troponin complex consists of three subunits of molecular mass of about 18, 23 and 37 kDa in rabbit (Pearson and Young, 1989). Electron microscopy has suggested a structure for the troponin complex containing both a globular and a rod-like portion that has a total length of approximately 26.5 nm, the rod-like portion being about 16.5 nm of this total. The total length of the polymerised troponin along the thin filament is approximately 40 nm. The 26.5 nm troponin complex would therefore react with two thirds of the total length of the tropomyosin molecule.

2.2 1,5-GLUCONO-δ-LACTONE

The cyclic ester, 1,5-glucono-δ-lactone (GdL) is widely used in the food industry as an acidulant. GdL hydrolyses gradually in water to form gluconic acid



The lactone, GdL is used as an additive in such foods as yoghurt, sausages, frankfurters, various dessert mixes (Trop and Kushelevsky, 1985), various cheeses and baking powder (Trop, 1984). Its purpose is generally to slowly lower the pH of the system. Several protein systems have been shown to form gels upon acidification induced by GdL hydrolysis. These include soymilk and soybean 11S protein (Nishinari *et al.*, 1991; Kohyama *et al.*, 1992; Yoshida *et al.*, 1990, 1992), skim milk powder and whey protein isolate and concentrate

(Aguilera and Kessler, 1989; Aguilera and Kinsella, 1991). However, only milk has been shown to gel without denaturing the protein by preheating and/or heating during gelation (Trop, 1984; Kim and Kinsella, 1989).

2.3 GELATION.

Glicksman (1982) defined gelation as the association or crosslinking of randomly dispersed polymer chains in a solution to form a three-dimensional network which immobilizes liquid in the interstitial structures and which resists flow against pressure. Variation in network flexibility depends on the number and nature of crosslinks, attraction and repulsion forces between filaments, and interaction with the solvents (Rees, 1972). Theories of gelation are abundant, but are largely based on the theories of Flory (1941, 1953) and Ferry (1948). Many of these recent theories are reviewed by Clark (1992).

Flory's proposed network theory of gelation states that the critical concentration of crosslinker is the minimum amount required to connect all the polymers into one continuous network (Flory, 1941, 1953). Mathematically, it has been expressed as:

$$P_c = \frac{V_c}{N} = \frac{1}{\overline{X}_n}$$

where P_c is the number density of crosslinkers at the point of incipient gelation, V_c is the number of crosslinked or branched monomers, N is the total number of monomers in a polymer and X_n is the weight average degree of polymerisation. This theory describes a covalent condensation of polyfunctional monomers either in an organic solvent or more commonly with the monomer itself as the solvent. It was concluded that the gelation corresponds to a situation where a macroscopically large branched polymer molecule becomes present spanning the entire volume of the sample. At the early stages of production of the gel "molecule", not all of the monomers originally involved form part of this network. A "sol" fraction consisting of free monomers and small aggregates co-exists with the network, being intimately dispersed through it. As crosslinking proceeds, the crosslinking fraction diminishes

in amount and the solid character becomes greater. As the process moves to completion, most of the free material is connected into the network. The emergence of the gel fraction is a sudden event occuring only when the degree of crosslinking reaches a critical value. Prior to this point there is a lag period during which the system becomes increasingly viscous as simple molecular condensation takes place. At the gel point where the critical crosslinking threshold is reached, the viscosity diverges to infinity.

Ferry's proposed mechanism explains gelation of thermally-induced protein gels (Ferry, 1948). This two step mechanism can be written as:

$$xP_N \rightarrow xP_D \rightarrow (P_D)x$$

where x is the number of protein molecules, P_N is the native protein and P_D is the denatured protein. This mechanism proposed that the final gel state corresponds to aggregates of partly denatured protein. The overall process requires that the protein unfold initially and that the second step, the aggregation, proceed more slowly than the first to allow the denatured protein molecules time to orient themselves and interact at specific points, thus forming a three-dimensional network. When the conditions of heat gelation are extreme, the molecules may not have time to align in an ordered fashion resulting in poorly hydrated aggregates or precipitates lacking the continuous matrix of gels.

Hermansson (1979) elaborated on Ferry's mechanism, stating that contrary to coagulation where aggregation of the protein molecules is random, gelation involves the formation of a continuous network exhibiting a certain degree of order. Furthermore, when aggregation is suppressed prior to denaturation, the resulting network can be expected to exhibit a higher degree of elasticity than if random aggregation and denaturation occur simultaneously, or if aggregation precedes denaturation. The slower the second step relative to the first, the better the denatured chains orient themselves and the finer the gel network.

Kauzman (1959) defined denaturation as a process or sequence of processes in which the spatial arrangement of the polypeptide chains within the molecule is changed from that typical of the native protein to a more disordered arrangement. Denaturation can be restricted to the continuous process of native protein structural changes involving the secondary, tertiary or

quaternary structure in which alterations of hydrogen bonding, hydrophobic interactions, and ionic linkages occur during the transition to the denatured state (Anglemeier and Montgomery, 1976). Ferry's mechanism stresses the importance of understanding and controlling the denaturation process.

In many systems such as polysaccharides, gelation is brought about by a change of pH, addition of reactive ions, lowering of temperatures or the mixing of interactive polymers. In protein systems it is more often brought about by denaturation by heat, surface forces or chemical reagents. This is followed by reassociation of the protein by intermolecular rather than intramolecular bonds, ultimately forming an extended network. The mechanisms underlying the formation of the three-dimensional networks characteristic of protein gels are not fully understood (Morrissey *et al.*, 1987). The general consensus of opinions is that polypeptide chains crosslink to form five or six crystalline regions per molecule during gelation. Other molecules can move in between these links or strands, and they account for the flexibility of the gel (Asghar *et al.*, 1985).

The ability of proteins to associate and coagulate, precipitate or gel, reversibly or otherwise, depends upon the protein, its amino acid composition, molecular weight and net hydrophobicity, its concentration, conditions such as heating rate and ionic strength, and the critical balance between attractive and repulsive forces (outlined in the review by Morrissey *et al.*, 1987). The characteristics of protein gels are affected by intra- and inter-strand crosslinking. This crosslinking combined with the fluidity of immobilized solvent gives gels their characteristic strength, elasticity and flow behaviour. Protein gels may be crosslinked by specific bonds at specific sites on the protein strands or by non-specific bonding occurring along the protein strands. The general types of crosslink bonds and their characteristics are summarised in Table 2.3. These bonds also contribute to the native structure and maintenance of a protein's conformation and can be viewed as important in protein denaturation.

In summary, Flory's theory requires a critical concentration of crosslinker as the minimum amount required to connect all of the polymers into one continuous network (Flory, 1941, 1953). This theory describes a covalent condensation of polyfunctional monomers to form a

Table 2.3. Structural bonding found in proteins and protein gels.

Type of Bond	Mechanism	Energy kJ/mol	Distance of interaction (Angstroms)
Covalent Bond	Electron Sharing	126-419	1-2
S-S[a]		266	
S-H[a]		368	
Ionic Bond	Coulombic attraction between oppositely charged groups	42-84	2-3
Hydrogen Bonding	Hydrogen sharing between two electronegative atoms (directional)	4-21	2-3
Hydrophobic Interaction (Van der Waals Attractive force)	Induction of dipole moments in apolar groups	4-13	3-5
Electrostatic Repulsive Force	Coulombic repulsion between similarly charged groups	$\frac{q_1 q_2}{r^2}$ [b]	
Van der Waals Repulsive Force	Repulsion between apolar groups in close proximity	$\frac{1}{r^6} - \frac{1}{r^{12}}$ [b]	

[a]Single bond enthalpies as taken from Pauling (1970)
[b]where q_1 and q_2 are charges (C) separated by a distance, r (m).
Adapted from Jones (1964), Whitaker (1977) and Alcock (1990).

macroscopically large branched polymer molecule spanning the entire volume of the sample. Ferry's mechanism explains thermal gelation, however this theory is applicable to any system where denaturation is a prerequisite to gelation (Ferry, 1948). It is considered a two-step mechanism, but, as with the crosslinker theory, there is a transitional phase where both denatured and gelled protein molecules exist (or in the case of Flory's theory, where free monomers and aggregates exist together). The slower the aggregation step relative to denaturation, the better the denatured chains orient themselves and the finer the gel network (Hermansson, 1979).

It is now widely accepted that the gelation of muscle proteins is largely responsible for the physical and chemical stabilization of fat and water in comminuted red meat and poultry

products and for binding at the junction between meat pieces in sectioned and formed products. The characteristic texture of various processed meat products may be a result of the protein matrix and its specific interactions with the continuous aqueous phase and dispersed fat (Ziegler and Acton, 1984a). In heat restructured meat products, Ferry's mechanism is likely to apply to myofibrillar proteins when sulfhydryl groups are not involved. At these temperatures (less than 70°C), binding is more likely to be result of hydrophobic and electrostatic interactions and hydrogen bonding, not covalent bonding.

2.4 RESTRUCTURING RESEARCH

2.4.1 Fresh meat restructuring

When a product is restructured its form is changed. It is not necessarily required that anything be removed or added as opposed to reconstitution of a product. Generally restructuring of meats refers to the binding together of meat pieces, ranging in size from small particles to large chunks, to form a cohesive mass. Any skeletal meat muscle can be restructured to a consumer-ready finished meat product. In the first meat products developed, binding of these meat pieces was generally achieved by solubilizing protein through mixing in the presence of salt and phosphate, bringing the soluble protein to the meat particle surface, putting meat particles in contact with each other, possibly reshaping, and heat setting these proteins during cooking (Smith, 1982). Today methods for restructuring include the use of enzymes and other additives such as algin in the presence of calcium, and do not necessarily require the application of heat to achieve bind.

In 1969, the University of Nebraska in the U.S.A. developed a prototype flaked steak and conducted some early work in the field of flaked steaks (Mandigo, 1982). Much of the research was aimed at developing institutional meat products. Primary criteria were to control shape, weight, composition of fat and protein and texture of the finished product. The products needed bind similar to intact muscle and uniform juiciness and tenderness. A variety of fresh and cured and smoked products was developed. Considerable research was conducted at other locations as well during that time (for example, Wilcox and Hafstad, 1968, 1970;

Japanese Leather Co., Ltd., 1970; Dechaine and Callaghan, 1968; Zakharov *et al.*, 1968; Armour and Co., 1968; Ralston Purina Co., 1968).

One of the problems with the flaked, formed and sectioned steak was that its texture was much like ground beef or pork sausage, not steak. As a result, efforts were begun to increase the particle size and bind by utilizing other techniques. Some concepts from sectioned and formed ham production were evaluated for use on the fresh meat system. The first major differences were in the substantially lowered salt content and the absence of nitrite in the fresh steaks. Concepts from work done by several investigations on protein binding were also incorporated into the change from the flaked steaks to the steaks made by chunking and forming or sectioning and forming. Today there are several products which have been developed ranging from steaks and chops to roasts and meat rolls. The binding of the proteins in these products is often fragile at refrigerated temperature, protected at frozen temperatures and finally realised when the proteins are coagulated during cooking (Smith, 1982).

Table 2.4 lists some of the research undertaken related to the bind of restructured meat products. This list is not comprehensive, but gives an indication of the extent of recent research in this area. A large amount of the research is primarily concerned with bind, however, there is also much reported work on other areas, including texture, colour, flavour, sensory evaluation, marketing and processing conditions. These cover a whole range of meat types, from different species at various ages to different muscle types. There is also a large amount of research on sausage and ground meat products. Much of this emulsion based work set the foundation for investigations using larger particle sizes, in particular, work on extraction of protein. A large body of work also exists on fish proteins, which, like the emulsion work, has played an important role in the research of restructured meat products. Both of these areas have also been major contributors to research of meat and meat proteins.

2.4.2 The function of muscle proteins in heat gelation

Most research on the binding of proteins has concentrated on heat-induced binding and until about 1985 it was thought that heat was required to produce effective gelation as raw meat

Table 2.4. Research related to bind of restructured meat products.

Researcher	Variables	Product
Froning (1965)	polyphosphate	chicken loaves
Torr (1966)	comminuted meat & salt with proteolytic digestion	poultry and/or animal meat
Maesso (1969)	NaCl, phosphate, egg albumen, poultry emulsion	poultry loaves
Maesso *et al.* (1970)	vacuum, pressure, pH	poultry loaves
Schnell *et al.* (1970)	beating, NaCl, phosphate, ribonucleic acid, dried meat	poultry loaves
Hwang and Carpenter (1975)	pork hearts or pH, with non fat dry milk, soy protein isolate & concentrate peanut flour & grits	pork or beef loaves
Pepper and Schmidt (1975)	NaCl, phosphate, blend time, hot boning	beef rolls
Moore *et al.* (1976)	NaCl, TPP, soy protein isolate, textured soy protein, modified whey solids	beef rolls
Schwartz and Mandigo (1976)	NaCl, TPP, storage	pork chops
Campbell *et al.* (1977)	cooking temperature, portion thickness	pork patties
MacFarlane *et al.* (1977)	myosin,actomyosin, sarcoplasmic protein	chunked beef
Ford *et al.* (1978)	myosin, sarcoplasmic protein	beef steakettes
Huffman and Cordray (1979)	salt, TPP	pork chops
Ibarra *et al.* (1979a,b)	non-skeletal muscle tissue	beef steaks
Siegel and Schmidt (1979a,b)	myosin, actomyosin, HC, NaCl, phosphate, pH, temperature, concentration	chunked beef
Turner *et al.* (1979)	myosin, NaCl, phosphate	chunked beef
Furumoto and Stadelman (1980)	NaCl	pork, chicken, beef, turkey rolls
Kyu-Pi Co., Ltd. (1981)	water soluble protein + $Ca_3(PO_4)_2$	fresh meat blocks
Chastain *et al.* (1982)	antioxidants, frozen storage period	beef and pork steaks
Hand *et al.* (1982)	KCl, NaCl, grind method, frozen storage period,	pork roast

Table 2.4. (cont.)

Researcher	Variables	Product
Kyu-Pi Co., Ltd. (1982)	egg white powder, soybean protein, gluten powder, $Ca(OH)_2$ or CaO powder, organic acid powder	fresh meat
Seideman *et al.* (1982)	hot boning, particle size	beef steaks
Terrell *et al.* (1982)	vital wheat gluten, egg albumen, isolated soy protein, plasma protein, sodium caseinate	pork slices
Tsai and Ockerman (1982)	emulsion coated cured pork	tissue
Coon *et al.* (1983)	NaCl, mixing times, tempering times	beef logs
Trout and Schmidt (1984, 1986a, 1987)	NaCl, phosphate, pH, fat, ionic strength, temperature	beef rolls
Vimini *et al.* (1983)	low frequency ultra-sound, NaCl	beef rolls
Brewer *et al.* (1984)	mechanical separation, NaCl, soy extenders, dried skim milk	lamb roasts
Berry *et al.* (1986)	pressure, NaCl, particle size	beef steaks
Means and Schmidt (1986)	algin/$CaCO_3$	beef steaks
Schmidt and Means (1986)	algin/calcium, GdL, citric acid lactic acid, sodium erythorbate	ground and chunked meat
U.S. Dept of Agriculture (1986)	sodium alginate, $CaCO_3$, lactic acid, calcium lactate	raw and cooked meat
Yoshimura and Hayashi (1986)	protein, polysaccharides, pulverised bone, egg shells, alkali earth metal hydroxides &/or NaOH, fats, fatty acids, surfactants, sugars, natural gums	meat pieces
Young *et al.* (1987)	NaCl, TPP	chicken patties
Johnson (1988)	algin/calcium, adipic acid	raw and cooked chunks
Bennett (1989)	sodium alginate/ encapsulated calcium	beef steak logs
Wijngaards and Paardekooper (1988)	fibrinogen, thrombin, transglutaminase	raw meat chunks

TPP = tripolyphosphate
GdL = glucono-δ-lactone
HC = heavy chains of myosin

pieces did not exhibit any bind (Vadehra and Baker, 1970; Asghar *et al.*, 1985). More recently, work has tended towards developing products requiring bind at raw meat temperatures, but much of the fundamental research lies with the heat gelation work. A lot of the earlier studies on meat used proteins of rabbit and chicken, whereas today other meat proteins, especially beef, are more commonly used.

2.4.2.1 Myosin and actin

Fukazawa *et al.* (1961a,b,c), Samejima *et al.* (1969) and Yasui *et al.* (1980) made several fundamental studies on the role of different muscle protein fractions in the gelation process. Fukazawa *et al.* (1961a,b) and Hashimoto *et al.* (1959) evaluated the binding characteristics of sausages prepared from myofibrils. These studies indicated that mainly myosin determines the binding quality in sausages and that bind strength displays a close relationship with the amount and the nature of myosin contained in and/or liberated as actomyosin from a meat structure. This view was substantiated by the findings of many researchers (including Maesso *et al.*, 1970; Schnell *et al.*, 1970; Vadehra and Baker, 1970; Nakayama and Sato, 1971b; Grabowska and Sikorski, 1976; Moore *et al.*, 1976; MacFarlane *et al.*, 1977; Ford *et al.*, 1978; Kijowski and Niewiarowicz, 1978; Siegel *et al.*, 1978a,b; Siegel and Schmidt, 1979a,b). From these studies it appears that the binding phenomenon depends solely on the extraction of proteins from myofilaments followed by gelation of these proteins during thermal processing.

Oosawa *et al.* (1959) reported that actin filaments form three-dimensional networks of linear polymers, or gels. Later studies on actin in a model system have revealed that actin alone did not exhibit any binding property (Fukazawa *et al.*, 1961b; Samejima *et al.*, 1969; Yasui *et al.*, 1980), but in the presence of different crosslinking proteins, actin filaments produced gels (Brotschi *et al.*, 1978; Bryan and Kane, 1978; Mimura and Asano, 1979; MacLean-Fletcher and Pollard, 1980a,b; Zaner and Stossel, 1983). By using different crosslinking proteins and several length-shortening proteins with actin, several researchers have observed Flory's equation (Flory, 1953) held to a fair extent (Nunnelly *et al.*, 1981; Yin *et al.*, 1980). However, in the case of actin, some crosslinkages were produced by direct actin-actin bonds (MacLean-Fletcher and Pollard, 1980a), whereas Flory's theory (Flory, 1941, 1953) assumes all bonds between polymers are formed only by crosslinking molecules. Whatever the

mechanism, in the presence of myosin, actin exerted a synergistic effect on gel strength under appropriate conditions of ionic strength (Samejima *et al.*, 1969).

Nakayama and Sato (1971a,b,c), Yasui *et al.* (1980, 1982), Samejima *et al.* (1982) and Ishioroshi *et al.* (1980) examined the rheological properties of heat set meat protein gel and reported that the binding strength of reconstituted and natural actomyosin was greater than that of myosin alone. In contrast, MacFarlane *et al.* (1977) found stronger binding force in myosin than actomyosin gels. Siegel and Schmidt (1979a) observed that a high proportion of myosin to actin within the ratio of 3 to 8 resulted in high binding ability. Yasui *et al.* (1982) resolved this disparity by showing that a specific myosin to actin ratio was essential to develop a stronger gel than that formed by myosin alone. Maximum gel strength was observed at a myosin to actin mole ratio of 2.7 which corresponds to a weight ratio of myosin to actin of about 15. At this ratio, 15 to 20% of the total protein exists as actomyosin complex and the remaining is free myosin. Asghar *et al.* (1985) suggested that the small amount of actomyosin complex that formed in the system acted as a crosslinker with the free myosin molecule on heating and may have been a prerequisite for actin-induced improvement in the gel formability of myosin. The free myosin to actomyosin ratio is therefore very important, the optimum ratio being 4 in 0.6 M potassium chloride at pH 6.0 and 65°C (Yasui *et al.*, 1982).

Ishioroshi *et al.* (1983) showed that the addition of actin to a myosin system in 0.2 M potassium chloride caused a progressive decrease in the heat-induced gel strength of myosin and it was assumed a result of blocking the actin binding site with actin which would otherwise be involved in network formation. Samejima *et al.* (1981) showed actin had no effect on gelation of myosin at 0.1 M potassium chloride, but at 0.6 M potassium chloride, gelation of myosin was accelerated. Yasui *et al.* (1980) found that the increasing effect of actin on the heat induced gel formability of myosin emerged only at the higher potassium chloride concentration range from 0.5 to 0.8 M potassium chloride. These findings suggested that the synergistic effect of actin on myosin was either ionic strength dependent or was determined by the state of the myosin at different ionic conditions.

Yasui *et al.* (1982) observed that,
Using scanning electron microscopy (SEM), myosin and actomyosin gels at 65°C in 0.6 M potassium chloride and at pH 6.0, showed a marked variation in pore size distribution, shape

of network structure and in the thickness of strands, depending on protein aggregation ranging from assemblies of irregular protein clumps to filamentous strands with entangled clusters.

Gel formed by an actomyosin system consisted of fine filaments with various degrees of crosslinking. Irregular chains of myosin were apparent characterised by non-homogenous particles cross-linked by tails to form networks of myosin-actomyosin systems at a weight ratio of 4. A ten-fold decrease in the myosin to actomyosin ratio formed a gel in which the network structure of actomyosin itself seemed to coexist. While high side-to-side crosslinkings between filaments appeared to prevail in a myosin-actomyosin system, intensive crosslinkages between free and bound myosin seemed to dominate.

Although the myofibrillar proteins as a group are known to be the major contributors to binding in meat systems, it is difficult to single out the contribution of the individual proteins. This is due to the fact that the different proteins interact with each other when binding (Yasui *et al.*, 1980) and that the method of extraction and purification of the proteins can have a profound influence on their binding ability (Siegel and Schmidt, 1979a).

2.4.2.2 Myosin subfragments and subunits

Samejima *et al.* (1981, 1988) and Ishioroshi *et al.* (1981) studied the contribution of different segments of the myosin molecule in the thermal gelation process. Intact myosin rods produced gels of approximately 25% of the strength of the myosin gels on an equimolar basis, but those formed by S-1 at the same protein concentration were weak by comparison and had poor water retaining ability. This difference was also reflected in the ultrastructure of the gel as seen in SEM (Samejima *et al.*, 1981). Myosin and myosin rods produced an extended three-dimensional network system, while S-1 fragments formed a bead-like aggregation upon heating. Samejima *et al.* (1984) also showed that heavy chains of myosin possessed the same gel strength potential as that of intact myosin molecules under similar conditions in a model system.

On combining S-1 and rod fragments, gel strength did not attain that of intact myosin molecules (Samejima *et al.*, 1981). Rigidity profiles of myosin rods as a function of protein concentration, pH or ionic strength have shown that the requirements for these variables were

essentially the same as for the intact myosin molecule to form a gel with maximum strength (pH 6.0 and an ionic strength of 0.6). However, gelation of S-1 fragments was found independent of pH and ionic strength (Samejima *et al.*, 1981; Ishioroshi *et al.*, 1981).

Ishioroshi *et al.* (1981) also studied the relative contributions of S-1 and S-2, LMM and HMM segments in the gelling process. Under conditions of 20 to 70°C at 0.6 M potassium chloride and pH 6.0, HMM gave about a five-fold greater gel strength than S-1 segments, whereas, LMM produced a gel of almost a quarter of the strength as formed by total myosin rods. The HMM subfragment of myosin showed pH and ionic strength dependency unlike that of S-1, and the maximum gel strength was observed at pH 5.0 and 0.1 M potassium chloride concentration. The rigidity of HMM gel increased with protein concentration exponentially depending on the pH value and the ionic concentration. The LMM subfragment produced a gel of maximum strength at pH 6.0 and 0.6 M potassium chloride.

Yasui *et al.* (1982) examined the thermogelling characteristics of intact myosin and its subfragments such as total rod, LMM, HMM and S-1 in the presence of actin and actomyosin. No increase was apparent in gel strength of myosin subfragments in the presence of actin, although HMM and S-1 did form complexes with actin. Samejima *et al.* (1984) found that myosin heavy chains formed stronger gels alone than in the presence of actin. LMM and total rod have no ability for interacting with actin. However, in the presence of actin, both showed the potential of forming a three-dimensional network structure on heating. None of the myosin subfragments exhibited any marked augmentation of thermogel strength in the presence of actin. Yasui *et al.* (1982) concluded that the heat-induced gel in sausages was formed possibly by crosslinking free and bound myosin molecules with actin involving tails so as to produce a mechanically more stable gel than formed by myosin alone.

Asghar *et al.* (1985) stated that the difference in gel characteristics of LMM and myosin rods may be ascribed to the aggregation behaviour at different ionic conditions. Moos *et al.* (1975) found large sheet-like paracrystals of myosin rods formed when the purified myosin rod was precipitated gradually by dialysis (from 0.5 to 0.1 M potassium chloride). Koretz (1982) observed that the best myosin rod aggregates were prepared in 0.05 to 0.15 M potassium chloride at pH 7.0. Under these conditions the rods formed unipolar sheets. Cohen *et al.*

(1985) showed that rods dispersed in 0.05 M potassium thiocyanate and then precipitated in 0.05 M calcium chloride or magnesium chloride, mainly formed ordered bipolar segments with occasional unipolar segments at low ionic strength. The LMM subfragment also aggregated in different forms in a low ionic strength environment, generally as paracrystals or tactoids (Chowrashi and Pepe, 1977; Koretz, 1982; Safer and Pepe, 1980; Bennett, 1981; Moos *et al.*, 1975), however other structures may have formed, such as ribbons, sheets, thin fibres, or square open lattices (Koretz, 1982).

2.4.2.3 Other muscle proteins

Nakayama and Sato (1971b) stated that like actin, tropomyosin also enhanced the binding strength of myosin gel. A greater increase in the viscosity (taken as a possible measure of bind quality) of myofibrillar protein was observed by addition of tropomyosin in the presence of pyrophosphate and magnesium chloride ($MgCl_2$) than in a tropomyosin-free system. It was also noticed that in the absence of pyrophosphate, only tropomyosin and myosin were related to the bind quality of fabricated meat products, whereas, in the presence of pyrophosphate, tropomyosin, actin and myosin determined the bind quality. According to other studies, the presence or absence of tropomyosin (Samejima *et al.*, 1982; Fukazawa *et al.*, 1961b) and troponins (Samejma *et al.*, 1982) in the actomyosin system made no difference to the microstructural characteristics of the resulting gel. Although it is the microstructure which determines the sensory and mechanical characteristics of a food (Stanley and Tung, 1976), the use of different characteristics could resolve an apparent contradiction in a lack of effect on the microstructural characteristics when an effect on bind quality measured as viscosity was evident. Asghar *et al.* (1985) noted as tropomyosin is a very heat-stable protein (Woods, 1969; Chrystall, 1970) it was logical to suggest that it would have little gelation value at normal cooking temperatures.

Fukazawa *et al.* (1961b) showed that the sarcoplasmic protein fraction contributed little to the myosin gel strength. However, Davey and Gilbert (1974) suggested that heat coagulation of sarcoplasmic protein resulted in the formation of a gel which can link structural elements of meat. Most of the sarcoplasmic proteins are coagulated at temperatures from 40 to 60°C (Hamm, 1966; Laakkonen *et al.*, 1970) although the heat denaturation of some proteins of this

group extends up to 90°C (Davey and Gilbert, 1974). MacFarlane *et al.* (1977) using a model system capable of measuring the cohesive forces between meat pieces for comparing the binding power of different muscle proteins indicated that in the absence of salt, sarcoplasmic protein enhanced the binding strength of myosin. However, with increasing ionic strength, a deleterious effect was exerted on the myosin binding strength, possibly resulting from either salt-induced denaturation or precipitation of the sarcoplasmic protein. The beneficial effect of the sarcoplasm was ascribed to the ionic contribution that it made to the system. The sarcoplasmic fraction also contains many enzymes, including proteases and phosphatases (Morita and Yasui, 1978; Morita *et al.*, 1983a, b), which are not inactivated until the temperature reaches over 60°C (Hamm, 1966; Neraal, 1975). These enzymes are likely to degrade the structural proteins and hence the gelling strength is expected to be lowered.

Samejima *et al.* (1969) showed that the addition of myosin-free connective tissue proteins to myofibril protein solutions in a 0.6 M potassium chloride, pH 6.0 system caused a reduction in the gel strength. These studies were performed at 60°C, therefore collagen could not be expected to show any significant positive response as it requires higher temperatures for transforming to a soluble gel (Asghar *et al.*, 1985), although it denatures at 35 to 40°C (Bendall, 1964).

To summarise, there is much evidence to support the findings that myofibrillar proteins and in particular myosin, are responsible for the heat-induced gelation of meat. Actin, although it was unable to gel on its own (Fukazawa *et al.*, 1961b; Samejima *et al.*, 1969; Yasui *et al.*, 1980), may play a role in gelation in the myosin system by forming actomyosin which may act as a crosslinker (Asghar *et al.*, 1985). Studies on the subfragments of myosin have provided information about the roles of the various units in gelation. These are discussed in the next section (2.4.2.4) when the physicochemical changes in myosin upon gelation are considered. Intact myosin rods produced gels of 25% of the strength of myosin gels on an equimolar basis (Samejima *et al.*, 1981, 1988; Ishioroshi *et al.*, 1981). The gels formed from the S-1 subfragment and at the same protein concentration were weak and had poor water retaining ability. The heavy chain possessed the same gel strength as that of intact myosin molecules under similar conditions (Samejima *et al.*, 1984). At 20 to 70°C, 0.6 M potassium chloride and pH 6.0, HMM gave five-fold greater gel strength than S-1; the LMM fragment

gave a gel of almost one quarter the strength as formed by myosin rods (Ishioroshi *et al.*, 1981). Combining S-1 and rod did not attain the gel strength of intact myosin (Samejima *et al.*, 1981). The rigidity profiles of rods generally showed the same requirements of concentration, pH and ionic strength as for intact myosin, however, S-1 gelation was independent of pH and ionic strength (Samejima *et al.*, 1981; Ishioroshi *et al.*, 1981). Actin did not affect gel strength of myosin subfragments (Yasui *et al.*, 1982).

In addition, Nakayama and Sato (1971b) observed an enhancement of myosin gel strength in the presence of tropomyosin. Samejima *et al.* (1982) and Fukazawa *et al.* (1961b) noticed that in an actomyosin system, tropomyosin and troponin did not contribute to the gel microstructure. Davey and Gilbert (1974) suggested that sarcoplasmic protein may also have a beneficial effect on myosin gel strength in the absence of salt. MacFarlane *et al.* (1977) ascribed the enhancement of gel strength to the electrostatic contribution the sarcoplasm makes to the system. However, this electrostatic effect became deleterious to the gel strength as the ionic strength is increased.

2.4.2.4 Physicochemical changes in myosin molecules during gelation

Many researchers have studied the heat-induced transitional temperature changes in viscosity (Jacobson and Henderson, 1973), helical content (Burke *et al.*, 1973a), pH value and "thermoprofiles" of myosin and its subfragments in solution (Swenson and Ritchie, 1980; Wright *et al.*, 1977; Yasui *et al*, 1966). Myosin has been reported to have from one to six transitions due to the denaturation of specific domains, commonly called transition temperatures (T_m). Table 2.5 lists some of the results of studies undertaken investigating the denaturation of myosin and its subfragments.

Wright *et al.* (1977) using differential scanning calorimetry (DSC) data reported one to three T_m for myosin representing three discrete molecular regions differing in thermal stability, dependent on pH and ionic strength of the medium. Three T_m (43, 49.5 and 60.5°C) were observed for myosin at pH 6.1 and an ionic strength of 1.0, while only one T_m (55°C) was observed at pH 7.0 and an ionic strength of 0.046. It was proposed that the formation of filaments at low ionic strength provided extra stability to the molecule accounting for the

Table 2.5. Transition temperatures of myosin.

Source	Protein	Technique[a]	Conditions		Transition Temperature (°C)			Order[b]	Reference
			pH	M or I	T_{m1}	T_{m2}	T_{m3}		
rabbit	myosin rod	OR	7.2	0.5 KCl	44	55			Burke *et al.* (1973a)
	LMM				44	52			
	S-2				39	50			
rabbit	myosin	pH	5.9	0.5 KCl	41				Goodno and Swenson (1975a)
	myosin rod				39.7				
	LMM				39.7				
rabbit	myosin rod	OR,CD	7.0	0.6 KCl	47.5	55			Samejima *et al.* (1976)
rabbit	myosin	DSC	6.0	1	43	49.5	60.5		Wright *et al.* (1977)
	myosin		7.0	0.05		55			
	actomyosin		7.0	0.05	51.5	60	73[c]		
rabbit	myosin	SM	6.0	0.6 KCl	43	55		(H+T)-T	Ishioroshi *et al.* (1979)
rabbit	myosin	SM	6.0	0.6 KCl	43	55		H-T	Ishioroshi *et al.* (1981)
	myosin rod					53			
	LMM					53			
	HMM				45	54			
	LMM	OR,CD				50-55			
rabbit	myosin	SM	6.0	0.6 KCl	43	55		H-T	Samejima *et al.* (1981)
	myosin rod					53			
	S-1				43				
	myosin rod	OR,CD	6.0	0.6 KCl	43	54			

Table 2.5. (cont.)

Source	Protein	Technique	Conditions		Transition Temperature (°C)			Order	Reference
			pH	M or I	T_{m1}	T_{m2}	T_{m3}		
rabbit	myosin	DSC	7.0	0.6 KCl	46	52.5	57	T-H-T	Samejima *et al.* (1983)
	LMM				45		56		
	HMM					52.5			
	S-1					51			
	S-2					52			
	myosin rod				45		56		
	myosin			0.1 KCl		53.5	58.5		
	LMM					55	58		
	HMM					54			
	S-1					52.5			
	S-2					53			
	myosin rod				48.5		58.5		
rabbit	heavy chain	SM	6.0	0.6 KCl	42	55		T-H	Samejima *et al.* (1984)
rabbit	myosin	DSC	5.0	0.01 KCl	54	67		T-H-T	Wright and Wilding (1984)
				0.212 KCl	52	59		1.0 M	
				0.962 KCl	49	64			
			6.0	0.012 KCl	56	64		H-T-T	
				0.212 KCl	54			0.1 M	
				0.962 KCl	43	50	59		
			7.0	0.012 KCl	50	55			
				0.212 KCl	45	53			
				0.962 KCl	44	52	52		
			8.0	0.012 KCl	47	55			
				0.212 KCl	46	54			
				0.962 KCl	45	51	51		

Table 2.5. (cont.)

Source	Protein	Technique	Conditions		Transition Temperature (°C)						Order	Reference
			pH	M or I	T_{m1}	T_{m2}	T_{m3}	T_{m4}	T_{m5}	T_{m6}		
rabbit	myosin	DSC	6.0	0.012 KCl	54	64						Wright and Wilding (1984) (cont.)
	LMM				60	64						
	rod				61	65						
	HMM				53							
	S-1				52							
	myosin			0.962 KCl	44	50	59					
	LMM				41	60						
	rod				43	60						
	HMM				47	57						
	S-1				47							
bovine	actomyosin	LS	6.0	0.6 KCl	58.5	57.5					H-T	Ziegler and Acton (1984b)
rabbit	rod (average)	decon	6.5-9.0	0.5 KCl	46	51	55	59	62	68		Lopez-Lacomba *et al.* (1989)
	LMM (average)	DSC	6.5-9.0		46	52	55	60	(65)[d]			
	S-2 (average)		6.5-9.0		47			57	(61)[d]			

[a]DSC = differential scanning calorimetry; LS = light scattering; SM = shear modulus; OR = optical rotary dispersion; CD = circular dichroism; pH = adsorption of protons as measured by pH.

[b]H-T denotes head denatured, then tail. Where there is a molar concentration given after the order, the order was noted at that molar concentration.

[c]attributed to actin.

[d]few samples gave readings, these numbers represent the averages of those that gave readings.

decrease in the number of T_m and for shifting the T_m to higher temperatures. It was suggested that the three T_m might correspond to the unfolding of the trypsin sensitive region (the hinge region), the head and the tail of myosin. In actomyosin, Wright *et al.* (1977) observed three T_m; the first two were assigned to myosin (51.5 and 60°C) and the last to actin (73°C). In actomyosin, myosin exists as individual molecules attached to F-actin polymers. This structural arrangement was the basis for explaining the presence of two myosin-derived transitions in actomyosin thermograms under conditions of pH and ionic strength where myosin alone affords only a single transition.

Melting of the subfragments of myosin has enabled the transitions to be assigned to various regions of the myosin molecule. The transition temperatures of myosin and its helical fragments, the rod (LMM and S-2) and LMM, have been found to be similar (Burke *et al.*, 1973a; Goodno and Swenson, 1975a; Ishioroshi *et al.*, 1981; Samejima *et al.*, 1983; Wright and Wilding, 1984) which would suggest that the changes occurred in a region of the myosin molecule common to all three. Samejima *et al.* (1976, 1981) using optical rotary dispersion (OR) and circular dichroism (CD) measurements reported that myosin rods experience two transition temperatures during a heat-induced denaturation process. The major one was approximately 44°C and was not pH dependent. This transition was thought to possibly be due to the α-helix to random coil transformation accompanied by the destruction of hydrogen bonds. The minor transition at 55°C was pH dependent and it was suggested this transition was due to electrostatic forces. Burke *et al.* (1973a) reported similar results using OR. In addition to the helix to coil transformation, Driezen and Richards (1972) found that the light chain subunits dissociated from the myosin heavy chains at approximately 40°C.

The thermal denaturation of a protein is normally accompanied by a net uptake or release of protons caused by changes in the environment of ionizable residues. Denaturation is manifested by a relatively abrupt shift in the pH of unbuffered protein solutions; this shift may be characterised by a transition temperature. Goodno and Swenson (1975a,b) employed this concept to investigate ion-induced variations in the conformational stability of myosin and its helical subfragments. They reported that T_m values for both myosin and myosin rod decreased from 44 to 34°C as the potassium chloride concentration was increased from 0.15 to 2.9 M (pH 6.0). Ionic strength below 0.15 eliminated the adsorption of protons upon heating; this

suggested a structural change upon filament formation that resembled those which occurred during denaturation. Ziegler and Acton (1984a) using light scattering reported similar results.

Goodno and Swenson (1975b) also observed that decreasing the pH from 7.0 to 5.4 (0.5 M potassium chloride) reduced the myosin T_m from 43 to 37°C, and suggested that the binding of either H^+ or other monovalent cations (Na^+, K^+) altered the charge on the myosin molecule and disturbed the balance between electrostatic forces which maintain the native structure of the protein. With the balance of forces already disturbed, less thermal energy was required for denaturation, and T_m became correspondingly lower. Goodno and Swenson (1975b) concluded that the globular S-1 of the myosin molecule had either a T_m identical to that of rod (LMM and S-2) or made no contribution to the melting curve generated by the pH method.

Several other studies have shown at least two conformational transitions in myosin molecules or filaments during heating; one at about 43 to 47°C and the other at 53 to 55°C (Samejima *et al.*, 1969, 1976; Burke *et al.*, 1973a; Ishioroshi *et al.*, 1979). The transition occuring at 55°C was regarded the most critical as this is where the myosin attained the maximum binding strength (Yasui *et al.*, 1982). Ishioroshi *et al.* (1981) and Samejima *et al.* (1981) with evidence provided by myosin subfragments, assumed that during the gelation of myosin, head-to-head interaction took place at 43°C while subsequent unfolding of the rod segment occurred at 55°C. In contrast, some workers cited by Wright *et al.* (1977) and Wright and Wilding (1984) correlated the T_m at 43 and 53°C with the changes in the heavy and light chains in myosin, respectively. These conclusions are similar to early suggestions of Samejima *et al.* (1976).

In later studies using DSC, Samejima *et al.* (1983) showed three transitional phases during heating myosin monomers in a high salt buffer at neutral pH. The major transition was at 52.5°C, and the two minor ones were at 46 and 57°C. The first corresponded to the composite transitions of S-1 (51°C) and S-2 (52°C) fragments of myosin. The minor transitions were shown to represent the changes in myosin tail portion. Other studies have also indicated that the helix to coil transition occurs in two phases during heat denaturation of a protein in solution. First the destruction of electrostatic and hydrophobic interactions occurred, followed by unfolding of the helical structure (Potekhin and Privalov, 1982; Williams and Swenson,

1981). While Samejima *et al.* (1983) noted little change in T_m of S-1, S-2 and HMM fragment with a change in pH or ionic strength of the medium, the T_m of LMM and rod were significantly affected by these variables and the order of melting regions changed from tail-head-tail to head-tail-tail as the ionic strength decreased from 0.6 to 0.1 M potassium chloride. These changes were ascribed to the formation of paracrystal of LMM under various conditions of pH and ionic strength. Besides unfolding, depolymerisation of the LMM may (Woods, 1969; Yasui *et al.*, 1971) or may not (Siegel and Schmidt, 1979a; Samejima *et al.*, 1976) have occurred depending on whether LMM was prepared by enzymic or non-enzymic treatment, respectively. Wright and Wilding (1984) showed that myosin followed similar trends to LMM when the ionic strength was decreased from 1.0 to 0.1 M potassium chloride.

Using deconvoluted DSC thermograms, Lopez-Lacomba *et al.* (1989) were able to determine six discrete melting regions for myosin rod. These were investigated at pH values from 6.5 to 9.0 and at ionic strengths up to 1.0. The LMM subfragment of myosin was found to have four to five T_m similar to myosin rod and S-2 had two to three.

A change in inter- and intramolecular bonds in muscle protein accompanies conformational change during heating. Hamm (1966) mentioned that the helical parts of the protein molecules in meat unravelled into random coils, which were then crosslinked by hydrogen bonds and electrostatic interactions. Significant formation of intermolecular disulfide bridges did not occur on heating meat up to 70°C (Hamm and Deatherage, 1960). Jacobson and Henderson (1973) also reported no change in the titrable sulfhydryl groups of myosin upon heating from 19 to 60°C. Hamm and Hofmann (1965) concluded that heat coagulation of myofibrils and actomyosin was not due to the oxidation of the sulfhydryl group, but it was associated with other types of intermolecular linkages. Samejima *et al.* (1969) substantiated this work when neither reducing agents, thioglycol or sodium sulfite nor a sulphur-blocking agent, p-hydroxy-mercuribenzoate prevented solutions of rabbit myosin from coagulating, but rather accelerated the reaction up to 60°C.

In contrast, some studies indicated the formation of disulfide bonds between S-1 fragments (Ishioroshi *et al.*, 1980; Samejima *et al.*, 1981) during heat-induced gelation of myosin molecules. Ishioroshi *et al.* (1980) concluded that the active sites on both myosin and actin

molecules played a role in the development of myosin gels in the presence of actin and that sulfhydryl groups were oxidised to form the disulfide linkages. However, the disruption of stabilizing forces that occurred in LMM on heating consisted of non-covalent linkages such as hydrogen bonds and electrostatic and hydrophobic interactions without involving disulfide formation. Liu *et al.* (1982) substantiated these views when oxidation of sulfhydryl groups was observed during the heat induced gelation of Atlantic croaker. Although fish protein have shown characteristics which differ to those of mammalian protein, it should be noted that the gel formed was solubilised with 2% sodium dodecyl sulphate, suggesting that hydrophobic interactions were the predominant force that brought about aggregation. When heated beyond 70°C, myosin showed a marked decrease in the number of titrable sulfhydryl groups (Hofmann and Hamm, 1978) and suggested the formation of disulfide bonds at these higher temperatures.

In summary, the transition temperatures of myosin and its helical subfragments, the rod (LMM and S-2) and LMM, were found to be similar (Burke *et al.*, 1973a; Samejima *et al.*, 1983; Ishioroshi *et al.*, 1981; Wright and Wilding, 1984; Goodno and Swenson, 1975a) which suggested that the changes occurred in a region of the myosin molecule common to all three. The S-1 fragment of the myosin molecule appeared to have either a T_m identical to that of rod (LMM and S-2) or made no contribution to the melting curve (Goodno and Swenson, 1975b). Little change was noted in S-1, S-2 and HMM fragments with change in pH or ionic strength of the medium (Samejima *et al.*, 1983). Myosin, LMM and rod T_m were significantly affected and the order of melting regions changed from tail-head-tail to head-tail-tail as the ionic strength decreased from 0.6 to 0.1 M potassium chloride. This could explain some of the differences in the order of melting among different workers. Generally results from the various methods were similar although some workers assigned different melting temperatures to different stages of melting, for example, Samejima *et al.* (1983) assigned T_{m1} and T_{m3} for the values obtained for the melting of LMM at the higher ionic strength values, whereas Wright and Wilding (1984) assigned these values to T_{m1} and T_{m2} (refer to Table 2.4). It appears that sulfhydryl groups were not involved in the gelation of myosin at temperatures less than 70°C, but at greater temperatures may become significant.

Jacobson and Henderson (1973) suggested that in many studies of heat-denaturation of myosin and actomyosin, it is possible that some of the changes reported were due, at least in part to

the presence of oxygen or to trace metal ion impurities present in reagent grade salts. The changes were loss of ATPase activity and the presence of titrable sulfhydryl groups. Asghar *et al.* (1985) suggested that the use of different animal species, pH and ionic strength would explain varying results obtained by different workers. It is also possible that the inclusion of troponin and tropomyosin, as contaminants in the actomyosin and crude myosin preparations, could affect experimental results. In the Szent-Györgyi (1947) preparation of natural actomyosin these contaminant proteins are not completely removed (Jacobson and Henderson, 1973).

2.4.3 Factors affecting heat-induced gelation of myosin in model systems

2.4.3.1 Myosin concentration

Wu *et al.* (1991), in an investigation of chicken breast myosin gels, found that higher myosin concentrations increased the rate of shear modulus development and the final values of shear modulus were higher. Siegel and Schmidt (1979b) showed that the level of protein had an increasing linear effect on the binding ability of bovine myosin. This was attributed to a tighter matrix of interweaving fibres formed from the higher concentration of protein. Generally relationships between myosin concentration and gel strength in the presence or absence of actin generated a rate of change of gel strength of 1.7 to 2.0 (Ishioroshi *et al*, 1979; Yasui *et al.*, 1982). This suggested that the heat-induced gel strength of myosin increased proportionally with the square of the protein concentration, irrespective of the ionic environment and whether or not actin was present in the system.

The effect of concentration on the morphology of myosin filaments is uncertain. Somewhat divergent findings have been reported regarding the influence of protein concentration on filament formation. Katsura and Noda (1973) reported the length of reconstituted filaments to be independent of myosin concentration to as low as 5 μg/ml. At intermediate concentrations the results of Koretz *et al.* (1982) were similar to Katsura and Noda (1973), however as the protein concentration was reduced to 20 μg/ml Koretz *et al.* (1982) noted a linear decrease in filament length.

2.4.3.2 Myosin isoforms

Asghar *et al.* (1984) in comparative studies of red and white broiler muscle, found white muscle always exhibited far greater gel strength than red muscle under similar conditions of ionic strength, pH or protein content. Much of the difference in isoforms of myosin was attributed to the nature of the light chains in the S-1 fragment (Asghar *et al.*, 1985).

There is much experimental evidence for the existence of polymorphism in myosin (for example, Gauthier and Lowey, 1979; Gauthier *et al.*, 1982, 1983; Holt and Lowey, 1977). Mammalian skeletal muscles contain at least three types of motor units, namely fast-twitch fast-fatigue, fast-twitch fatigue-resistant and slow-twitch fatigue resistant motor units (Burke *et al.*, 1973b). These differences in types of muscle fibres are reflected in isoforms of different proteins (Salviati *et al.*, 1982), for example fast-twitching posterior *L. dorsi* and *pectoralis* muscle of chicken were found to contain three isozymes, whereas slow-twitching anterior *L. dorsi* contained only two isozymes of myosin (Hoh, 1978, 1979). Other studies have indicated the presence of three classes of light chains in fast-twitching white muscle from rabbit and chicken, but slow-twitching red and cardiac muscle contained only two classes of light chains with molecular weights of 27000 and 19000 (Lowey and Risby, 1971; Sarkar *et al.*, 1971; Yagi *et al.*, 1975). Possibly these variations explain the difference in the rate of ATPase activity (Heilmann *et al.*, 1977), and stability of myosin from fast- and slow-twitching muscle under different pH conditions (Sreter *et al.*, 1966).

When referring back to Figure 2.5 it is noted that the heads of the two myosin molecules are not necessarily identical (Pearson and Young, 1989). Adult skeletal myosin may contain two or more populations of myosin that differ in their light chain composition. There are three light chains, the DTNB light chain and two alkali light chains. Immunoabsorption experiments showed that each native myosin molecule contains two A1 light chains or two A2 light chains. No hybrid myosin molecules, containing one A1 light chain and one A2 light chain are present (Holt and Lowey, 1977), however, different amounts of the light chain combinations may exist in different muscles.

Sharp and Offer (1992) using electrophoretic results concluded that the mechanism of heat-induced head-to-head aggregation depended on which light chains were dissociated from the myosin molecule after heating at 30 to 60°C. Loss of light chains caused head-to-head aggregation by exposing hydrophobic patches on the heavy chain surface (Bagshaw, 1980). Sharp and Offer (1992) provided a possible explanation for the different types of aggregation by suggesting that when the DTNB light chains were dissociated, a hydrophobic patch was exposed only at the base of the heads, but owing to the curvature of the heads, the two patches in a single molecule could not come together. When the alkali light chains were dissociated, the hydrophobic patch that was exposed was more extensive and the position of the patch with regard to the curvature of the head may have allowed both intra- and intermolecular head association to occur.

Maesso *et al.* (1970) reported the binding ability of poultry leg meat to be weaker than breast. This variation was not investigated, but it was suggested by Asghar *et al.* (1985) to be a result of the different myosin isoforms present. Further comparisons of chicken leg and breast meat and myofibrillar protein have shown differences in the gelation characteristics of the different muscles (Xiong and Brekke, 1991). Young *et al.* (1992) observed differences in the rheology of bovine myofibrillar gels made from three classes of muscle defined by myosin type; fast twitch, slow twitch and heart. Montejano *et al.* (1984) observed differences in gelation characteristics of muscle from different species; fish, beef, pork and turkey. These differences may also have been a result of different isoforms of myosin present in the different muscles and muscles of different species. Parsons and Knight (1990) drew conclusions that the salt concentration required for the extraction of myosin from myofibrils under conditions similar to those of meat processing depended on the myosin isoform content of the myofibrils, however consideration had to be given to whether the result was also influenced by factors such as other proteins or post-mortem events.

2.4.3.3 Ionic Strength

Ishioroshi *et al.* (1979, 1983) evaluated the strength of myosin gels that formed on heating at 65°C, pH 6.0 and at ionic strengths from 0.1 to 0.6 M potassium chloride. Highest shear modulus was attained between 0.1 and 0.2 M potassium chloride concentrations. Beyond 0.4

ionic strength the gel strength decreased to a minimum; and thereafter remained steady with increases in potassium chloride concentration up to 0.6 M. A relatively finer three-dimensional network developed at 0.2 M potassium chloride than at 0.6 M as viewed under SEM. Asghar *et al.* (1985) ascribed this difference to the fact that at low ionic concentration the myosin molecules assembled into filaments (Huxley, 1963; Kaminer and Bell, 1966), and on heating the filaments formed three-dimensional structures. At high ionic strength the molecules existed as monomers, which on heating produced head-to-head aggregates. Asghar *et al.* (1985) also noted that the probability of random collisions due to Brownian motion was likely to be much greater among free myosin monomers than if they were immobilized into the built-in architecture of myofilaments. Hermansson *et al.*, (1986) used this information to explain the difference in gel structure at pH 5.5 to 6.0. A fine stranded gel structure was formed at 0.25 M potassium chloride where filaments existed prior to gelation and a coarsely aggregated structure formed at 0.6 M potassium chloride where the myosin was in a monomeric form prior to gelation. It was concluded that the conditions required for the formation of the different gels were present prior to heating. The fine stranded structure had a higher rigidity than the coarsely aggregated structure.

Samejima *et al.* (1985) found that increasing the concentration of sodium chloride up to 0.5 to 0.6 M, increased the protein concentration of the liquid phase of the beef myofibrillar gels at pH 6.0. At ionic strengths greater than 0.25, thick filaments were progressively depolymerised to myosin molecules (Hasselbach, 1953; Hanson and Huxley, 1953, 1955; Huxley, 1963; Josephs and Harrington, 1966) the action of the salt being to weaken the secondary bonds between neighbouring myosin tails in the shaft of the thick filament. It was also noted that myosin heavy chains at 1 mg/ml formed precipitates below an ionic strength of 0.3 and above this strength became soluble (Samejima *et al.*, 1984). On heating to 65°C and at protein concentrations less than 0.2 mg/ml, myosin heavy chains lost their solubility and formed aggregates even at 0.6 M potassium chloride (Samejima *et al.*, 1984).

Sarkar (1950) noted that the isoelectric point (IEP) of salt free myosin was 5.4, but with the addition of 0.025 M potassium chloride increased to pH 6.9. At higher potassium chloride concentration it fell, reaching pH 5.4 in 0.2 M potassium chloride and pH 3 in 0.8 M potassium chloride. It was therefore concluded that at lower potassium chloride

concentrations, potassium ions preferentially bound to myosin, but at higher concentrations, more chloride ions than potassium ions were bound.

2.4.3.4 The pH value

Ishioroshi *et al.* (1979) examined the dependence of heat-induced myosin gel strength on the pH in the range of pH 5 to 8. Myosin solution exhibited much greater gel strength at 4.5 mg/ml protein in 0.6 M potassium chloride or sodium chloride and at pH 6 than those formed at other pH values. At high pH, gels appeared relatively translucent; at low pH, gels exhibited syneresis. A study by Yasui *et al.* (1980) also indicated pH 6.0 as the optimum pH from the range of pH 5.0 to 8.0, for achieving maximum gel strength on heating to 60°C at 0.5 to 0.8 M potassium chloride. However, Yasui *et al.* (1980) showed that the pH value at which peak gel strength was achieved shifted towards the acidic side (i.e. pH 5.5) if the myosin to actin ratio was decreased from a molar ratio of myosin to actin of 2.7 to a ratio of 0.33, or if the ionic strength was kept above 0.2 M potassium chloride (Ishioroshi *et al.*, 1983). The charge distribution on the protein surface is affected by a change in pH and hence may partly explain the difference in the heat-induced gel strength of myosin at different pH values.

Young *et al.* (1992) noted that with the myofibril system the results obtained were similar to those in dilute myosin solutions. Rigidity rose as pH decreased and the gel texture in heated myofibrils appeared to hinge on whether the structure of the gel was aggregated at low pH between 5.2 and 5.7 (brittle) or was dispersed at a higher pH of 5.7 to 7.2 (elastic).

The differences in the heat-induced gel characteristics of myosin at varying pH and ionic strength of the system may partly be ascribed to the morphology of myosin filaments or aggregates that may form under different conditions (Asghar *et al.*, 1985). Studies on myosin gelation in model systems have generally been performed on reconstituted myosin filaments, rod aggregates or LMM paracrystals. Asghar *et al.* (1985) suggested it would be appropriate to take an account of those factors which affect the morphological traits of myosin during reconstitution.

2.4.3.5 Cations

There is little information available on the influence of different cations on heat-induced gelation of myosin. Ishioroshi *et al.* (1979) observed no difference in myosin gel in the presence of Na^+ or K^+ cations. The presence of divalent cations shifted the IEP of myosin in actomyosin solutions to a more alkaline pH range (greater than 5.76; Young *et al.*, 1968). Koretz (1982) reported that in the presence of Ca^{2+} ions, myosin monomers formed ordered non-filamentous aggregates. In view of the findings of Sanger (1971) that a relative difference in the binding strength of monovalent cations with myosin existed, a change in gel strength in the presence of different cations can be expected.

2.4.3.6 Anions

Phosphates are common additives to meat products, particularly pyrophosphate (PP) and tripolyphosphate (TPP), usually added at concentrations of about 0.3% to enhance the uptake of additional water (Bendall, 1954; Hamm, 1960; Sherman, 1961; Ranken, 1976). The effect of phosphates on water holding capacity of meat has been reviewed by several authors as listed by Asghar *et al.* (1985) and Sofos (1989). The improvement in the binding property of sausages by various phosphates such as PP, TPP and hexametaphosphate (HMP) was shown by Fukazawa *et al.* (1961c) and Gillet *et al.* (1978). The mechanisms by which phosphates brought about improvement in a myofibrillar or meat system, were assigned to:

1) the quantity of extracted protein by addition of the ion, PO_4^{3-} (for the denatured state of myofibrillar protein),
2) the increase in the concentration of the "light" components rather than the quantity of extracted protein in the presence of 0.1 M sodium chloride (provided the myofibrils are not denatured),
3) the decrease in the viscosity due to the dissociation of actomyosin (Fukazawa *et al.*, 1961c).

Light components were taken as being myosin and the dissociable components of natural actomyosin.

The binding of strongly negative polyvalent anions, such as ATP, ADP and PP, with myosin prevented precipitation of the myosin. This was taken as evidence (Morita and Shimizu, 1969; Tonomura *et al.*, 1963) that ATP or PP, on binding changed to some extent the secondary and tertiary structure of the myosin molecule. However, Gratzer and Lowey (1969) studied myosin and HMM using optical rotary dispersion (OR) and far UV absorption spectroscopy in the presence and absence of ATP and PP and found no change by either method. It was concluded that any change in the α-helix content was less than 0.2%. There were changes in the binding capacity for bromothymol blue, supporting the view that a small conformational change occurred.

The compounds, ATP and PP have been known as a substrate and a competitive inhibitor for myosin ATPase, respectively (Ishioroshi *et al.*, 1980). Both are also known to dissociate the actomyosin complex to myosin and actin in solutions of high ionic strength (Ishioroshi *et al.*, 1980). Based on this knowledge, Ishioroshi *et al.* (1980) supposed that the F-actin effect on the heat-induced gel formability of myosin was suppressed by dissociating the complex to individual components if actomyosin was a prerequisite for the effect. The presence of F-actin enhanced the gel formability of myosin solutions containing high concentrations of neutral salt. Ishioroshi *et al.* (1980) observed that incubating actomyosin (composed of a 15:1 w/w ratio of myosin to actin) in 0.6 M potassium chloride at 25°C for 1 to 24 h before heating to 65°C gradually decreased the depressing effect of ATP on gel strength, but only partially. During incubation myosin ATPase converted the ATP to inorganic phosphate (Pi) and ADP which is a competitive inhibitor of the ATPase, but does not dissociate the complex. Almost complete restoration of gelling ability only occurred with the addition of adenylate kinase, an enzyme which catalyses the conversion of ADP to ATP. This ATP was then hydrolysed back to ADP and Pi by myosin ATPase already in the system. This result indicated that ADP exerted a great influence to suppress the actin-induced effect on the heat-induced gelation of myosin. The inhibitory effect of PP on actomyosin (15:1 ratio of myosin to actin) gels was not eliminated on incubation (Ishioroshi *et al.*, 1980) possibly due to the fact that the system lacked the enzyme activity necessary for hydrolysing PP (Tonomura, 1972). Hydrolysis of PP by adding pyrophosphatase (PPase) to the system resulted in a decrease in the inhibitory effect of PP on actomyosin gel formation.

Similar investigations of the heat-induced gel strength were also undertaken on actomyosin of a 4.5:1 w/w ratio of myosin to actin (Ishioroshi *et al.*, 1980) which was closer to the stoichiometry of myosin binding with actin (Tonomura, 1972). With the addition of PP, the heat-induced gel rigidity decreased, but was restored with the addition of PPase. When ATP was added to the actomyosin, the rigidity decreased and continued to do so in spite of hydrolysis of ATP to ADP during incubation at 25°C. Reassociation of myosin and actin did take place during this stage. It was suggested that in the *in vitro* ATP containing system, as far as the heat-induced gelation was concerned, the suppressing effect of ATP on the rigidity enhancement by the presence of F-actin, which initially stems from ATP-induced dissociation of actomyosin complex to myosin and actin, was taken over and strengthened by the product of the ATPase reaction, ADP. This occurred in spite of reassociation of myosin and actin to actomyosin complex during incubation at 25°C. The enhancement effect of F-actin on the rigidity was a result of the ATP-induced dissociation of actomyosin complex to myosin and actin.

Where the optimal myosin to actin mixture was used (15:1) and partial recovery of the rigidity was observed with conversion of ATP to ADP instead of the continuous fall of rigidity as with the stoichiometric mixture of myosin to actin (4.5:1), the crosslinking of actomyosin complex with myosin upon heating was suggested to be responsible for the high rigidity of the heat-induced gels, over-riding negative contributions from an alteration of electrostatic nature of myosin due to ADP binding (Ishioroshi *et al.*, 1980).

Heat-induced myosin gels also showed immediate decreases in rigidity with the addition of ATP and PP, but to a lesser extent (Ishioroshi *et al.*, 1980). It was suggested that these findings implied an electrostatic contribution to the gelation process upon heating at 65°C due to ligand-binding with myosin. Magnesium-ATP had two effects on rigidity - initially on binding and then to its hydrolysis and the binding of the product, ADP, whereas PP showed a single change due to the simple binding to one of the binding sites on myosin (Ishioroshi *et al.*, 1980). The reduced rigidity was decreased either by addition of PPase or adenylate kinase.

Ishioroshi *et al.* (1983) noted that addition of ATP to the myosin system at 0.2 M sodium chloride, instantly caused a decrease in the rigidity of the heat-induced gels produced. The suppressing effect of ATP on the gelation of fresh myosin in 0.2 M potassium chloride, was diminished with the time of incubation at 25°C before heating. The restoration of the initial value paralleled the liberation of phosphate from ATP during incubation. After complete hydrolysis of the added ATP, the suppressing effect of ATP on gel strength completely levelled. The addition of PP showed no adverse effect on myosin gelation in 0.2 M potassium chloride, and the presence of ADP had no influence. It was suggested that this inhibitory effect was ATP-specific and may have been closely related to complex formation at the enzymic active site(s) of the myosin head with ATP.

Upon addition of ATP, actin filaments dissociated from myosin filaments and subsequently with hydrolysis of ATP, re-associated to superprecipitate (contract; Ishioroshi *et al.*, 1983). Since the actin binding sites as well as the enzymic active sites are located in the head region of myosin (Tonomura. 1972; Margossian and Lowey, 1978), it was presumed that both of these sites were involved in the interfilamental head-to-head interactions for network formation in the system upon heating. This presumption was based upon the specific inhibitory effect of actin and ATP on the heat-induced gelation of myosin filaments. The specific binding of actin and ATP to the head made the condition favourable for head-to-head interactions between myosin filaments.

The changes in light scattering intensity and viscosity of myosin preparations on incubation implicated the formation of aggregates without the exposure of hydrophobic residues of the myosin molecules. The loss of the characteristic filament formability of the stored myosin during incubation followed by the depression in heat-induced gel formability of myosin filaments was ascribed to the progressive polymerisation of myosin monomers (Ishioroshi *et al.*, 1983).

Robe and Xiong (1992) found that the effect of TPP on myosin gelation was not solely a result of increased ionic strength. Tripolyphosphate appeared more effective than sodium chloride in modifying the electrostatic forces in proteins, thereby altering the salt soluble aggregation patterns. Muhlrad (1991) and Muhlrad *et al.* (1991) conducted myosin phosphate

binding studies and reported that PP and TPP bound to myosin S-1 at the same binding sites. The larger cyclic molecule, HMP, possibly had less affinity for myosin and hence less effect on the thermal transitions of salt soluble proteins. The smaller molar ratio may also have accounted for the lesser effect of HMP. An investigation of the binding of phosphates showed myosin and HMM bound two moles of PP per mole of myosin (Nauss *et al.*, 1969). Subfragment 1 bound only one mole PP per mole of the fragment. Actomyosin bound only one mole of PP per mole of myosin and the addition of actin to myosin reduced the binding to one mole of PP per mole of myosin. In the case of HMM complete suppression of PP binding could be obtained with actin. This suggested that one PP binding site was the same as or near the actin binding site and was masked with the addition of actin. Myosin also appeared to have a stronger affinity for actin than for PP. Siegel and Schmidt (1979b) noted that phosphate (2% TPP) linearly increased the binding ability of myosin. This was attributed to the specific interaction between polyphosphate and protein said to work by dissociating the actomyosin contaminating the crude myosin preparations.

The observation of the two binding sites of HMM, but only one site for actomyosin and one site for myosin plus actin, would suggest that only one of the binding sites was the same as or near the actin-binding site of myosin. This would further suggest that at least one of the binding sites is not affected by actin. The binding of only one PP to S-1 would suggest that the second binding site is on the rod of myosin. However, addition of actin to HMM resulted in complete suppression of PP binding. These results are not explained.

Egelandsdal *et al.* (1985) investigated the effects of potassium octanoate (KC_8), decanoate (KC_{10}), dodecanoate (KC_{12}) and sodium dodecyl sulphate (SDS) on the thermal stability and gel formability of bovine myosin. Sulphate and carboxylates destabilized myosin as determined from DSC. Increasing the aliphatic chain length decreased the thermal stability of myosin. All detergents examined, especially KC_{10} and KC_{12}, were enhancers of gel strength. However in the case of KC_{12} and SDS, enhancement could only be detected after a prolonged (> 2 h) interaction time with myosin. It was suggested that binding of these amphiphiles led to an increased repulsion between protein chains and thus to a more ordered aggregation (gelation) upon heat treatment.

In addition to their effect on heat-induced gelation, inorganic salts have been shown to induce myosin gelation in the absence of added heat. Migita and Okada (1954a,b) and Umemoto *et al.* (1954) reported that such anions as the cyanate anion, were found to be highly powerful in fish meat setting. It is known that a high concentration of inorganic salt, such as potassium thiocyanate or lithium bromide, affects the secondary structure of protein and destroys the α-helix (Hamaguchi, 1968, 1976). Tonomura *et al.* (1962) found that the α-helical content and the reduced viscosity of poultry myosin decreased sharply and reversibly within a narrow range of concentrations of lithium bromide, lithium chloride, potassium iodide or potassium thiocyanate. In potassium chloride solution (0.6 to 5.1 M), however, the helical content and reduced viscosity were almost unchanged. It was concluded after sedimentation studies that inorganic salt induced the transition of myosin from a fibrous long molecule of helical structure to a globular molecule of random coil. Nakayama *et al.* (1983) showed 4.0 and 5.1 M potassium thiocyanate induced gelation of chicken breast myosin. Gelation also occurred with 3.0 M lithium bromide and further increases in ion concentration induced the opposite effect on dynamic viscosity. The helical content decreased with increasing concentrations of potassium thiocyanate or lithium bromide and a sharp decrease occurred at 3.0 M lithium bromide. It was concluded that a local melting of helical structure could be related to gelation. In the case of heat-induced myosin gelation (Samejima *et al.*, 1981), the gelation began when about half of the helical structure had unfolded. In the case of salt-induced myosin gelation (Nakayama *et al.*, 1983) the most elastic gel was formed when half of the helical structure was unfolded. Stafford (1985) observed that while increasing the salt concentration stabilized the major portion of the coiled-coil, the chloride ion destabilized a domain near the S-2 and LMM junction.

2.4.3.7 Temperature

Yasui *et al.* (1979) investigated the influence of heating temperature (20 to 70°C) on myosin gelation in a model system at pH 6.0 or 7.0 and 0.6 M potassium chloride. The rigidity of the gels increased linearly in the region of 20 to 35°C; thereafter the rise was exponential and reached a maximum at 55 to 60°C. Beyond these temperatures little change was apparent in the gel strength. Samejima *et al.* (1981) reported a similar trend for the gelation of myosin as well as for its subfragments, S-1 and rod in 0.6 M potassium chloride and pH 6.0. The

turkey breast temperature profiles of Arteaga and Nakai (1992) at pH 6.0 and 12.0 showed small changes in the helical content from 0 to 30°C, major changes from 30 to 50°C and thereafter levelling off. At pH 2.0, a gradual change in the helical content was observed over the temperature range. Wu *et al.* (1991) found that gelation of chicken breast myosin followed second order kinetics. The rate constants changed in a complex manner with respect to temperature. Gels formed at low temperatures (44 to 56°C) developed greater shear modulus values and were more elastic than those formed at higher temperatures (58 to 70°C). A study by Ishioroshi *et al.* (1983) indicated that the gelation profile of myosin of different temperatures was influenced by the ionic strength and the pH value of the system and suggested that the interactions which occurred in the heat induced-gelation of the myosin filaments at low salt concentrations (0.2 M potassium chloride) were different from those which were responsible for the gelation of myosin monomers at high salt concentrations (0.6 M potassium chloride).

Myosin gels of 3 and 6 mg/ml formed at linear heating rates (12 and 50°C/h) increased in rigidity between 50 and 70°C, whereas gels formed under constant heating tended to decrease in rigidity at higher temperatures (Foegeding *et al.*, 1986). For myosin heated to 70°C the 12°C/h rate produced the most rigid gels. The greater amount of time in a denatured state could have allowed for more aggregation to occur. Constant heating at 70°C and 3 mg/ml did not allow gel structure to develop, that is, the constant heating rate resulting in the most rapid temperature rise favoured more random protein-protein interactions rather than ordered interactions which would have lead to development of a three-dimensional gel network. Gels did develop at 6 mg/ml suspensions under the constant heating regime indicating both random and ordered protein-protein interactions occurred, but at 3 mg/ml there apparently were not enough ordered interactions to develop a gel skeleton. Egelandsdal *et al.* (1986) found that decreasing the heating rate from 2.5 to 0.1°C/min had a large positive effect on the storage modulus (G') of myosin gels at 10 mg/ml and 75°C. The slower the rate of heating, the earlier the network started to develop, eventually resulting in more elastic and stronger gels at high temperatures for the lowest heating rates of 0.1, 0.5 and 2.5°C/min studied. Gels formed at the lower heating rates remained stronger throughout the heat treatment.

2.4.3.8 Storage

The pattern of changes in the gel formability of myosin stored in 0.1 or 0.6 M potassium chloride at 0°C for 1 to 15 days was studied by Ishioroshi *et al.* (1979). They found no significant change in the strength of gel produced by myosin suspension, which was previously stored in 0.1 M potassium chloride for up to 10 days. However, myosin stored in 0.6 M potassium chloride was not stable and started losing its gel-forming potential soon after storage. This change was prominent only when gel formability was tested between 0.1 and 0.3 M potassium chloride, but the change was not obvious at high ionic strength (0.4 to 0.6 M potassium chloride) where the shear modulus was minimal in both cases. The gelling power of stored myosin appeared to precede the decrease in quantity of myosin monomers and actin activated Mg^{2+}-ATPase activity (Ishioroshi *et al.*, 1983). Changes in light scattering intensity and viscosity of stored myosin indicated aggregation of myosin monomers with increased storage time without exposure of hydrophobic residues. Pinset-Härström and Whalen (1979) showed that extracted rat skeletal muscle, on aging, tended to lose the DTNB light chain, possibly due to proteolytic action, which led to a general loss in bipolar filament forming ability. If so, Asghar *et al.* (1985) suggested that this may explain the decrease in gel strength of stored myosin as observed by Ishioroshi *et al.* (1983).

2.4.3.9 Pressure

Generally studies of the effects of pressure on myosin have usually been carried out on myosin solutions of ionic strength of about 0.5 M. Ivanov *et al.* (1960) found that after subjecting myosin to a pressure of 405.2 MPa, its molecular weight had increased to 1.5 to 2.0 times. O'Shea *et al.* (1976) from measurements of light scattering of myosin in 0.6 M potassium chloride, pH 6.5, concluded that pressure treatment resulted in an increase in molecular weight with the molecules arranged as overlapping dimers. O'Shea and Tume (1979) concluded that pressure treatment of myosin in 0.6 M potassium chloride at pH 6.5 had an effect over the entire surface of the myosin molecule, resulting in the exposure of hydrophobic tyrosyl residues.

The effect of pressure treatment on the heat-setting characteristics of myosin up to 150 MPa at 0 to 40°C was assessed by measuring samples after heating to 70°C (Suzuki and MacFarlane, 1984). The response was dependent upon ionic strength, pH of the myosin suspension and the intensity and duration of the pressure treatment. It was most pronounced at pressures of 75 MPa or greater applied for some minutes to myosin in 0.2 to 0.3 M sodium chloride at approximately pH 6. It was suggested that the alteration in heat-setting properties was due to depolymerisation, under pressure, of myosin filaments accompanied by a conformational change of the monomer so that it reaggregated in a different manner upon release of the pressure.

In the study by Suzuki and MacFarlane (1984) the heat-setting characteristics of myosin in the presence of 0.5 M (or greater) salt solutions and pH 6 to 7 were not affected by pressure treatment. Therefore it was thought unlikely that the changes at 0.5 M and greater concentrations of salt solutions were responsible for the changes in heat-setting characteristics observed at the lower ionic strengths.

2.4.4 Acid-induced Gelation

Until 1985, studies of changes in myosin conformation had generally been limited to pH values greater than the isoelectric point of myosin, pH 5.4 (Szent-Györgyi, 1951). Reported findings showed either that thermal stability decreased when the pH was lowered to 5.5 (Goodno *et al.*, 1976; Wright and Wilding, 1984) or remained essentially unaffected (Samejima *et al.*, 1983). In a study by Ishioroshi *et al.* (1981) it was found that heat-induced development of turbidity in dilute solutions of HMM showed a strong dependence on pH occurring at a much lower temperature at pH 5.0 than at 6.0.

As a result of these findings Fretheim *et al.* (1985) studied the effect of lowering the pH of solutions of myosin. It was found that solutions of myosin (10 mg/ml) formed gels at 5°C if the pH was decreased slowly, by dialysis, to a value in the region of 2.5 to 5.5. Salt (potassium chloride) concentration was found to affect gel strength positively and linearly. Wright and Wilding (1984) found that myosin denaturation enthalpy was decreased when pH was lowered to 5.5 and Fretheim *et al.*, (1985) revealed that the myosin of acid-induced gels

absorbed no thermal energy by DSC, when heated. Acid-induced denaturation was implicated as the basis of gel formation. Hermansson *et al.* (1986) in an investigation of the effect of pH and ionic strength on thermally-induced myosin gels also observed that myosin solutions (7 to 9 mg/ml) dialysed against pH 4.0 buffers containing 6.0 M potassium chloride, formed gels spontaneously at 4°C. Investigations of SEM revealed the structure to be strand-type. These results were considered interesting since the gel was formed from the association of myosin molecules that had not undergone any heat-induced conformational changes.

Fretheim *et al.*, (1985) concluded that the results of the DSC experiments demonstrated that a conformational change was involved in the acid-induced gelation. Prolonged exposure to low pH values eliminated the proteins absorption of heat in the calorimeter. Acid-induced denaturation had taken place prior to calorimetry, making gel formation in the cold possible. A comparison with heat-induced denaturation at a neutral pH value was made to explain some aspects of the acid-induced gel formation. This analogy was supported by investigations of the addition of dithiothreitol (DTT) to the myosin gels. With addition of DTT to the myosin system, the gel strength was decreased and implied the formation of disulphide bonds was involved in network formation. This suggested that an element of head-to-head aggregation was involved in gel formation and was compared to the work of Samejima *et al.* (1981), who proposed sulfhydryl groups are involved in gel formation. Although contrary to evidence of other workers (Hamm and Deatherage, 1960; Jacobson and Henderson, 1973; Hamm and Hofmann, 1965; Samejima *et al.*, 1969), Fretheim *et al.* (1985) used this information to provide further support to the proposed analogy of heat- and acid-induced denaturation.

The increase in gel strength observed with decreasing pH values (to pH 4.5) was compared to the observation that the higher the temperature, the stronger the heat-induced gel (up to about 60°C; Ishioroshi *et al.*, 1979). In both cases, stronger denaturing conditions (lower pH or higher temperature) led to stronger gels. The strength of heat-induced gels reached a plateau when gel formation took place at a sufficiently high temperature (>60°C; Ishioroshi *et al.*, 1979). Fretheim *et al.* (1985) noted that the situation was more complex with acid-induced gelation. The lowering of the pH from the isoelectric point, also increased the positive charge on the myosin molecules and thereby the repulsion between them. It was presumed that the gel network formed initially was dynamic and remained dynamic as the pH

was lowered from 4.5 to 4.0. This allowed the increased intermolecular repulsion to have a deleterious effect on the strength of the network as the pH was lowered to 4.0.

The positive correlation between final potassium chloride concentration (from 0.1 to 0.6 M potassium chloride) and gel strength prompted Fretheim *et al.* (1985) to question the role of filaments in the formation of gels at 4°C. In an investigation of the structures of bovine myosin in diluted suspensions, Hermansson and Langton (1988) showed that at pH 4.0 and 0.6 M potassium chloride the filaments often interacted approximately at right angles, which resulted in a denser network than that observed at pH 5.5 and 0.25 M potassium chloride. The efficient network formation at pH 4.0 which gave rise to spontaneous gel formation upon dialysis, did not change in network character upon heating (Hermansson *et al.*, 1986). The gels formed at pH 4.0 and 0.6 M potassium chloride had a strand-type gel structure. No differences could be detected between the unheated and heat-treated gels at low magnifications. At higher magnifications it was seen that heating resulted in a loss of details of the filaments at both pH values and ionic strengths. The shape of the myosin heads was lost, and the heads fused together on the filament backbone. Hermansson *et al.* (1986) concluded that at pH 4.0 the repulsive balance was such that the myosin filaments could interact spontaneously to form a gel without heating.

In addition to the experiments resulting in the acid-induced formation of myosin gels, investigations on the effect of lowering the pH of meat or myofibrillar protein systems has been undertaken with an emphasis on the effects of marinading. The addition of acid in the marinading of meat was reviewed by Offer and Knight (1988). Jordan-Lloyd (1916, 1933) observed a steep rise in water uptake from pH 5 to 4 and at pH 4.0 the muscle swelled to approximately 2.7 times its original weight. Wenham and Locker (1976) found that muscle did not swell uniformly in all three dimensions, but like Rao *et al.* (1989a) swelling increased along and across the muscle fibre axis between pH 5.1 and 4.4. For large meat pieces a pH gradient from inside to outside resulted (Wenham and Locker, 1976). It was noted that acid swollen muscle was generally turgid, scattered less light than untreated meat and was glassy in appearance (Offer and Knight, 1988; Jordan-Lloyd, 1916; Grau *et al.*, 1953). A possible problem with the marinading of meat with a large quantity of marinade was the loss of protein

and other components from the meat into the marinade (Gault, 1985, 1984; Howat *et al.*, 1983).

The underlying cause of swelling of meat at low pH values is the swelling of myofibrils (Bowman, 1840). Myosin is known to be soluble at low ionic strength at pH values of 4 or less (Erdos and Snellman, 1948). When myofibrils are irrigated with a solution of 0.1 M sodium chloride and 10 mM acetic acid - sodium acetate at pH 4.0, the myofibrils swell laterally greatly and the A-band is rapidly extracted. With prolonged exposure, the I-bands are also extracted leaving strings of Z-discs.

Hamm (1960, 1975, 1981, 1982) accounted for the swelling of myofibrillar proteins by enlargement and eventually solubilization of the gel network. Offer and Trinick (1983) pointed out that Hamm did not account for the fact that only part of the myofibril was solubilised (the A-band) and the site of water uptake was not clearly specified. The highly ordered structure of myofibrils was not taken into account, but was expressed in general terms of gel networks. Offer and Trinick (1983) concluded in general terms that water uptake by myofibrils at varying pH occurs by expansion of the filament lattice brought about by an increased electrostatic repulsive force and/or a diminution in one or more of the constraining forces. Both effects would require binding of ions to the myofibrillar proteins. Constraints of myofibrillar proteins to swelling include the sarcolemma, Z and M lines (Matsubara and Elliot, 1972, Millman and Nickel, 1980) and the crossbridges of actin and myosin (Goldman *et al.*, 1979; Millman, 1981). Wilding *et al.* (1986) showed that if the endomysial sheath surrounding a fibre was damaged at one point, much more swelling occurred there and it was concluded that the endomysium acted as a mechanical restraint to swelling.

Hamm (1960) favoured the electrostatic theory of maximal swelling first applied to protein systems by Tolman and Stearn (1918) and thus supposed that the swelling effect of acids and bases on meat systems was influenced by changes in protein charge. Thus the addition of acid below the IEP gradually protonated negatively charged carboxyl groups in the protein molecules which were thought to break some of the electrostatic bonds (salt linkages) with protonated amine groups on adjacent protein chains. The increase in the net positive charge was thought to result in a repulsion between protein groups of similar charge and thereby

created space for the immobilisation of extra water. At the point of maximal swelling, around pH 3.5, it was assumed that all the available carboxyl groups within the muscle system were protonated. The subsequent addition of further acid was then considered to induce an increase in the binding of anions (for example, chloride anion in the case of hydrochloric acid), thereby screening the positive charges on the protein molecules and decreasing the repulsion between protonated groups. Consequently, the molecular structure shrunk again and explained the reduction in swelling below pH 3.5.

In contrast to the electrostatic theory used to explain the swelling of meat, a theory based on osmosis was proposed by Proctor and Wilson (1916). Neither of these theories has displaced the other. However, the osmotic theory is only suitable for situations with complete ionisation of the protein compound formed (Gault, 1991). The concept of osmotic swelling (Gault, 1991) is that essentially the addition of acid to the protein systems results in the protonation of carboxyl groups. It supposes that the corresponding anions of the acid form an ionized salt with the available cationic groups on the protein molecules. This results in an unequal distribution of free ions between the proteins and the surrounding acid solution giving rise to a difference in osmotic pressure. The inherent tendency of the diffusible anions to move away from the proteins into the surrounding fluid is inhibited by their close association with the protein molecules whose movement is restricted by the cohesive forces associated with their structural organisation. Consequently water is induced to flow into the system to reduce the concentration of anion associated with the protein molecules and the system swells. As the concentration of acid increases, all of the available carboxyl groups on the protein molecules become discharged by protons. At this stage, the excess of diffusible anions associated with the proteins is now at a maximum and the pH of maximum swelling of the system has been reached. With the further addition of acid to the system, the overall concentration of the protons and anions in the external fluid starts to increase while the concentration of diffusible anions associated with the protein remains the same. Consequently, with decreasing pH the difference in anion concentration between the two phases is reduced and swelling is depressed.

Rao *et al.* (1989b) undertook studies on the ultrastructure of beef muscle at pH below the IEP. Discs of muscle were incubated in solutions of acetic acid. At pH values greater than 4.5 the

myofibrillar structure was apparent and swelling was observed as the pH was lowered. With this swelling was noted a progressive extraction of the A-band, fragmentation of the thin filaments in the Z-line region and disorganisation of Z-line structure. Below pH 4.5, swelling became much more pronounced with further extraction of myosin filaments. Most of the actin filaments were extracted and the myofibrils fused together giving an amorphous appearance. Z-lines were partly extracted. At pH 3.92, the H-zones were also lost indicating complete depolymerisation of myosin filaments. It was noted that the ultrastructural changes brought about by the acidification of the samples over the pH range 4.5 to 3.9 clearly indicated the severe disruptive nature of low pH on the myofibrillar components of meat. Hamm (1960) stated that the pH changes between 4.5 and 7.0 were completely reversible. Acid denaturation did not start until pH 4.0.

The presence of salt greatly decreased the extent of swelling of meat, especially in acidic media (Jordan-Lloyd, 1916). The uptake of water when meat pieces were treated with sodium chloride ranged from 0 to 40% depending on product or amount of water added (Offer and Knight, 1988). The amount of swelling depended on the concentration of sodium chloride and went through three phases. The physiological ionic strength of muscle is approximately 0.2 and at this ionic strength the muscle volume was at a minimum. At ionic strengths of less than 40 mM and in the absence of divalent ions, the maximum volume in distilled water was twice that of the volume at physiological ionic strength (Bozler, 1955). As the concentration increased above the physiological ionic strength, progressive increases in the amount of swelling were observed with a maximum at approximately 1 M (Hamm, 1957; Callow, 1930, 1932). At higher ionic strengths, less swelling was noted, and shrinkage occurred above 4.5 (Callow, 1930, 1931). At ionic strengths of greater than 3.5 M, the initial shrinkage was due to osmotic pressure of the ions, then the swelling was not strong enough to counteract the shrinkage (Callow, 1932; Körmendy and Gantner, 1958)

On the acidic side of the IEP of muscle, the electrostatic repulsion between positively charged groups of protein was reduced by the binding of anion as a result of the decrease of protein nett positive charge (Cann and Phelps, 1955). This resulted in a tightening of the protein structure and a decrease in water holding capacity (WHC). The stronger the ion was bound (or in other words, the more the positive charges of the protein are "screened" by the anion

of the salt) the greater the reduction of WHC. The higher the salt concentration, the more effectively it inhibited swelling (Gault, 1991).

In terms of osmotic swelling, the suppression of swelling by neutral salts was explained by the fact that the presence of neutral salt increased the amount of free anions in the external solution. This reduced the difference in anion concentration between the solid protein phase and the external solution under the prevailing acidic conditions and thus the swelling potential was decreased (Gustavson, 1956).

2.5 SUMMARY

Myofibrillar proteins, in particular myosin, have been shown to be primarily responsible for the binding quality of thermally-induced restructured meat products. Ferry's theory of gelation states that heat-induced denaturation precedes gelation and in these meat products denaturation was induced by heat (Ferry, 1948). There is little research on acid-induced gelation, however, it is proposed that denaturation could be induced by lowering the pH and subsequent gelation could occur at chilled temperatures. Lowering of the pH via dialysis has been shown to result in the formation of myosin gels at chilled temperatures (Fretheim *et al.*, 1985; Hermansson *et al.*, 1986). Comparison of acid-induced gelation with thermally-induced gelation could provide a useful parallel to determine the mechanism of gelation under acidic conditions.

It is suggested that if meat myofibrillar proteins or myosin alone could be modified in some consumer acceptable way to enable it to preform restructured meat muscle at low temperatures a range of fresh chilled meat products could be developed. The probable existence of a market for a range of chilled restructured meat products has been shown (Jolley *et al.*, 1988; Field, 1982; Mandigo, 1982). Slow lowering of the pH via GdL addition, could possibly mimic conditions of dialysis and therefore induce denaturation and subsequent gelation at chilled temperatures. Binding is a function of gelation and therefore an understanding of the mechanism of this acid-induced binding could enable the production of a chilled restructured meat product to fulfil the perceived need for such a range of products. Determination of the effects of sodium chloride and phosphate, additives commonly used in thermally-induced meat products today to enhance WHC and product bind, could aid in the determination of the

mechanism of binding and perhaps enhance these characteristics under acidic conditions providing reason for addition to the final product. Investigation of the reversibility or otherwise of the binding of meat products under acidic conditions could illustrate the stability of the gelation network formed, provide further information pertaining to the mechanism of formation of the gel network and illustrate the effect of increasing the pH after gelation, for example for taste purposes.

CHAPTER THREE

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Meat

Meat used for minced meat, myofibrillar protein and myosin preparations was obtained from local freezing works within 1 h of slaughter. Bovine *m. cutaneus trunci* was used. The meat used for laser diffraction experiments was bovine achilles tendon or 'gravy beef' obtained from a local supermarket.

3.1.2 Water and washing materials

Water used had been passed through a Milli-Q reagent water system (Millipore Corporation, Bedford, Massachusetts) unless stated otherwise.

Pyroneg^{R} detergent was supplied by Diversey-Wallace Ltd. (Papatoetoe, New Zealand).

3.1.3 Chemicals

Aniline (extra pure) and glucono-δ-lactone (GdL; 99.5%) were obtained from Merck (Darmstadt, Germany).

Acrylamide (electran), ammonia solution (35%), Coomassie Brilliant Blue R250 (biochemical), concentrated hydrochloric acid (35.4 %; AR), disodium hydrogen orthophosphate (AR), dithiothreitol (DTT; molecular biology grade), ethylenediaminetetraacetic acid (EDTA; AR), methanol (AR), N,N'-methylene-bis-acrylamide (bis; electran), potassium chloride (AR),

potassium cyanide (AR), potassium ferricyanide (AR), sodium dihydrogen orthophosphate (AR), sodium dodecyl sulfate (SDS; biochemical), sodium hydroxide (AR), sodium nitrite (AR), sodium potassium tartrate (AR), N,N,N',N'-tetramethylethylenediamine (TEMED; electran) and tetrasodium pyrophosphate decahydrate (TSPP; AR) were all obtained from BDH Laboratory Supplies Ltd. (Poole, England).

Glycerol and sodium chloride (AR) were obtained from Scientific Supplies Ltd. (Auckland, New Zealand).

Cupric sulphate (AR), glycine (AR), sodium hydrogen carbonate (AR), sucrose (AR) and tripolyphosphate (AR) were obtained from Ajax Chemicals (Sydney, Australia).

Bovine serum albumin (BSA), β-naphthol, ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA; 97%) and p-hydroxy-mercuribenzoic acid (PCMB) were obtained from the Sigma Chemical Company (St. Louis, Missouri, U.S.A.).

Hemin was obtained from New Zealand Pharmaceuticals Ltd. (Linton, New Zealand).

Hexametaphosphate, magnesium chloride hexahydrate and sodium azide were obtained from Riedel-de Haën AG (Seelze-Hannover, Germany).

Tris(hydroxymethyl)aminomethane (Tris; enzyme grade) was obtained from United States Biochemical Corp. (USB, Cleveland, Ohio, U.S.A.).

Ammonium persulfate (ultrapure electrophoresis grade) was obtained from Bethesda Research Laboratories (BRL, Gaithersburg, Madison, U.S.A.).

Glacial acetic acid and isopropanol were obtained from Rhône-Poulenc Chemicals (Avonmouth, Bristol, England).

8-Anilino-1-naphthalenesulfonic acid (ANS; 97%) was obtained from Aldrich Chemical Company, Inc. (Milwaukee, Wisconsin, U.S.A.).

Bromophenol blue and SDS-PAGE high molecular weight standards were obtained from Bio-Rad Laboratories (Richmond, California, U.S.A.).

3.2 METHODS

3.2.1 Cleaning of glassware

All glassware was washed in hot Pyroneg^{R} solution, rinsed in tap water, rinsed in distilled water and then hot air dried.

3.2.2 Minced meat

3.2.2.1 Gel preparation

Bovine *m. cutaneus trunci* was trimmed of excess visible fat and connective tissue and cut into approximately 3 cm cubes. Samples of the meat (approximately 300 g) were vacuum packaged and heat sealed using a chamber type vacuum packaging machine. These samples were stored at -20°C in PVDC nylon polyethylene polymer bags with low oxygen permeability and low moisture permeability. The meat was used within 5 months of preparation.

Meat was thawed at 4°C overnight and was then minced in a Bauknecht electric mincer (Type A2-1, Germany) with a hole plate diameter of 3 mm. Minced meat (50 g) was removed and water (10 ml) was added with GdL (0.00, 1.00, 2.00, 3.00 g). In some samples sodium chloride (1.25 g) and/or TSPP (0.15 g) were also added at the same time as the GdL. The samples were well mixed. Samples were transferred to PVC tubing (20.0 mm depth, 17.5 mm i.d.) or to ring molds (29.0 cm e.d., 19.0 cm i.d., 1.2 cm depth) and placed under vacuum using a chamber type vacuum packaging machine. The ends were levelled and covered with polyethylene all-purpose food film. The gel preparations were stored at 4°C, at 2.5 h after the minced meat samples had been removed from the freezer.

3.2.2.2 Young's Modulus, springiness, penetrometer and tensile tests.

The degree of gelation of minced meat was estimated from the force strain relationship for the uniaxial compression of cylinders of the material using an Instron Universal Testing Machine (model 4502, Instron Ltd High Wycombe, England) equipped with a Phillips Computer (model Pro9CM082, Taiwan). A two bite program was used with a constant displacement of 7.5 mm, a crosshead speed of 39.6 mm/min and a 1 kN load cell. The surfaces in contact with the samples were lubricated with soya oil. Young's Modulus and springiness (Bourne, 1978) were calculated from averages of force displacement curves generated from four replicates. Displacement for Young's Modulus calculations was less than 1 mm.

Penetrometer tests were undertaken using the Instron Universal Testing Machine. A flat probe of 3 mm diameter was used at a crosshead speed of 50 mm/min and with a 1 kN load cell. The surfaces in contact with the samples were lubricated with soya oil. Force displacement curves were generated from four sample replicates.

Tensile tests were also undertaken on the Instron Universal Testing Machine. Rings of minced meat were suspended from horizontal probes and tensile tests were undertaken by extension of the sample at a rate of 50 mm/min. A 10 N load cell was used and extension of the sample was taken to destruction. Force displacement curves were generated from four sample replicates.

3.2.2.3 Measurement of pH

Direct pH measurements were taken upon completion of tests, at 20°C using a standard pH meter (digital ionalyzer, model 701, Orion Research Inc, U.S.A.) equipped with a glass electrode. Measurements of pH were calculated from the average of 4 replicates.

3.2.2.4 Laser diffraction

A thin piece of bovine achilles tendon or 'gravy beef' was cut with a scalpel, placed on a microscope slide and covered with water or a solution of 1.12 M GdL. The diffraction pattern was obtained by shining a laser beam (Helium-Neon 4 mW, class IIIb laser product, Model 102-3, Spectra Physics Inc., California, U.S.A.) through the sample immediately upon addition of the solution. The pattern was monitored and further solution was added without disruption of the sample to prevent complete evaporation of the solution. In some instances, a piece of meat was stirred vigorously for 15 min in a solution of 0.1 M potassium chloride and then transferred four times more to fresh beakers of 0.1 M potassium chloride each time stirring for 15 min and then the above method was followed. Diffraction was monitored visually.

3.2.3.1 Myofibrillar protein

3.2.3.1 Preparation of myofibrillar proteins

Bovine *m. cutaneus trunci* was trimmed of excess visible fat and connective tissue, cut into approximately 3 cm cubes and stored at 4°C overnight. Ice and tap water was added to the cubed meat (1:1:1, w/w/w) in a Jeffco Wet Disintegrator (model 291, Jeffress Bros Ltd, Brisbane, Australia). The mixture was stirred for 5 min. The meat slurry was poured into a motorised mixer with a further addition of iced tap water to slurry (2:3, w/w). The mixture was stirred for 10 min during which time collagen was removed with a spoon. The meat slurry was strained through a 3 mm stainless steel mesh to remove residual collagen. Discs were removed from a Westfalia Separator AG (model LWA 205, Westfalia Oelde, Germany) which was operated at 12000 rpm. The slurry was added slowly to the separator until a maximum amount of myofibrillar protein, which collected on the inner surface of the bowl, was retained. Liquor was collected and run once through the separator. The myofibrillar protein was mixed in a Kenwood Chef Cakemixer (model A703C, Australia) to achieve homogeneity. The protein was then vacuum packaged and heat sealed using a chamber type vacuum packaging machine. It was stored as discs, less than 5 mm thick, at -20°C in PVDC nylon polyethylene polymer bags with low oxygen permeability and low moisture permeability. Myofibrillar protein was used within 5 months of preparation.

3.2.3.2 Proximate Analysis

Moisture, fat and protein content was determined by the Food Technology Research Centre, Palmerston North and M.I.R.I.N.Z., Hamilton. Collagen was determined by M.I.R.I.N.Z., Hamilton and the AgResearch Analytical Services Laboratory, Palmerston North. The method used was of hydroxyproline content measured after acid hydrolysis in 6 M concentrated hydrochloric acid and a conversion factor of 7.14 was assumed.

3.2.3.3 Hydrochloric acid dialysis into myofibrillar protein

Myofibrillar protein (100 g) was ground in a mortar and pestle for 30 s. Water (30 ml) was mixed into the protein for 30 s and the samples were syringed into PVC tubes (20.0 mm high, 17.5 mm i.d.). The ends of the tubes were levelled and covered with dialysis membrane (viscose cellulose, molecular weight cut-off approximately 12000 to 14000, Union Carbide 453105). The samples were suspended in a 0.17 M hydrochloric acid solution at 4°C for 18 h.

3.2.3.4. Gel Preparation

Myofibrillar protein (100 g, 80% moisture) was thawed for 30 min at room temperature (approximately 20°C) and then ground in a mortar and pestle for less than 30 s. Water (10 ml) or a solution of sodium chloride (2.50 g) and/or TSPP (0.30 g) in water (10 ml) was added to the myofibrillar protein sample approximately 1 h 20 min after removal of the protein from the freezer. The mixture was stirred for 30 s. A solution of GdL (0.00, 1.00, 2.00, 4.00 g) in water (20 ml) was added to the protein mixture 5 min later. The mixture was stirred for 30 s. Samples were syringed into PVC tubing (20.0 mm high, 17.5 mm i.d.) and placed under vacuum using a chamber type vacuum packaging machine. The ends were levelled and covered with polyethylene all-purpose food film. The gel preparations were stored at 4°C, at 2.5 h after the myofibrillar protein samples had been removed from the freezer. The time measurements at which samples were removed for testing were taken from the addition of GdL to the protein.

These results give a sodium chloride concentration of 2.3% w/w and a TSPP concentration of 0.3% in the water phase of the myofibrillar protein system. In all subsequent references to percentage concentrations, the calculations are based on a weight per weight ratio with the water phase of the system studied; myofibrillar protein or myosin.

Gels prepared with GdL (6.00, 8.00, 10.00, 12.00 g) had the GdL added as slurries. Solutions of GdL were prepared just prior to addition to the protein samples. Increased concentrations of sodium chloride were added to the protein at the same time as the water. Increased concentrations of TSPP were dissolved in water before addition. When combined addition of sodium chloride and TSPP was investigated, mixing of these additives at increased concentrations of TSPP prior to addition to myofibrillar proteins resulted in a precipitate. Hence, a modification of the above method was used in which the sodium chloride and TSPP were dissolved separately and the solutions were added to the protein at the same time.

Gels were also prepared using concentrated hydrochloric acid (4.9, 9.8, 19.6 ml) instead of GdL. These were molar concentrations of ten times those of GdL (1.00, 2.00, 4.00 g) in myofibrillar protein (100 g).

In experiments in which temperature was a variable (0°C, 10°C, 20°C, 30°C), samples were stored in incubators at the appropriate temperatures 2.5 h after the addition of GdL (2.00 g).

3.2.3.5 Young's Modulus measurements

Young's Modulus was measured by compression tests as for minced meat samples (Section 3.2.2.2).

3.2.3.6 Measurement of pH

The measurement of pH was a direct measurement as for the minced meat samples (Section 3.2.2.3).

3.2.3.7 Soluble protein measurements

The given method for gel preparation of myofibrillar protein was followed (Section 3.2.3.4). Immediately and 24 h after addition of GdL, samples of myofibrillar systems (4 x 15 g) were centrifuged at 20000 rpm for 1 h (Sorvall RC-5C automatic superspeed refrigerated centrifuge, Du Pont Company, Delaware, U.S.A.). The supernatant was decanted immediately and kept at 4°C. The concentration of the protein was determined by moisture content analysis (Section 3.2.3.7.1) and biuret reaction (Section 3.2.3.6.2). Myoglobin content was determined by the Drabkin's test for protein content (Section 3.2.3.6.3) and an average concentration was subtracted from the results of the biuret test.

3.2.3.7.1 Moisture content analysis

Moisture content analysis of the solution drained from the myofibrillar protein was determined. Three moisture dishes per soluble protein preparation were dried (at least 2 h) at 105°C and cooled at room temperature in a desiccator (2 h). The dishes were weighed and supernatant (approximately 1 g) was accurately weighed into each dish. These samples were dried for 24 h at 105°C and then transferred to a desiccator at room temperature for 2 h. The weight of the sample was accurately measured and moisture content of the supernatant was calculated.

3.2.3.7.2 Biuret test for analysis of protein concentration

The concentration of the protein in the supernatant was determined based on the procedure described by Gornall *et al.* (1949). Standards of BSA were used.

The biuret reagent was made as follows: cupric sulphate (1.500 g) and sodium potassium tartrate (6.000 g) were measured into a dry one litre volumetric flask. Water (500 ml) was added and the solution was well shaken. A 10% (w/v) sodium hydroxide solution (300 ml) was added and the solution made up to 1.0 l with water.

Supernatant obtained from soluble protein measurements was measured into plastic test tubes. At least two amounts of the supernatant were analyzed for each sample to ensure consistency in the measurements and samples. Aliquots (0.0, 0.5, 1.0, 1.5,2.0 ml) of 10 mg/ml BSA were used as standards. The supernatant samples and the standards were made up to 2.0 ml using a 10% (w/v) sodium hydroxide solution. Biuret reagent (8.0 ml) was added and the solutions were vortexed. After standing for 30 min at room temperature, the absorbance was measured on a Philips UV/Visible Spectrophotometer (PU 8625, England) at a wavelength of 540 nm using 1 cm plastic cuvettes.

3.2.3.7.3 Drabkin's test for myoglobin concentration

Drabkin's solution was made as follows: ammonia (4.00 ml) was measured into a one litre volumetric flask using water. Potassium cyanide (50.0 mg) and potassium ferricyanide (0.200 g) were dissolved in the ammonia solution and made up to 1.0 l with water.

Hemin (0.010 g) was dissolved in Drabkin's solution (100 ml) to give a standard solution of 100 mg/ml hemin. The solution was filtered (Whatman number 1) on a buchner funnel. Aliquots (0.0, 0.5, 1.0 and 2.0 ml) of hemin solution were diluted to 2.0 ml in plastic test tubes with water. These solutions were used as standards for the protein determination. At least two amounts of each supernatant were used to ensure consistency. Supernatant was used from gels of myofibrillar protein (100 g) with added GdL (1.00 g) centrifuged immediately, with GdL (1.00 g) and TSPP (0.30 g) centrifuged after 24 h, with GdL (2.00 g) and TSPP (0.30 g) centrifuged after 24 h. These samples all resulted in red, translucent supernatants after centrifugation. Drabkin's solution (3.0 ml) was added to the samples and standards, and all were vortexed. Absorbance was measured on a Philips UV/Visible Spectrophometer (PU 8625, England) at a wavelength of 540 nm, using 1 cm plastic cuvettes.

3.2.3.8 Transmission electron microscopy (TEM)

Transmission electron microscopy was undertaken by the Electron Microscopy Laboratory, Hort+Research, Palmerston North.

Small pieces of myofibrillar gels were fixed in a primary fixative of 3% glutaraldehyde and 2% formaldehyde in 0.1 M phosphate (Na_2HPO_4/KH_2PO_4) buffer (pH 7.2) for 2 h at room temperature. The pieces of gels were then washed three times in the buffer at room temperature. The gel pieces were placed in a secondary fixative of 1% osmium tetroxide in phosphate buffer, (pH 7.2) for 1 hr at room temperature. Dehydration was undertaken using a graded acetone series (25%, 50%, 75%, 95%, 2 x 100%) in the buffer. Infiltration of resin into the gels was undertaken in an acetone:resin (50:50) mixture (Polarbed 812 epoxy resin). The mixture was stirred overnight at room temperature. The pieces of gels were placed in 100% resin and stirred for 7 hr at room temperature. The gel fragments were embedded in fresh resin in a silicone rubber mould and cured at 60°C for 48 hours.

Sections of 90 nm (pale gold interference colour) were cut from the trimmed blocks using a diamond knife and a Reichert Ultracut E ultramicrotome. Sections were grid mounted and double stained using saturated uranyl acetate in 50% ethanol, followed by lead citrate (Venable and Coggeshall, 1965). Sections were studied using a Philips 201c Transmission Electron Microscope.

3.2.3.9 Analysis of Data

All effects of the various treatments on Young's Modulus and springiness were determined by analysis of variance for a factorial arrangement of treatments in a completely randomized design using a Stats-Packets Statistical Analysis Package.

3.2.4 Myosin

3.2.4.1 Myosin preparation

The method of preparation of myosin was based on the procedure described by Dudziak and Foegeding (1988).

Hasselbach-Schneider solution consisted of 0.6 M potassium chloride, 1 mM magnesium chloride hexahydrate, 0.1 M phosphate (KH_2PO_4/K_2HPO_4) buffer (pH 6.4), 20 mM EGTA, 10 mM TSPP.

The preparation of myosin was undertaken at 4°C. All containers and utensils except the blades of the Kitchen Whizz were plastic. Meat (approximately 300 g) was minced in a Sunbeam Kitchen Whizz (model LC-AX, type 272, France) that had been rinsed with 20 mM EDTA (pH 7.0). The meat was mixed with 20 mM EDTA (pH 7.0) at a weight ratio of 1:2 of meat to EDTA solution. The suspension was stirred manually with a spatula for 15 min. The suspension was centrifuged at 7010 rpm (Sorvall RC-5C automatic superspeed refrigerated centrifuge, Du Pont Company, Delaware, U.S.A.). These steps of addition of EDTA and centrifugation were repeated twice on the pellets obtained from centrifugation. The supernatants were discarded. The resultant pellet was then mixed with Hasselbach-Schneider solution at a weight ratio of 1:2 of pellet to solution. The suspension was stirred for 15 min and centrifuged for 30 min at 8000 rpm. The supernatant was retained and strained through a plastic food strainer. The pellet was discarded. The supernatant was added to water to give an approximate ionic strength of 0.03 assuming uniform removal of ions (67-70x dilution). The precipitate was allowed to settle overnight. Liquid was carefully siphoned from the settled precipitate and the resultant suspension was centrifuged at 8000 rpm for 30 min. Supernatant was discarded and the pellet was resuspended in 0.5 M Tris (pH 7.0), 1.5 M potassium chloride that had been diluted with water to an ionic strength of approximately 0.10 to 0.15 (33.3 ml Tris/potassium chloride in 500 ml total, about 15 times dilution). The suspension was centrifuged at 18500 rpm for 1 h and the supernatant was discarded. Solutions of 3.0 M potassium chloride and 20 mM phosphate (Na_2HPO_4/NaH_2PO_4) buffer (pH 7.0), 0.5 M sodium chloride were mixed at a weight ratio of 1:1 and then diluted (200 ml) with water (100 ml). The resultant solution was used to resuspend the pellet. The suspension was centrifuged at 18500 rpm for 1 h. The pellet was discarded. The supernatant was diluted ten-fold with water. The suspension obtained was centrifuged at 8000 rpm for 30 min and a crude myosin preparation was obtained. The protein was stored in a plastic container at 4°C. Sodium azide (0.01%, w/w) was added to the protein preparation to slow microbial degradation. The protein content was determined by the biuret test and moisture content analysis. Electrophoresis and densitometry determined the purity of the preparation.

3.2.4.1.1 Biuret Test

Determination of the protein content was as for soluble myofibrillar protein concentration determination (Section 3.2.3.6.2) except the samples were of myosin, weighed into the plastic test tubes. This required that the samples be well mixed with the biuret reagent to ensure complete solubilisation of the protein.

3.2.4.1.2 Moisture content analysis

Three moisture dishes per myosin preparation were dried (at least 2 h) at 105°C and cooled at room temperature in a desiccator (2 h). The dishes were weighed and myosin (approximately 1 g) was accurately weighed into each dish. These samples were dried for 24 h at 105°C and then transferred to a desiccator at room temperature for 2 h. The weight of the sample was accurately measured and the moisture content of the myosin preparation calculated.

3.2.4.1.3 Electrophoresis

The procedure for preparation of electrophoresis gels and sample preparation were taken from the methods described by Locker and Wild (1984) and Porzio and Pearson (1977). A Hoefer Mighty Small II Slab Gel Electrophoresis Unit was used (SE 250, Hoefer Scientific Instruments, San Francisco, U.S.A.) to run the electrophoresis gels. The gels were cast in a Hoefer Dual Gel Caster (SE 245, Hoefer Scientific Instruments, San Francisco, U.S.A.) using a 10-well comb of 1.0 mm thickness.

For the 6.5% acrylamide (100:1) casting gel system, a stock solution was prepared by dissolving acrylamide (47.5 g) and bis (0.475 g) in water (231 ml). This solution was stored at 4°C. Additional stock solutions were prepared as 2.0 M Tris/glycine (0.50 M Tris:1.50 M glycine, pH 8.8), 50% v/v glycerol, 2.5% SDS/2.5 mM EDTA and 1% TEMED (stored at 4°C). A 10% ammonium persulfate solution was made up fresh for each electrophoresis experiment. The casting solution consisted of stock solutions in the following proportions: 10 volumes acrylamide/bis, 5 volumes tris/glycine, 2.5 volumes glycerol solution, 1 vol

SDS/EDTA and 4.5 volumes water. Immediately before casting, 1 volume of TEMED and 1 volume of the initiator, ammonium persulfate, were added. After the gels were loaded, the surface of the solution was layered (0.2 ml) with layering solution of 400 mM Tris/glycine (pH 8.8), 0.04% TEMED, 0.1% SDS and 0.004% persulfate. After polymerisation was completed in 45 to 90 min, the layering solution was decanted, the surface of the gel was rinsed and water was removed from the surface using a Whatman number 1 filter paper.

For the 4.5% acrylamide (100:1) stacking gel system, a stock solution was prepared by dissolving acrylamide (11.25 g) and bis (0.113 g) in water (100 ml). This solution was stored at 4°C. An additional stock solution was prepared as 2.0 M Tris/glycine (0.50 M Tris:1.50 M glycine, pH 6.8). The stacking gel was made as for the casting gel, but 4.5% acrylamide/bis and 2.0 M Tris/glycine (pH 6.8) were used instead of 6.5% acrylamide/bis and 2.0 M Tris/glycine (pH 8.8), respectively. The gel was loaded onto the casting gel. After 45 to 90 min polymerisation was completed and the surface of the stacking gel was washed and water was removed with filter paper.

A stock solution of concentrated heating buffer was made consisting of 25 mM Tris, 75 mM glycine, 2 mM EDTA, 2% SDS. A stock solution of 0.02 M DTT (1.54 g) in water (10 ml) was stored at 4°C. Myosin samples were diluted to concentrations of 0.40 mg/ml myosin with water. At least one dilution of the 0.40 mg/ml solution of myosin was made and sampled to ensure consistency between samples and measurements. Concentrated heating buffer (0.1 ml), 0.02 M DTT solution (0.1 ml) and water (0.8 ml) were added to the myosin samples (1.00 ml). High molecular weight standards were prepared by adding concentrated heating buffer (0.02 ml), 0.02 M DTT (0.02 ml) and water (0.16 ml) to the standard (10 µl). Samples and standards were vortexed and then heated at 50°C for 20 min. After removal from the water bath, a sucrose/bromophenol blue solution (25%, w/v sucrose, 0.0025%, w/v bromophenol blue) was added (0.05 ml to the standards, 0.5 ml to the samples). The solutions were vortexed.

A stock solution of reservoir buffer consisting of 200 mM Tris/glycine (0.05 M Tris and 0.15 M glycine, pH 8.8), 5% glycerol and 0.1% SDS was made. The chambers were filled and aliquots (14 µl) of the samples and standards were syringed into the stacking gels using a

Hamilton syringe. The gels were run for 45 to 60 min at 20 mA (approximately 150 V) per gel using a 500/200 V power supply (serial number 8676, PET Department, Massey University, New Zealand). The gels were cooled with tap water throughout the runs.

The gels were rinsed with water then transferred to a fixing solution of 25% (v/v) isopropanol and 10% (v/v) acetic acid, for 2 h. The gels were then rinsed with water and stained in a solution of Coomassie Brilliant Blue R250 (1.25 g) in water (242 ml), methanol (242 ml) and acetic acid (46 ml) which had been filtered through a Whatman number 1 filter paper on a Buchner funnel. After 2 h of staining, the gels were washed with water and transferred to a destaining solution of 7.5% (v/v) acetic acid and 5% (v/v) methanol for at least 48 h. The gels were recorded by photography and scanned using a densitometer to determine the purity of the preparation.

3.2.4.1.4 Densitometry

Gels were scanned on a LKB Produker AB Ultrascan XL Laser Densitometer (Part number 90 02 2351, Serial number 235, Bromma Sweden) with a Computer Display (Panasonic Model number TR90T1G, Matsushita Electric Industrial Co., Ltd., Japan). Purity of the sample was calculated from the total components present in a sample. The protein bands were assigned based on those observed in Porzio and Pearson (1977) and Sender (1971). A typical gel is shown in Figure 3.1. Quantitation of the proteins assumes that the integrated optical density of stained gel bands is proportional to the mass of the protein present; the affinity of the dye for the different proteins may however be variable (Sender, 1971). Therefore, calculating the purity of the myosin preparation by comparison of components in one sample is not strictly correct as different proteins have different extinction coefficients (Samejima *et al.*, 1992). However, the procedure used appears to be an accepted method of determination (for example, Locker and Wild, 1984; Porzio and Pearson, 1977; Samejima *et al.*, 1992).

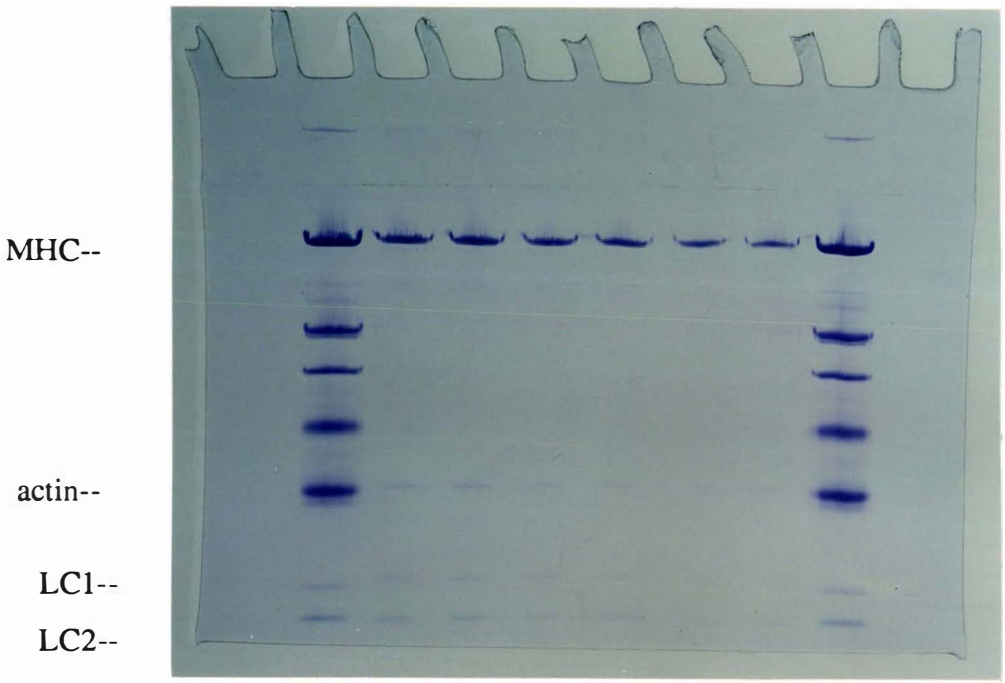


Figure 3.1. A typical SDS-PAGE gel. Assignment of the protein bands was based on Sender (1971) and Porzio and Pearson (1977). MHC = myosin heavy chain, approximately 200 kDa; LC1 = alkali light chain 1, approximately 25 kDa; LC2 = DTNB light chain, approximately 18 kDa. Actin has a molecular mass of approximately 44 kDa.

3.2.4.2 Washed myosin

Water (1.5 l) was added to myosin (30.0 g, 34.58 mg/ml) and the suspension was stirred. The suspension was then centrifuged at 8000 rpm for 30 min. The biuret test was used to analyze the protein concentration as in Section 3.2.4.1.1.

3.2.4.2.1 Atomic absorption analysis

The atomic absorption analysis of supernatant of the washed myosin was undertaken by the Department of Chemistry, Massey University, Palmerston North.

3.2.4.3 Gel preparation

Myosin (4.50 g, 20 mg/ml) was measured into a plastic beaker. Where sodium chloride (0.011, 0.110, 0.170 g) and/or TSPP (0.006, 0.011, 0.017 g) were added, these compounds were added directly to the myosin and stirred well. Where GdL (0.015, 0.030, 0.060 g) was added to the myosin, it was added immediately after mixing the sodium chloride and/or TSPP solution if added, and the GdL was added just prior to addition of the gel to the Bohlin rheometer. The mixture was stirred well after GdL addition. The pH of the myosin samples was measured concurrent to the rheological measurements.

Washed myosin was also formed into gels using this preparation method.

Additional phosphates were investigated and were added in the place of TSPP. These phosphates were orthophosphate ($NaH_2PO_4.2H_2O$, 0.0037 g), tripolyphosphate ($Na_5P_3O_{10}$, 0.0093 g) and hexametaphosphate (0.015 g).

Sucrose (0.45 g) was added to myosin suspensions (4.50 g) as a hydrogen bond competitor. The sucrose was added 24 h prior to measurement of the rheological characteristics and the myosin was stored at 4°C. This myosin-sucrose suspension was used instead of myosin in the gel preparation.

The sulfhydryl blocking agent, PCMB (0.0004, 0.0008, 0.0040 g) and the reducing agent, DTT (0.0002, 0.0005, 0.0020 g), were added to myosin (4.50 g, 20 mg/ml) 24 h prior to the measurement of rheological characteristics. These suspensions of myosin plus agent were used instead of myosin in the gel preparation. The methods were based on those of Ishioroshi *et al.* (1980), Samejima *et al.* (1988) and Taguchi *et al.* (1987).

3.2.4.4 Rheological investigations

The Bohlin VOR Rheometer System (Bohlin Rheologi AB, Lund, Sweden) was used in the oscillatory mode. The torque measuring shaft was supported by an air bearing. The torque bar (0.307 g cm) rigidity constant combined with the inertia of the shaft gave a natural frequency to the detector of about 10 Hz or more. No measurements at a frequency above 1 Hz, except in the frequency sweep, were reported.

During gelation experiments, the protein suspension was covered with a layer of paraffin oil in order to prevent evaporation of water. All experiments were conducted at 4°C. The temperature was maintained by an in-built cooling system of the Bohlin VOR Rheometer System. The sample cell consisted of serrated parallel plates (40 mm diameter) with a plastic containing ring (3.0 mm diameter) and a 1 mm gap between the plates. Oscillation tests, frequency scans and strain sweeps were run. The parameters G', G", δ and dynamic viscosity were measured.

3.2.4.5 Measurement of pH

The measurement of pH was a direct measurement as for the minced meat samples (Section 3.2.2.3).

3.2.4.6 Fluorescence

The method used was a modification of the method of Nakai and LiChan (1988). A solution of ANS (0.0048 g) in water (2.00 ml) was vortexed until the ANS had dissolved. Relative

fluorescence intensity (RFI) was standardized by adjusting the reading of the fluorometer to 30% full scale for ANS (20 µl) in methanol (4.00 ml).

Myosin (30 ml, 0.2 mg/ml) was measured into a plastic beaker fitted with a glass pH probe. While stirring the myosin solution, ANS (0.15 ml) was added and the pH allowed to settle. When added, sodium chloride (0.0039, 0.0075, 0.0150, 0.0375 g), TSPP (0.0003, 0.006 g) and/or GdL (0.0039, 0.0075, 0.0150, 0.0375 g) were added to the stirring solution. An aliquot (4.00 ml) of the myosin solution was then removed to a glass cuvette and measurement of fluorescence was commenced 2.5 min after the addition of GdL, TSPP and/or sodium chloride. Fluorescence was measured with a Sequoia-Turner Digital Fluorometer (Model 450-000, Serial number B002370TV, Sequoia-Turner Corporation, California, U.S.A.). The wavelengths of excitation and emission were 360 and 475 nm, respectively; excitation and emission slits were 0.58 and 2.00 mm, respectively. The pH of the solution was monitored continuously, concurrent to the fluorescence measurements by a Metrohm Herisau pH meter (E350B, Switzerland).

Higher concentrations of TSPP (0.0015, 0.0036 g) resulted in the formation of aggregates of myosin as the pH decreased. The presence of aggregates resulted in data which was not reproducible.

3.2.4.7 Light microscopy

Gels were prepared as in Section 3.2.4.3, but were set in plastic test tubes. Samples of the gels were removed with a spatula and were placed on glass microscope slides. Glass cover slips were placed over the samples. The samples were viewed and photographed under a Reichert Diapan Microscope (serial number 340172, Austria) with Nomarski Differential Interference Contrast (NDIC).

3.2.4.8 Transmission Electron Microscopy (TEM)

The procedure for TEM was as for myofibrillar gel preparations (Section 3.2.3.7).

3.2.4.9 Van Slyke reaction

The reaction described by Vogel (1961) was followed.

3.2.5 Dialysis

3.2.5.1 Myosin, myofibrillar protein and minced meat dialysis

Gels of minced meat were prepared as in Section 3.2.2.1 and gels of myofibrillar protein were prepared as in Section 3.2.3.3. Two types of gels were made of each system. One group had the ends of the PVC tubes covered with dialysis membrane (viscose cellulose, molecular weight cut-off approximately 12000 to 14000, Union Carbide 453105) instead of the polyethylene all-purpose food film. The second group of gels were made in the dialysis membrane, without the use of the PVC tubing and were tied at the ends with cotton.

Myosin gels were prepared as in Section 3.2.4.3, but were not put onto the Bohlin rheometer. Some gels were set in dialysis tubing as round 'sausages' and the ends were clipped with weighted plastic dialysis clips. Other samples were placed in dialysis tubing suspended between two vertical parallel perspex plates which were separated by a 1.00 mm gap. The tubing was clipped at the base using metal bulldog clips. The suspensions were forced into the tubing to fill the gap and stored for a given time in this position, at 4°C. Prior to removal from the plates, the tubing was clipped at the top with metal bulldog clips. Several myofibrillar samples were also dialysed as thin strips in dialysis tubing as described for myosin.

The samples in PVC tubing, dialysis tubing or without any constraining surface were placed in water, water of pH adjusted with sodium hydroxide or hydrochloric acid, or solutions of sodium hydrogen carbonate. The liquid used was at least a volume of 1.0 l, was changed at least once during the dialysis process, and dialysis occurred for at least 24 h. Sodium azide (0.01%) was added to the dialysis liquid to slow microbial degradation of the sample. The dialysis was undertaken at 4°C and the liquid was stirred slowly to ensure continual movement of the solution over the exposed surfaces of the samples. The myosin samples in dialysis

tubing with bulldog clips at the ends were weighted by the clips and rested on the bottom of the containers instead of being suspended. These samples were rocked instead of stirred.

3.2.5.2 Tensile tests

Tensile tests were undertaken on the Instron Universal Testing Machine (model 4502, Instron Ltd High Wycombe, England) equipped with a Phillips Computer (model Pro9CM082, Taiwan).

Myosin and myofibrillar protein samples dialysed as thin strips and their undialysed counterparts were cut into samples of 23.0 mm length and 7.5 mm width. The samples were suspended from bulldog clips attached to horizontal probes and tensile tests were undertaken by extension of the sample at a rate of 25.4 mm/min. A 10 N load cell was used and extension of the sample was taken to destruction. Force and displacement at the point of destruction were recorded. Many of the samples, in particular the undialysed samples, were too fragile to be suspended from the clips and no data was able to be obtained.

3.2.5.3 Measurement of pH

Measurement of the dialysed samples was direct as for the minced meat samples (Section 3.2.2.3).

3.2.5.4 Inductively coupled plasma (ICP) emission spectrometry

The ICP analysis of dialysate was undertaken by the Analytical Services Laboratory, AgResearch, Palmerston North. The dialysate was not changed, instead one volume of water was used to dialyse the myosin samples for 43 h.

CHAPTER 4

GdL-INDUCED GELATION OF MYOFIBRILLAR PROTEINS

4.1 INTRODUCTION

The most important feature of restructured meat products is the ability of the protein matrix formed to effectively bind the meat pieces together. Effective binding is essential for the product to retain its structural integrity during subsequent handling and slicing (Schmidt and Trout, 1982). Initial research in the area of bind was directed towards studying the thermally-induced binding involved in meat patties and sausages (Hashimoto *et al.*, 1959; Fukazawa *et al.*, 1961a,b,c; Nakayama and Sato, 1971a,b) and has since developed to include chunked and formed meat products (e.g., Schnell *et al.*, 1970; MacFarlane *et al.*, 1977; Ford *et al.*,1978; Siegel and Schmidt, 1979a; Wijngaards and Paardekooper, 1988). Fukazawa *et al.* (1961a,b) and Hashimoto *et al.* (1959) showed that myofibrillar proteins, in particular myosin, were primarily responsible for the binding quality in restructured meats and that bind strength displayed a close relationship with the amount and nature of myosin contained in and/or liberated as actomyosin from a meat structure. These findings have been substantiated by many workers (for example, Maesso *et al.*, 1970; Schnell *et al.*, 1970; Vadehra and Baker, 1970; Nakayama and Sato, 1971b; Grabowska and Sikorski, 1976; Moore *et al.*, 1976).

Many of the meat products available today incorporate salt (NaCl) to aid in product binding, flavour and preservation. Extraction and solubilization of myofibrillar proteins are functions of sodium chloride (NaCl) concentration contributing to meat particle binding, fat emulsification and water holding capacity (WHC) and thus reducing cook losses and improving quality and texture (Schmidt *et al.*, 1981; Acton *et al.*, 1983). The possible involvement of sodium consumption in the development of hypertension has prompted public health and regulatory authorities to recommend reduction in the dietary intake of NaCl (Sofos, 1989). Certain meat products contain relatively high amounts of sodium and are, therefore,

major targets for lowering ingoing levels of NaCl (Sofos, 1986a,b). As sodium chloride levels are reduced, however, meat cohesion, water retention, product quality and shelf life may be compromised (Sofos, 1983, 1985a,b, 1986b). The meat industry has therefore turned to the use of various phosphates, which can partially replace sodium chloride and restore product quality and identity (Keeton, 1983; Madril and Sofos, 1985; Puolanne and Terrell, 1983a,b; Seman *et al.*, 1980; Sofos, 1985a,b; Trout and Schmidt, 1984, 1986b; Whiting, 1984).

Much research has been undertaken investigating fundamental aspects of thermally induced binding and gelation of meat and its protein fractions. As a result Vadehra and Baker (1970) stated that binding between meat pieces was a heat mediated reaction since no binding was observed in the raw state. However, Fretheim *et al.* (1985) found that solutions of myosin (10 mg/ml) formed gels at 5°C if the pH was decreased slowly, by dialysis, to a value in the region of 2.5 to 5.5. Potassium chloride concentration was found to affect gel strength positively and linearly. Differential scanning calorimetry revealed that the myosin of acid-induced gels absorbed no thermal energy when heated, implicating acid-induced denaturation as the basis of gel formation. Hermansson *et al.* (1986), in an investigation of the effect of pH and ionic strength on thermally-induced myosin gels, also observed that myosin solutions dialysed against pH 4.0 buffers in 0.6 M potassium chloride formed gels spontaneously at 4°C.

The aim of this study was to investigate the characteristics of gels of bovine skeletal muscle and myofibrillar proteins formed at 4°C as a result of the slow lowering of pH through the addition of GdL. The effects of the presence of salt (NaCl) and/or tetrasodium pyrophosphate (TSPP) were investigated at levels commonly used in the meat industry. The collection of data pertaining to the gelation characteristics of myofibrillar protein could aid in the formulation of a mechanism to explain acid-induced gelation.

4.2 RESULTS

4.2.1 Preparation of myofibrillar proteins

Large quantities of connective tissue were recovered from the meat with a spoon while stirring. As a result, the collagen content was approximately 0.21% of the wet weight. In a

typical adult mammalian muscle, the collagen content is approximately 1% of the wet weight (Lawrie, 1974) and, therefore, approximately 80% of the collagen was removed. Quantities of fat were recovered on the centrifuge spindle so that the fat content of the recovered myofibrils was less than 0.33% of the wet weight. This left approximately 13% of the 2.5% fat in a typical adult mammalian muscle (Lawrie, 1974). The moisture content was determined to be 81.5% and protein content, 17.76% of the wet weight.

4.2.2 Minced meat gelation

No quantitative data was obtained because the variation between replicates was greater than the difference between samples. Visually and to the touch, the minced meat (50.0 g) formed gels with GdL (2.0, 3.0 g) after 10 h and had a grey translucent appearance with final pH values of 4.1 and 3.8, respectively. With lower GdL (0.5, 1.0 g) concentrations no gel formed and at higher GdL (4.0, 7.0 g) concentrations the samples gelled immediately upon addition of the lactone. High local acid concentration around solid GdL particles resulted in uneven gelling with insufficient time to mix the slurry into the system.

4.2.3 Myofibrillar protein gelation with hydrochloric acid

When samples of myofibrillar protein were dialysed against a solution of 0.17 M hydrochloric acid for 18 h, gels were formed. Direct addition of hydrochloric acid resulted in the immediate gelation of myofibrillar protein. However, due to high local hydrochloric acid concentration which resulted in rapid gel formation, a mixture of non-gelled and gelled myofibrillar protein resulted. Much of the hydrochloric acid at 8.22% was not incorporated into the gel structure and a liquid phase around the myofibrillar protein resulted. Slow lowering of the pH by dialysis resulted in a more homogeneous gel of pH 1 with a solid white core of about one third of the volume of the gel, and with a grey, glassy and tacky outer layer of similar pH.

4.2.4 Myofibrillar protein gelation with GdL

At concentrations of 1.8 and 3.6% GdL, myofibrillar protein gels of appreciable Young's Modulus were obtained (Figure 4.1). At 0.9% GdL concentration, no significant differences were observed with the addition of the lactone compared to the sample without added GdL. The majority of the modulus of elasticity was obtained within the first 2 to 5 h after addition of GdL. The rate of gelation increased with increasing rate of fall of pH. Increasing rate of fall of pH was a result of greater GdL concentration, which when hydrolysed, would have resulted in a greater number of protons at higher GdL concentrations. Figure 4.2 illustrates the springiness of the gels which unlike the graph of Young's Modulus, showed that the gel formed using 3.6% GdL did not decline to values less than that of 1.8% GdL. Again, at 0.9% GdL the springiness was similar to that of the myofibrils alone. The addition of 1.8% GdL resulted in increased springiness, which after 10 h was similar to that at 3.6% GdL. As with the Young's Modulus results, the rate of formation of springiness increased with increasing rate of fall of pH. Table 4.1 shows the subjective characteristics and final pH measurements of the gels. As the pH progressed from 5.8 to 4.5 (corresponding to the samples of myofibrils alone to the final pH attained with the addition of 1.8% GdL) the gel visually changed from a pink/white crumbly mass which when pushed out of the tube did not maintain the shape of the tube, to a red, glassy, sticky gel which held its shape for at least several days. At 0.9% GdL (final pH 4.8), the visual characteristics were the same as the myofibrils alone (pH 5.8). Figure 4.3a is a photograph of the myofibrils without added GdL illustrating the slumping of the gel when removed from the tube. The gel appeared crumbly, and moist. Figure 4.3b illustrates the gel formed at 3.6% GdL. The red glassy appearance and the smoothness in texture of this gel are evident in comparison with the myofibrils alone.

The concentration of GdL was increased to levels of 5.5, 7.3, 9.1 and 10.9%. At concentrations of greater than 3.6%, the GdL did not fully dissolve in the water and was added to the myofibrils as a slurry. Gels formed almost immediately upon addition of the GdL to the proteins. With these increasing concentrations, the stickiness of the gels caused difficulties in manipulation. After 10 min the pH ranged from 4.6 at 5.5% GdL to 4.3 at 10.9% GdL. The final pH values were 3.0 to 3.5. All samples appeared red/grey and glassy,

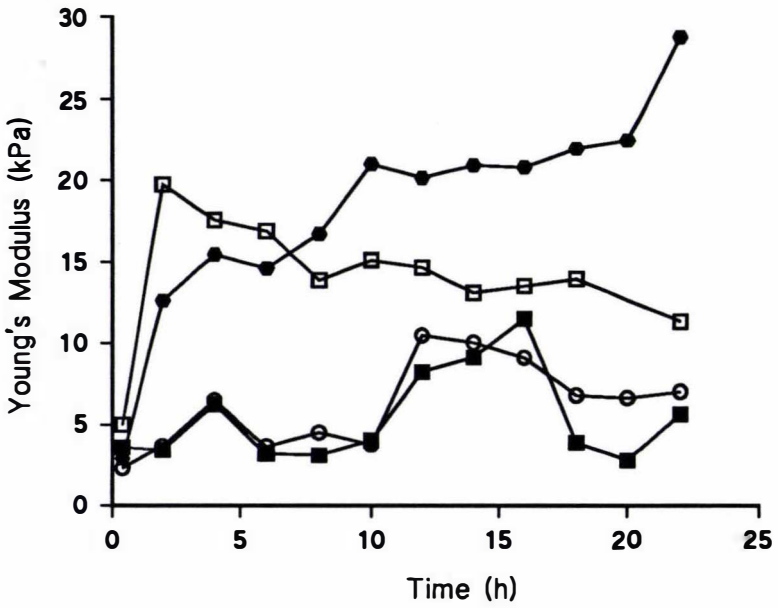


Figure 4.1. Time development of Young's Modulus. No added GdL (■), 0.9% GdL (○), 1.8% GdL (●), 3.6% GdL (□).

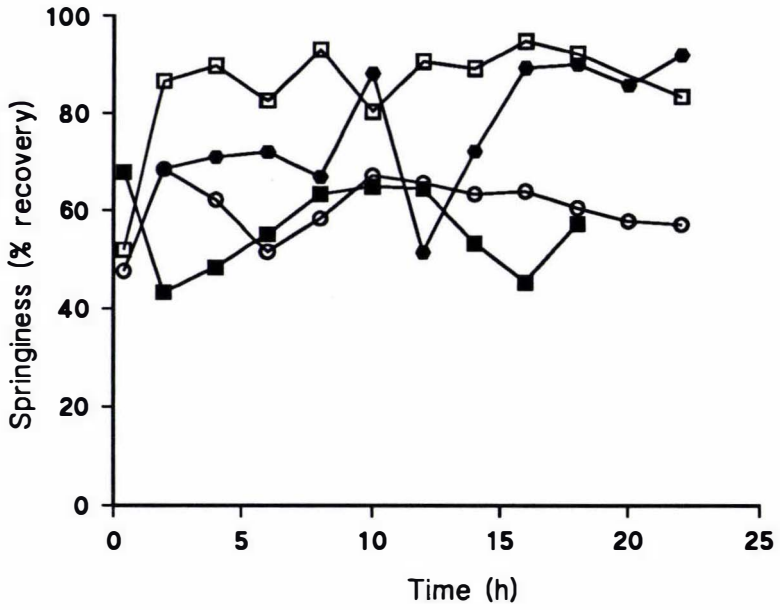
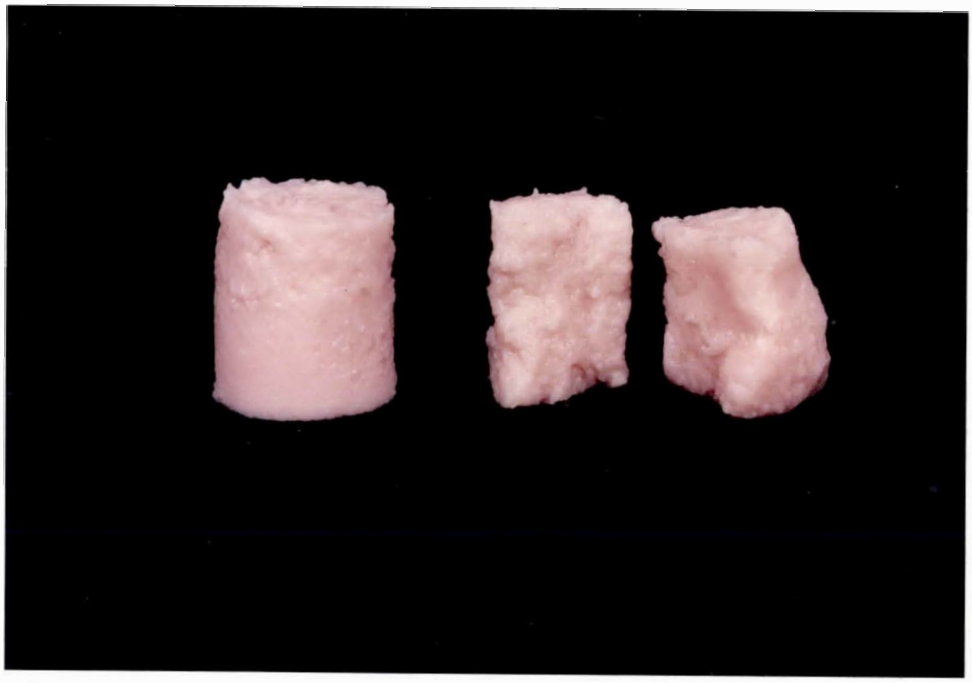


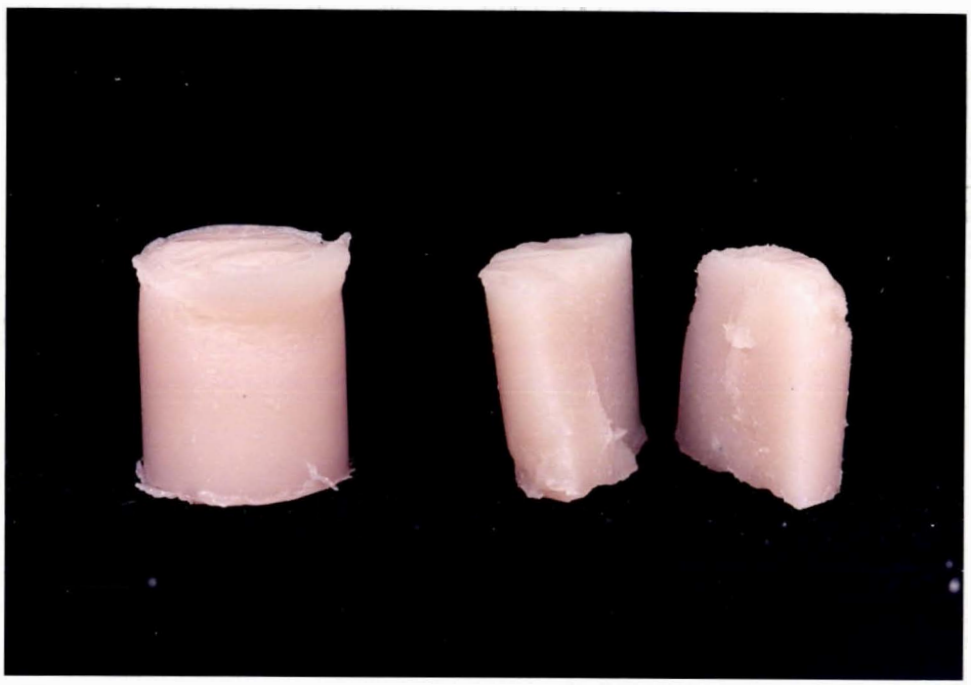
Figure 4.2. Time development of springiness. No added GdL (■), 0.9% GdL (○), 1.8% GdL (●), 3.6% GdL (□).

Table 4.1. Qualitative characteristics and final pH measurements of myofibril gels.

Appearance	GdL conc (%)	NaCl (%)	TSPP (%)
pink/white, shape is not stable, pH 5.8	0	0	0
red, glassy, initially sticky, pH 5.6	0	2.5	0
pink, becomes crumbly, initially shape is not stable, pH 6.1	0	0	0.3
red, glassy, sticky, pH 5.8	0	2.5	0.3
pink/white, crumbly, shape is not stable, pH 4.8	1	0	0
red, glassy, initially sticky, loses liquid upon compression after 6 hours, pH 5.0	1	2.5	0
pink, crumbly, initially shape is not stable, loses liquid upon compression, pH 5.1	1	0	0.3
red, glassy, initially sticky, pH 5.1	1	2.5	0.3
red, glassy, becomes sticky, initially shape is not stable, pH 4.5	2	0	0
red, fades to pink, granular, loses liquid upon compression, pH 4.5	2	2.5	0
pink, crumbly, loses liquid upon compression, initially shape is not stable, pH 4.7	2	0	0.3
red, glassy, inner lumpy texture, loses liquid upon compression after 12 hours, pH 4.8	2	2.5	0.3
initially pink and crumbly, becomes red, glassy and sticky, pH 3.9	4	0	0
red, becomes pink, crumbly, pH 3.8, loses liquid upon compression	4	2.5	0
initially pink and crumbly, becomes red, glassy and stodgy, pH 4.1	4	0	0.3
pink, becomes white/grey, crumbly, exudation occurs upon standing, pH 4.2	4	2.5	0.3

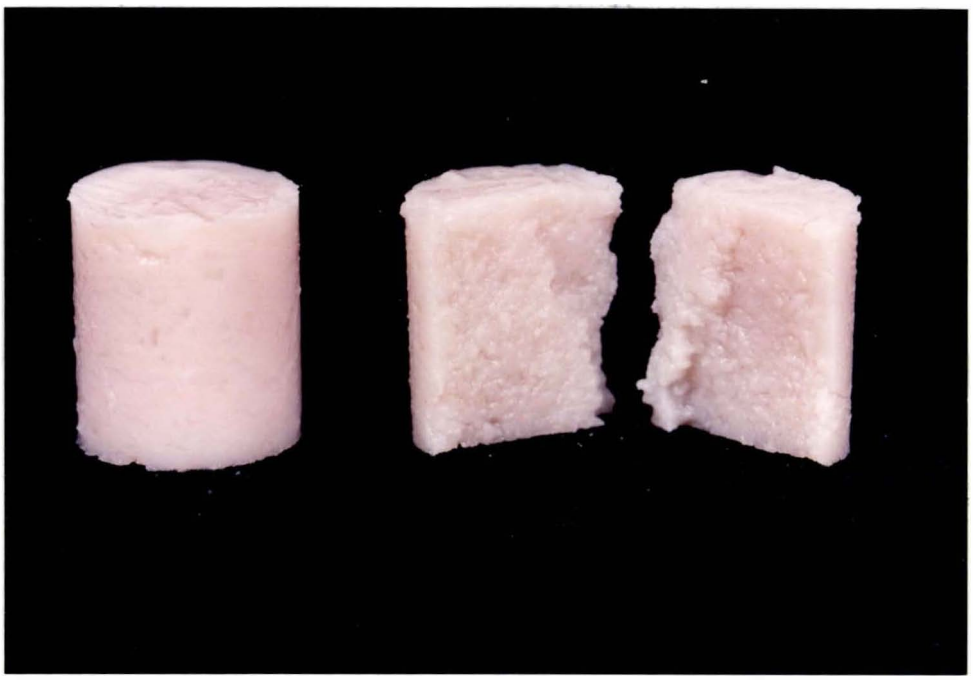


a

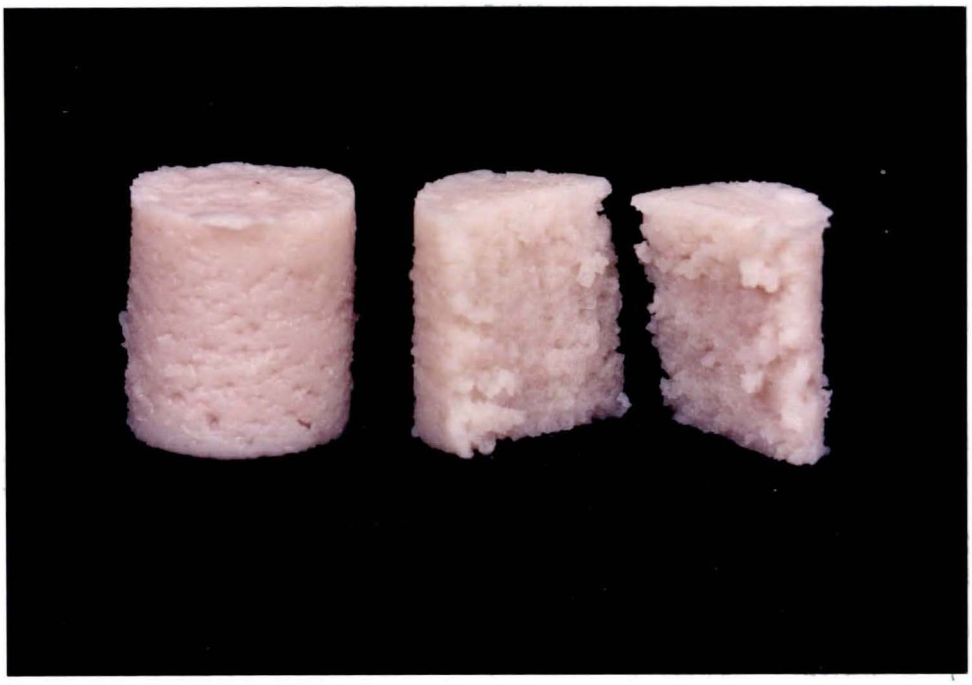


b

Figure 4.3 Gels of myofibrillar protein in the presence and absence of GdL, NaCl and TSPP. a) myofibrillar protein only, b) myofibrillar protein plus 3.6% GdL.



c



d

Figure 4.3 (cont.)
c) myofibrillar protein plus 3.6% GdL and 2.3% NaCl, d) myofibrillar protein plus 1.8% GdL, 2.3 % NaCl and 03% TSPP.

and had a sticky, smooth texture. In subsequent experiments, levels of GdL of 0 to 3.6% were used.

Samples stored at 0, 10, 20 and 30°C, at 2.5 h after the addition of 1.8% GdL, showed no significant ($p < 0.05$) difference in the Young's Modulus values up to 20°C. At 30°C, the Young's Modulus increased at a faster rate than at the lower temperatures and to a greater final value. The myofibrillar protein was chilled prior to use. Gel preparations were made over a maximum period of 2.5 h at room temperature, and thereafter remained at 4°C until measurements were taken. It is unlikely that the temperature of the gels would have exceeded 20°C, but if so it would have been for less than 1 h and at not more than 22°C.

4.2.5 The effect of sodium chloride on gelation

Addition of 2.3% sodium chloride (NaCl) on a weight basis to the myofibrillar proteins increased the Young's Modulus at all four GdL concentrations (Figure 4.4) compared to the samples without NaCl (Figure 4.1). Similar to the results without NaCl, at 0.9% GdL the Young's Modulus time course could not be distinguished from the control sample with NaCl and no added GdL. However, at these lower GdL concentrations (0 and 0.9% GdL), the Young's Modulus was 4 to 5 times greater in the presence of NaCl than its absence. There was no indication of the Young's Modulus decreasing at 3.6% GdL with NaCl. For all GdL concentrations the Young's Modulus had levelled within 5 to 10 h, possibly continuing to increase slightly. At 1.8 and 3.6% GdL, the Young's Modulus was 2 to 3 times that without NaCl. Increasing concentrations of GdL and therefore increasing rate of fall of pH resulted in a faster rate of increase of Young's Modulus. The springiness of the gels was enhanced by the addition of NaCl at 0 and 0.9% GdL (Figure 4.5), but was reduced at 3.6% GdL with NaCl when compared with gels formed without NaCl (Figure 4.2).

Immediately upon addition of NaCl the myofibrillar protein became red and glassy in appearance, visually similar to the gel formed at 1.8 and 3.6% GdL in the absence of NaCl. In the presence of NaCl, concentrations of 1.8 and 3.6% GdL induced a visual reversal to the original appearance of the myofibrillar protein within 8 and 2 h respectively. The pH at which this transition was noted was approximately 4.5 for both samples and exudation

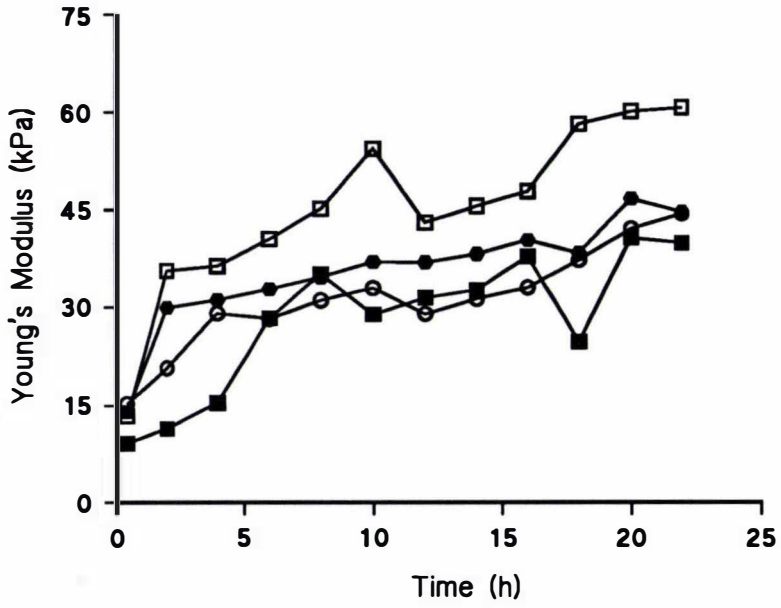


Figure 4.4. Time development of Young's Modulus with addition of 2.3% NaCl. No added GdL (■), 0.9% GdL (o), 1.8% GdL (●), 3.6% GdL (□).

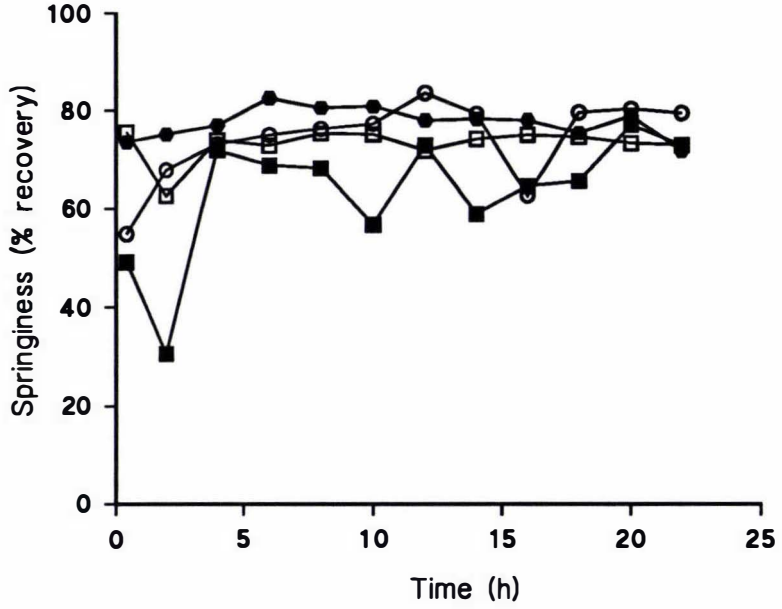


Figure 4.5. Time development of springiness with addition of 2.3% NaCl. No added GdL (■), 0.9% GdL (o), 1.8% GdL (●), 3.6% GdL (□).

occurred on compression at and below this pH. The visual characteristics of the gels are given in Table 4.1. Figure 4.3c illustrates the gel at 3.6% GdL in the presence of 2.3% NaCl. The gel appeared similar to the myofibrils alone, but maintained its shape for at least several days. The level of NaCl was increased in increments up to 20.5% at a concentration of 1.8% GdL. At 2.3% NaCl and greater, a visual change occurred immediately upon NaCl addition to the myofibrillar protein. This change was not evident at 1.4% NaCl and less. Below 2.3% NaCl the gels were pink and visually resembled myofibrillar protein. At 2.3% NaCl and greater the gels were red, glassy and increasingly water exuded from them upon compression. Above 16.4% NaCl, the water holding capacity (WHC) deteriorated so that visible exudation occurred even without compression and at 20.5% NaCl the gels became crumbly. The Young's Modulus rose with increasing concentrations of NaCl to approximately 10.9% where it reached a plateau (Figure 4.6). The springiness declined until 2.3% NaCl, and then increased with increasing NaCl concentration to approximately 5.9% NaCl (Figure 4.7). A sharp decline began at approximately 10.9% NaCl where the gel started to appear crumbly and exuded water upon compression.

4.2.6 The effect of TSPP on gelation

Tetrasodium pyrophosphate (TSPP) was added to the myofibrillar proteins at a concentration of 0.3% and appeared to show slight increases in Young's Modulus at 0 and 0.9% GdL (Figure 4.8) when compared with the Young's Modulus of samples without the added TSPP (Figure 4.1). However, these differences could be accounted for by the variation in sample replicates and, therefore, the differences are not significant. There was no observable difference in Young's Modulus between 1.8% GdL only and 1.8% GdL with TSPP. However, at 3.6% GdL, the Young's Modulus was approximately double that without TSPP. The presence of TSPP resulted in final pH values of 0.2 to 0.3 units greater than in the absence of TSPP. Subjectively, the samples resembled those without the added TSPP. Table 4.1 lists the characteristics of the gels. At 1.8% GdL (final pH of 4.7), the sample with TSPP was similar to the lower GdL concentrations. However, at 3.6% GdL with TSPP the gel was red and glassy after approximately 2 h (pH 4.3). No significant differences were observed in springiness measurements (Figure 4.9) compared to samples without the added TSPP (Figure 4.2).

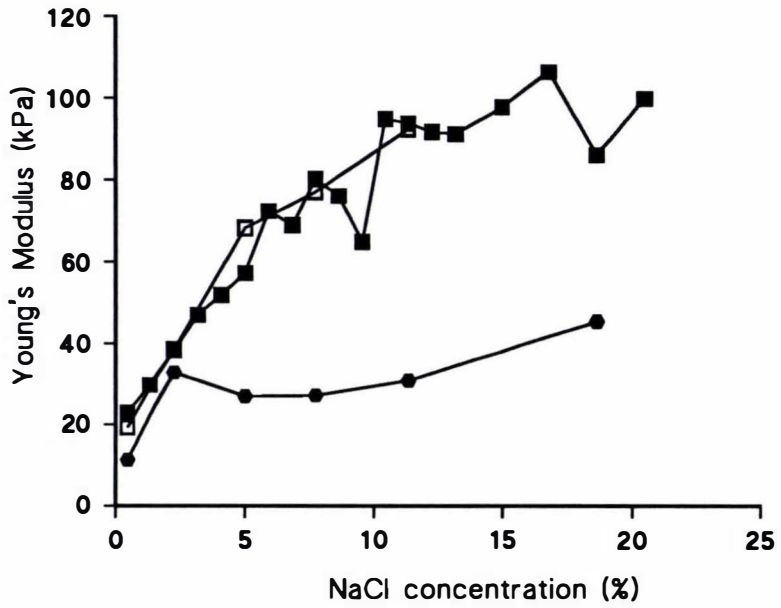


Figure 4.6. Development of Young's Modulus with increasing NaCl concentration. No added GdL (●), 1.8% GdL (■), 0.3% TSPP + 1.8% GdL (□).

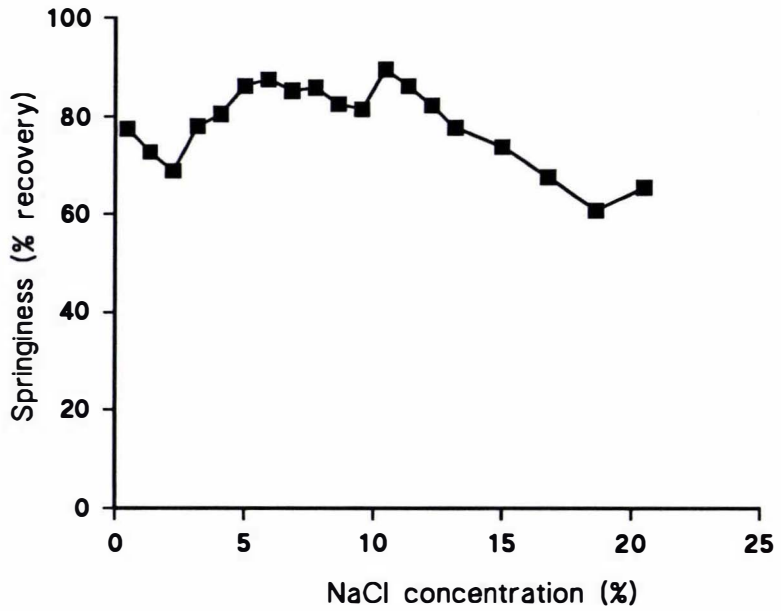


Figure 4.7. Development of springiness with increasing NaCl concentration.

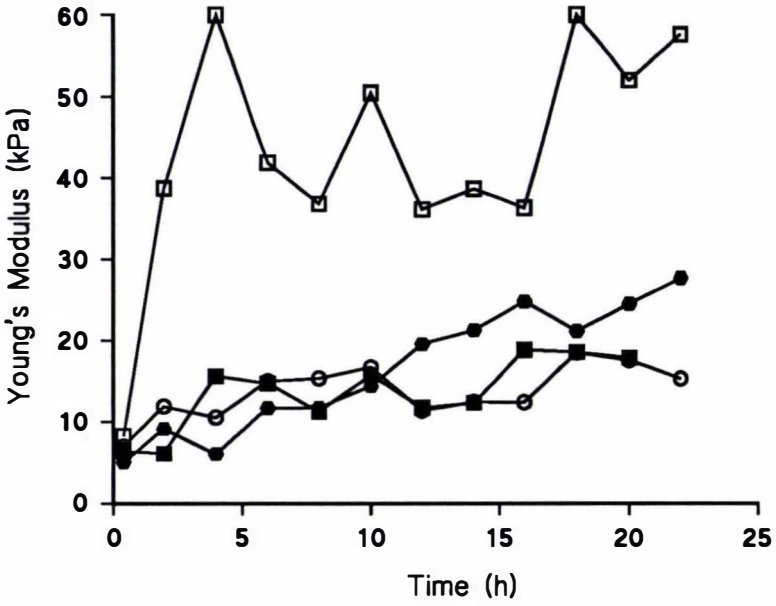


Figure 4.8. Time development of Young's Modulus with addition of 0.3% TSPP. No added GdL (■), 0.9% GdL (o), 1.8% GdL (●), 3.6% GdL (□).

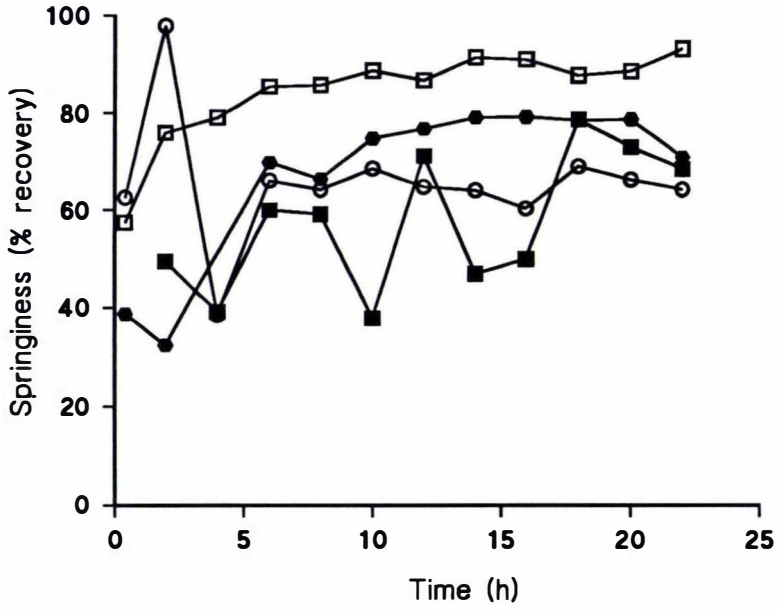


Figure 4.9. Time development of springiness with addition of 0.3% TSPP. No added GdL (■), 0.9% GdL (o), 1.8% GdL (●), 3.6% GdL (□).

Upon addition of increased levels of TSPP (0.5 and 1.1%), in the presence and absence of 1.8% GdL, no significant ($p < 0.05$) differences in the modulus of elasticity or springiness were observed. At these higher levels of TSPP, the final pH values of the samples did notdecrease below 4.5. The greater the concentration of TSPP added, the higher the final pH of the sample.

4.2.7 The effect of addition of NaCl and TSPP

The addition of TSPP with NaCl resulted in a decrease in Young's Modulus at levels of 0 and 1% GdL (Figure 4.10) when compared to gels with NaCl only added (Figure 4.4). This difference could be accounted for by the variance within sample replicates. The Young's Modulus time courses were similar to those without the added TSPP. At all GdL concentrations, Young's Modulus appeared 2 to 3 times greater than in the absence of NaCl and TSPP (compare Figures 4.1 and 4.10). At 0 and 1% GdL, in particular, the springiness was enhanced with the addition of NaCl and TSPP (Figure 4.11) compared to the myofibrils alone or with 1% GdL only (Figure 4.2). There was little notable change at 2% GdL, but at 4% GdL the springiness decreased with the addition of NaCl and TSPP (compare Figures 4.2 and 4.11). At all four concentrations of GdL, the springiness time courses of NaCl addition (Figure 4.5) were not significantly different to the springiness time courses of the samples containing both NaCl and TSPP (Figure 4.11). The visual characteristics of the gels are given in Table 4.1. Visually, the samples were similar to those with NaCl only added at the four given GdL concentrations. The photograph in Figure 4.3d illustrates the crumbly nature of the gel produced at 1.8% GdL in the presence of NaCl and TSPP, even compared to the myofibrils alone (Figure 4.3a) or the sample with 3.6% GdL and NaCl (Figure 4.3c). The final pH values were 0.1 to 0.3 units higher with TSPP present than without.

The Young's Modulus at levels of NaCl from 0 to 12.5% (Figure 4.5) and at 1.8% GdL was not affected by the presence of 0.3% TSPP. At 1.8% GdL and in the presence and absence of 2.3% NaCl, levels of TSPP of 0.6 and 1.2% showed no significant ($p < 0.05$) differences when compared to 0.3% TSPP. At these increased levels of TSPP, the pH of the preparations did not decrease below 5.0.

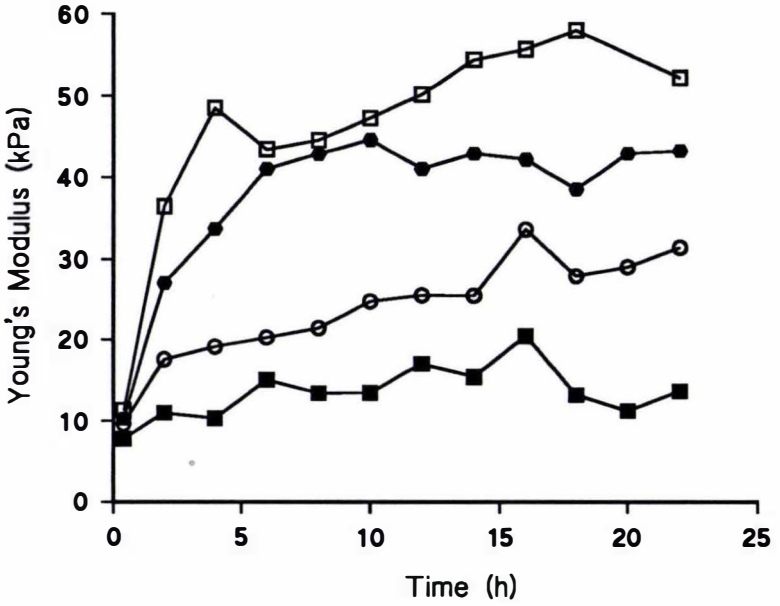


Figure 4.10. Time development of Young's Modulus with addition of 2.3% NaCl and 0.3% TSPP. No added GdL (■), 0.9% GdL (○), 1.8% GdL (●), 3.6% GdL (□).

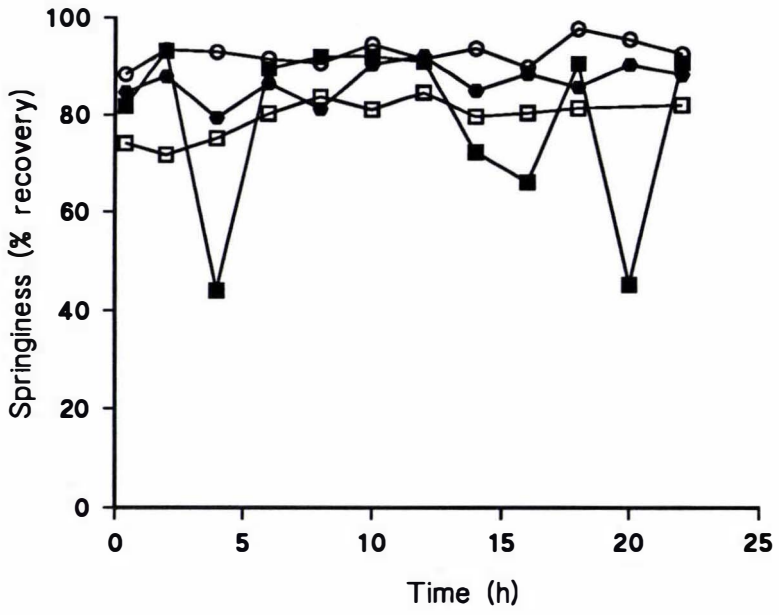


Figure 4.11. Time development of springiness with addition of 2.3% NaCl and 0.3% TSPP. No added GdL (■), 0.9% GdL (○), 1.8% GdL (●), 3.6% GdL (□).

4.2.8 Soluble protein measurements

In some gels it appeared that all of the solution was held within the gel structure and, on centrifuging, no supernatant was obtained. In these cases the soluble protein could not be determined. Although stated as immediate soluble protein measurements, the centrifuging began approximately 10 min after the addition of GdL, NaCl and/or TSPP. The biuret test was not undertaken until 1 h after mixing and therefore the readings were actually as measured 1 h after mixing and not immediately.

Immediate centrifugation:

Results are given in Figure 4.12. The myofibrils alone gave a soluble protein measure which was assumed to be a result of myoglobin when compared with the results of the Drabkin's test. The soluble protein content of the myofibrillar protein alone was subtracted from all other readings and taken as zero. The soluble protein concentration with GdL alone ranged from 3 to 6% of that of total protein. In the presence of TSPP approximately 7% of the total protein was solubilized regardless of the pH of the samples. The results were similar to those obtained with GdL in the absence of TSPP, but with myofibrils alone, TSPP induced about 7% protein solubilization. In the absence of GdL, the presence of NaCl induced approximately 22% protein solubilization which was greater than that of myofibrils alone or in the presence of TSPP. Solubilisation of the protein ranged from 24 to 17% in the presence of GdL and NaCl. In the presence of GdL, the soluble protein concentration in the presence of NaCl and TSPP combined ranged from 22 to 19% at 0 to 1.8% GdL and then decreased to 17% at 3.6% GdL.

Centrifugation after 24 h:

Results are given in Figure 4.13. A large increase in soluble protein content, from approximately 0.5 to 25%, was obtained with the addition of 0.9% GdL. Further increases in GdL concentration did not allow any solution to be obtained. In the presence of NaCl and absence of GdL, no solution could be obtained upon centrifugation. At 0.9% GdL in the presence of NaCl, the soluble protein content was about 11%, but with addition of increasing concentrations of GdL the soluble protein content decreased to 2% at 3.6% GdL. The addition of TSPP gave a soluble protein content of approximately 7% at 0% GdL and 12% at 0.9%

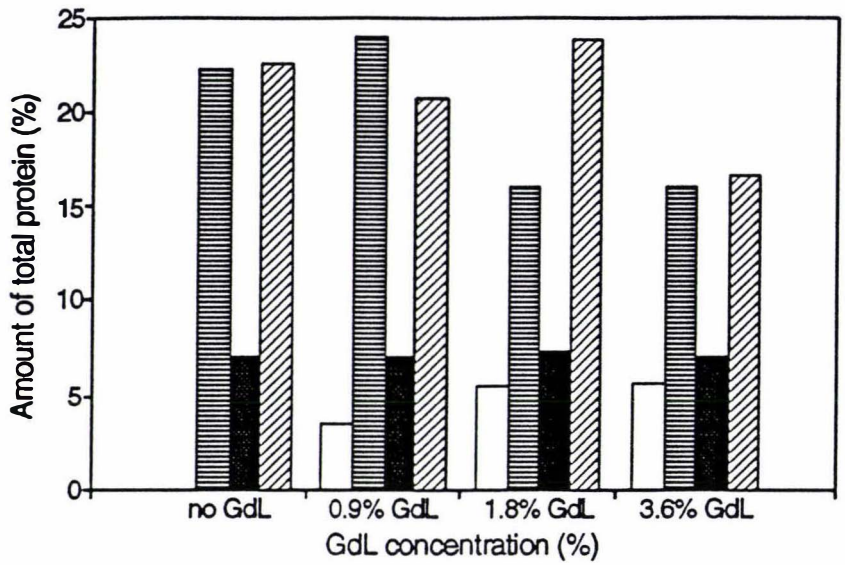


Figure 4.12. Immediate soluble protein content of myofibrillar gels. GdL only (□), 2.3% NaCl (▤), 0.3% TSPP (■), 2.3% NaCl + 0.3% TSPP (▨).

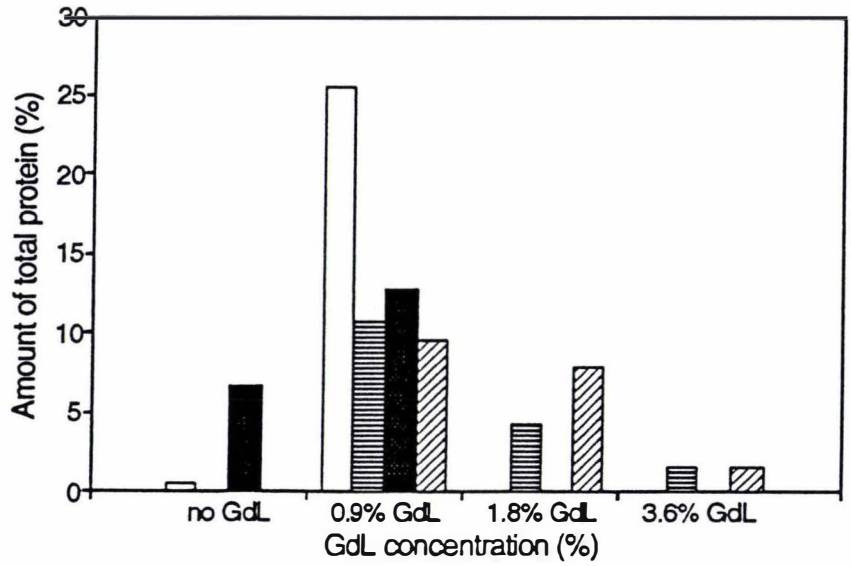


Figure 4.13. Soluble protein content of myofibrillar gels at 24 h. GdL only (□), 2.3% NaCl (▤), 0.3% TSPP (■), 2.3% NaCl + 0.3% TSPP (▨).

GdL. No solution was able to be obtained at 1.8 and 3.6% GdL concentration. The addition of TSPP and NaCl together was similar to NaCl addition at the given GdL levels. No solution could be obtained upon centrifugation in the absence of GdL. The soluble protein content in the presence of NaCl and TSPP was 14% at 0.9% GdL and decreased to 2% at 3.6% GdL.

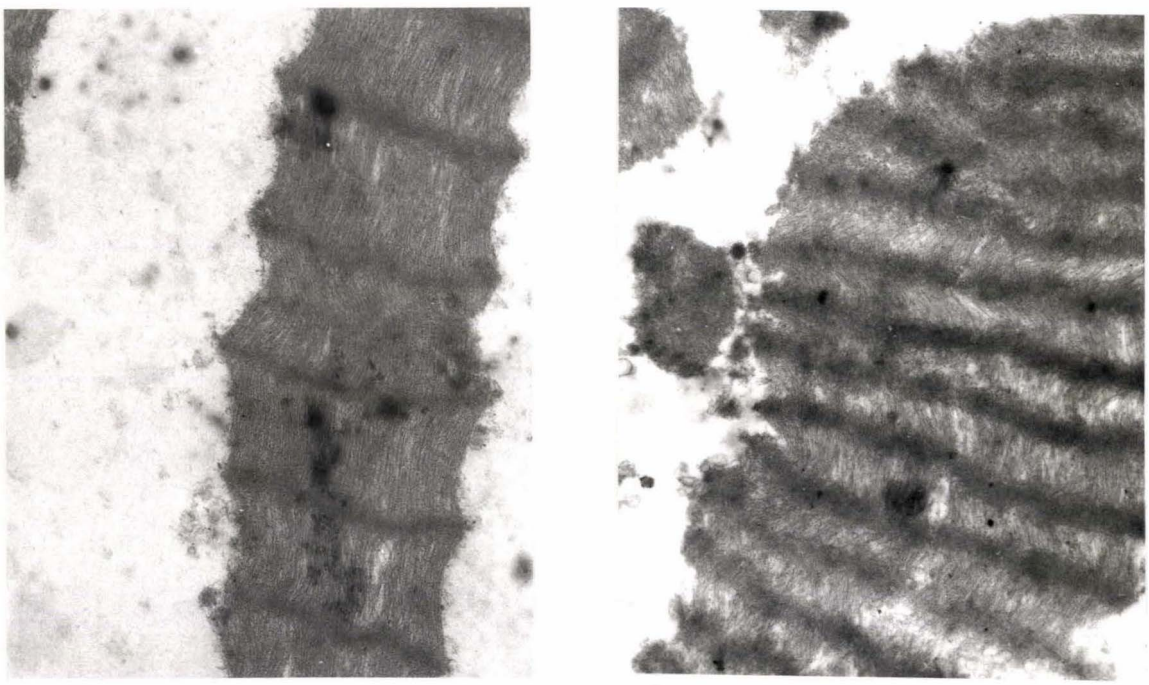
After 24 h, myofibrils alone had a soluble protein content of less than 1% of the total protein, not significantly different to the immediate soluble protein concentration of 0%. In all cases, immediately upon addition of NaCl, the concentration of soluble protein in the gel system increased. However, with time the concentration decreased, except in the absence of GdL where no solution was obtained by centrifugation after 24 h. The addition of TSPP showed little immediate effect on the concentration of soluble protein in the presence of GdL. When added with NaCl, centrifugation immediately and after 24 h gave results which resembled those of NaCl at the four GdL concentrations given.

4.2.9 Transmission electron microscopy (TEM)

Transmission electron microscopy was undertaken on four different gel systems:

- myofibrils only (pH 5.8; Figures 4.14a and b),
- with added 3.6% GdL (pH 3.8; Figures 4.15a to d),
- with added 2.3% NaCl (pH 5.6; Figures 4.16a to c),
- with added 2.3% NaCl and 3.6% GdL (pH 3.8; Figures 4.17a to c).

The gels containing 3.6% GdL only had a granular, amorphous appearance (Figures 4.15a-d) and there was no evidence of the myofibrillar structure (Figure 4.14a-b), except for possibly faint Z-disc lines (Figure 4.15a). The addition of NaCl with and without 3.6% GdL appeared to show little disruption of the myofibrillar structure (Figures 4.16a-c and 4.17a-c, respectively) compared to the myofibrils alone (Figures 4.14a-b). Extraction of the A-band was apparent in the samples containing GdL and NaCl alone. A small area around the Z-disc also appeared to have been extracted in the GdL plus NaCl sample (Figures 4.17a-c).



a b

Figure 4.14. Transmission electron micrographs of myofibrillar protein (pH 5.8). The micrographs are at magnifications of a) 15300x and b) 15300x.

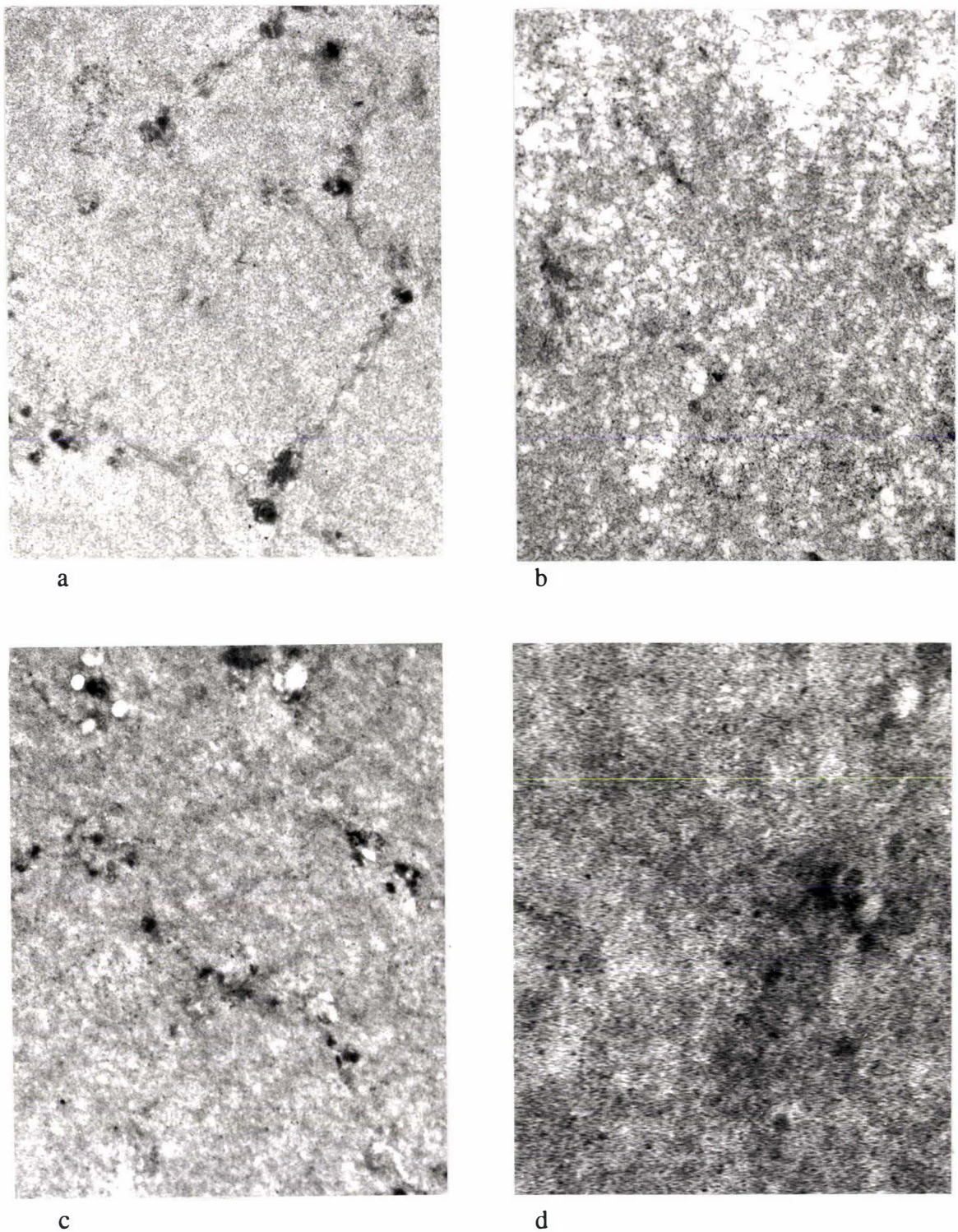
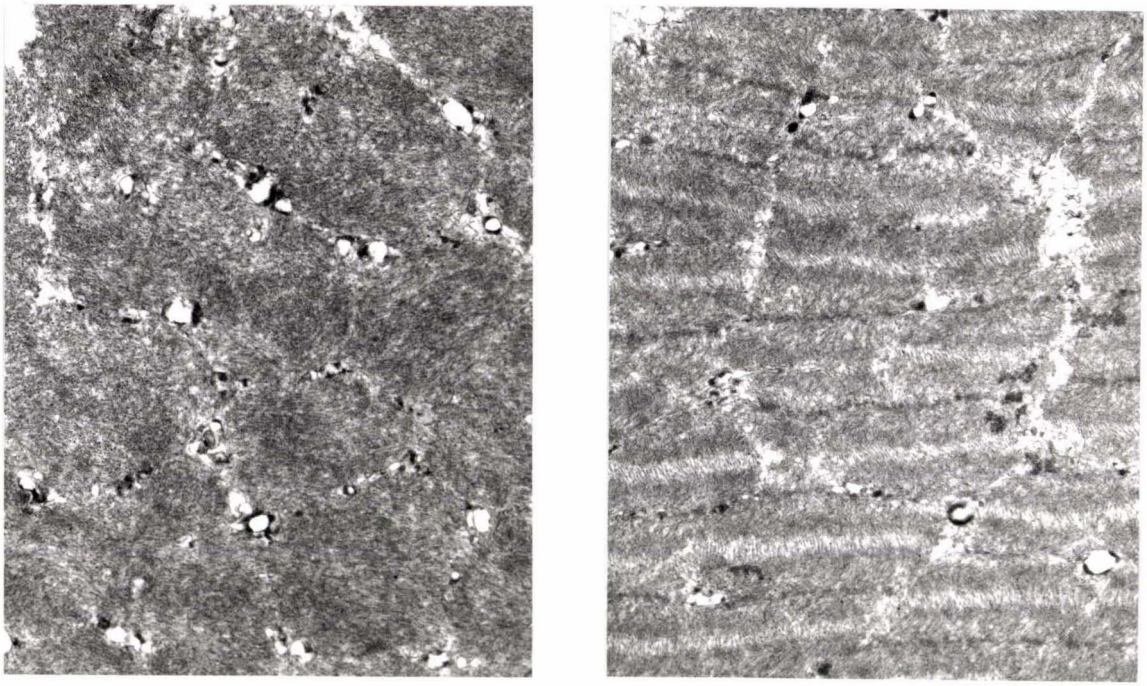
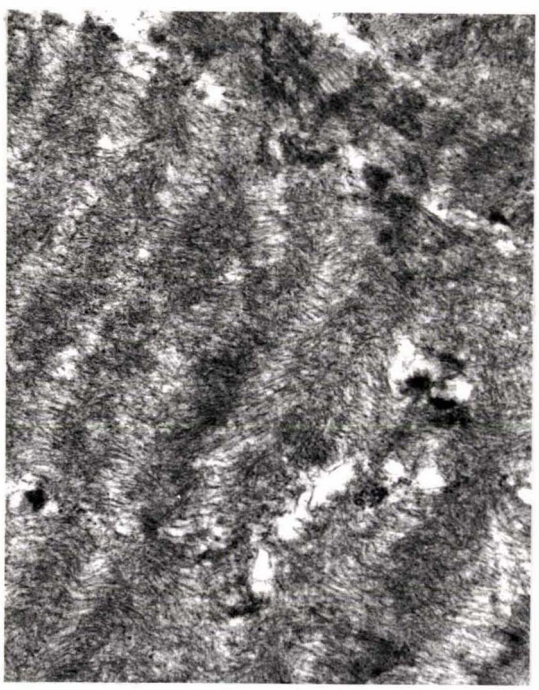


Figure 4.15 Transmission electron micrographs of myofibrillar protein with 3.6% GdL added (pH 3.8). The micrographs are at magnifications of a) 21200x, b) 21200x, c) 21200x, d)103600x.



a b



c

Figure 4.16 Transmission electron micrographs of myofibrillar protein 2.3% NaCl added (pH 5.6). The micrographs are at magnifications of a) 11200x, b)11200x, c) 21200x.

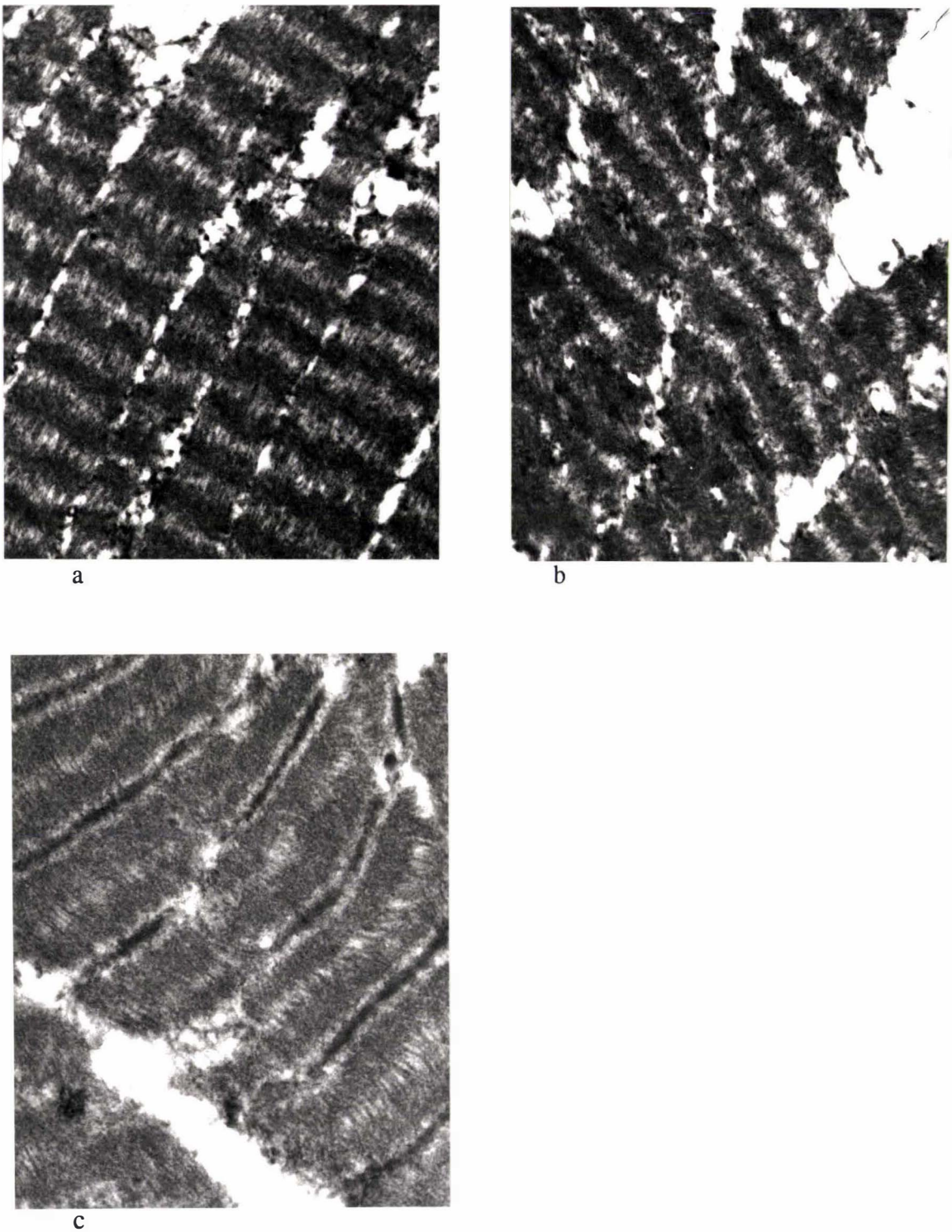


Figure 4.17 Transmission electron micrographs of myofibrillar protein with 2.3% NaCl and 3.6% GdL added (pH 3.8). The micrographs are at magnifications of a) 11200x, b) 11200x, c) 21200x.

4.2.10 Laser diffraction experiments

The diffraction pattern of meat samples in water remained present for at least 10 h. Addition of a 3.6% solution of GdL caused the pattern to disappear within 2 h. The disappearance was a gradual occurrence, the outer lines fading out first. The addition of 0.43 M NaCl (approximately 2.5% NaCl) to the meat sample did not affect the diffraction pattern. The addition of 1.8 and 0.9% GdL solutions resulted in the disappearance of the outer lines within 2.0 h, but the disappearance of the diffraction pattern was not evident until approximately 2.5 h for both concentrations. The pH of the solutions decreased rapidly to 3.6 and then at a slower rate to 2.4, 3.2 and 3.4, at 3.6, 1.8 and 0.9% GdL, respectively. After the diffraction patterns had disappeared, the samples were clear and swollen.

4.3 DISCUSSION

This discussion of results is directed towards:

1. gelation,
2. the phenomenon of events over the gelation time course, namely,
    - the development of Young's Modulus and springiness,
    - the changes in the visual appearance of the proteins,
    - the solubilisation of the protein,
    - the changes in TEM and laser diffraction results.

Section (2) applies to the addition of GdL only. This is followed by:

3. the effects of sodium chloride (NaCl) and tetrasodium pyrophosphate (TSPP).

Section (3) is discussed with regard to similar parameters as Section (2), that is, the Young's Modulus, soluble protein and TEM and laser diffraction results. Finally, a sequence of events is postulated to explain the results presented (Section 4).

4.3.1 Gelation

Fukazawa *et al.* (1961a,b) and Hashimoto *et al.* (1959) showed that myofibrillar proteins, in particular myosin, were primarily responsible for the binding quality in restructured meats and these studies have been substantiated by many researchers (including, Maesso *et al.*, 1970;

Schnell *et al.*, 1970; Vadehra and Baker, 1970; Nakayama and Sato, 1971b; Grabowska and Sikorski, 1976). This research involved thermally-induced gelation, however, it is a reasonable working hypothesis that the myofibrillar proteins, and in particular myosin, are also largely responsible for acid-induced gelation of meat. The studies of Fretheim *et al.* (1985) and Hermansson *et al.* (1986) substantiate this proposal in that the acid-induced gels formed were of myosin. In this study, the addition of GdL to minced meat resulted in the formation of gels within 10 h. However, because of the presence of connective tissue and fat, no useful results were collected from the present experiments using minced meat, as a large variance within sample replicates was obtained. Such complicating factors should be minimised by the use of myofibrillar protein and their method of preparation. Having shown the ability to form gels using a minced meat system, the progression to myofibrillar proteins, in order to gain quantitative data, was essential.

The formation of myofibrillar gels as a result of hydrochloric acid dialysis and the direct addition of hydrochloric acid would suggest that the formation of gels upon hydrolysis of GdL is an acid-induced mechanism and not a GdL- or a gluconic acid-specific interaction with the protein. Further support for this suggestion can be taken from the results of Fretheim *et al.* (1985) and Hermansson *et al.* (1986) who used buffers consisting of combinations of phosphate and citric acid to lower the pH of myosin solutions, via dialysis, resulting in the formation of gels. The slow introduction of the acid allowed a uniform gel to form. In the present experiments, the direct addition of hydrochloric acid to the myofibrillar protein system caused uneven gelation to occur immediately, because high local acid concentration did not allow sufficient time to mix the acid solution evenly into the proteins. This resulted in a mixture of gelled and non-gelled protein, in which the gelled protein was extremely sticky and difficult to manipulate. Increasing levels of GdL (>3.6%) resulted in a faster lowering of the pH due to a greater amount of protons present in solution. As the conditions of pH began to approach those of direct addition of hydrochloric acid, so too did the characteristics of the resultant gels. As the pH became more acidic, the gels became sticky and unmanageable

Glicksman (1982) defined gelation as the association or crosslinking of randomly dispersed polymer chains in a solution to form a three dimensional network which immobilizes the liquid in the interstitial structures and which resists flow against pressure. This is an academic

definition. The experiments in this study were not designed to show whether or not the system had truly gelled and for practical purposes the term "gel" has been used to describe the resulting solid, whether it be a crumbly, slumping mass as was the sample of myofibrils alone, or a sticky and smooth solid as was the myofibril sample with 1.8% GdL added.

4.3.2 The phenomenon of events over the gelation time course

It should be noted that while in this study Young's Modulus has been measured as a parameter of the gel systems produced, it is not an entirely appropriate parameter for characterising gels. As well, there is much subjectivity in estimating the slopes of force displacement graphs in the calculation of Young's Modulus. However, this method of measurement was chosen due to its ease of implementation, because it relates to the molecular structure of the material and because it is one of the major distinguishing parameters in gel systems (Glicksman, 1982). The strain for all Young's Modulus measurements was set at 0.05 to avoid the complexity of compression tests when the strain exceeds 0.05-0.10 (Culioli and Sherman, 1976). It must be noted that the Young's Modulus often did not correlate to the type of gel produced; for example, a high Young's Modulus resulted from compression of a crumbly mass of material, exuding much liquid and also from a cohesive, elastic-like gel. Therefore, Young's Modulus should not be taken as a measure on its own, but in conjunction with springiness and qualitative aspects.

Within several hours of addition of 1.8 and 3.6% GdL to the myofibrillar proteins, gels of appreciable strength had formed (Figure 4.1). At the same time the characteristics of the myofibrillar proteins at these levels had changed from a pink crumbly mass to a red glassy gel. At 3.6% GdL, a possible decline in Young's Modulus was evident after 2 h. However, this decline was not significant and may be accounted for by the variance within sample replicates. This levelling (or apparent decline) occurred as the pH fell below 4.5 to around 4.0 where both the pH and the Young's Modulus levelled off. Springiness time courses showed similar trends to Young's Modulus, but at 3.6% GdL, the springiness levelled at values greater than or equal to the springiness values for 1.8% GdL after 2 h (Figure 4.2). An explanation for gel formation at pH 4.5 to 4.0 can be taken from the work of Rao *et al.* (1989b) who investigated changes in the ultrastructure of beef muscle as influenced by acidic

conditions. Rao *et al.* (1989b) observed that there was evidence of partial extraction of myosin filaments at pH 4.48 which had not been apparent at pH 5.10 and greater. This suggested that the proton concentration at pH 4.48 caused a sufficient increase in the net positive charge of the M-line proteins to render them completely soluble. Depolymerisation of the myosin filaments was also likely at pH 4.48. When the pH was decreased to 4.3, the H-zones were completely lost which indicated complete depolymerisation of actomyosin and dispersion of myosin filaments (Rao *et al.*, 1989b).

From the assumption made earlier, that myosin is responsible for gel formation, it is reasonable to assume that gelation could not occur until some of the myosin in the myofibrils had been extracted. In the work of Rao *et al.* (1989b), this extraction was not evident until a pH value between 5.10 and 4.48 had been reached, and therefore, gelation in the present system was not expected until the pH was lowered to values less than 5.10. The partial extraction of myosin evidenced at pH 4.48 could lead to the formation of a myosin network about the myofibrillar structure resulting in an impregnated composite system. The myosin gel would reinforce the existing myofibrillar structure. In the present experiments, at pH 4.3 and less, complete depolymerisation of actomyosin and dispersion of myosin filaments indicated destruction of the myofibrillar network. Any resultant network could be a myosin network possibly incorporating other components, such as actin and water, into the structure.

The existence of myosin network formation at pH below the isoelectric point (IEP) is supported by Fretheim *et al.* (1985) and Hermansson *et al.* (1986). The addition of acid to a myosin system would be expected to protonate negatively charged carboxyl groups possibly breaking some of the electrostatic interactions with protonated amine groups. Fretheim *et al.* (1985) suggested that a "dynamic" gel network formed with the addition of acid. A maximum rigidity at about pH 4.5 was observed and it was suggested that as pH decreased from 4.5 to 4.0, increased intermolecular repulsion had a deleterious effect on gel strength. This suggestion, like the suggestion that the myofibrillar structure is broken down, could explain the decrease in Young's Modulus at 3.6% GdL in the present experiments. It must be noted that both the work of Fretheim *et al.* (1985) and Hermansson *et al.* (1986) differed from the present work, not only in that these workers used myosin, not myofibrillar proteins, but that the myosin used contained 0.6 M potassium chloride, at least prior to use. However, Fretheim

et al. (1985) did show that myosin formed gels at 0.1 M potassium chloride, although they were weaker gels at this lower ionic strength.

In order for gelation to occur, Ferry's gelation theory states that denaturation must have taken place (Ferry, 1948). Hamm (1960) suggested that muscle merely swelled with lowering of the pH to 4.0. At pH 4.0 and less, denaturation of the muscle occurred. Rao *et al.* (1989b) also observed swelling of the myofibrils in muscle as the pH was lowered. However, in addition, Rao *et al.* (1989b) observed extraction of the A-band proteins (M-line) and eventually the H-zone. Extraction of the A-band proteins was not observed by Hamm (1960) who did not report the use of TEM. Hamm's suggestion (Hamm 1960) is therefore superceded by the TEM observations of Rao *et al.* (1989b) which allowed a more detailed view of Hamm's vague "denaturation". The preferred sequence of events explaining the effect of acid on meat is then based on the work of Rao *et al.* (1989b). One could postulate that the denaturation at pH 4.0 reported by Hamm (1960) was the complete depolymerisation of actomyosin and the dispersion of the filaments observed by Rao *et al.* (1989b) at pH 4.0. Denaturation of the partially depolymerised myosin and therefore myosin extracted from the myofibrillar structure, at pH values of 4.5 and less may have occurred upon extraction. With this denaturation, gelation would then have been able to occur. The DSC experiments undertaken by Fretheim *et al.* (1985) indicated that a conformational change, that is denaturation, had taken place at pH 5.0 with prolonged exposure of the myosin to this pH (40 h). Within 16 h at pH 5.5 (acidic, but not below the IEP), a reduction in the apparent enthalpy of heat denaturation of the protein was noted.

Xiong and Brekke (1989) indicated that myofibrils will not form a gel unless some soluble protein is present prior to heating and Samejima *et al.* (1985) observed a positive linear correlation between protein solubility and heat developed gel strength in bovine myofibrils. In the present work, solubility experiments indicated an immediate increase in the percent of protein in solution at all levels of GdL (Figure 4.12). After 24 h the soluble protein content had increased at 0.9% GdL to five times the level measured immediately. Fretheim *et al.* (1985) also noted that gels retained considerable amounts of protein in the liquid phase at a final pH of greater than or about 5.0. The final pH of the sample at 0.9% GdL was 4.8. Therefore, the presence of soluble protein at a pH value close to the maximum would be

expected. At 1.8 and 3.6% GdL in the present experiments, no liquid could be obtained by centrifugation which suggested that all the solution present was incorporated into the gel structure or associated with the protein and indicated increased WHC at these higher levels of GdL. The visual characteristics corroborate this suggested increase in WHC. Because proteins are generally least soluble at their IEP, it was expected that decreasing the pH to values below the IEP would increase the content of soluble protein assuming no gelation or protein-protein interaction occurred. This could explain the increased solubility at 0.9% GdL after 24 h (Figures 4.12 and 4.13). Increased soluble protein could be an indication of extraction, probably partial extraction initially, of myosin as observed by Rao *et al.* (1989b). Extraction did not occur until pH 4.48 in the work of Rao *et al.* (1989b). In the present experiments, the final pH at 0.9% GdL was 4.8 indicating significant extraction of protein could have occurred, thereby increasing the soluble protein content. The fact that no solution could be obtained at 1.8 and 3.6% GdL provides further support for the suggestion of gel formation, whereby the soluble protein was incorporated into the gel structure and water was bound to protein or held in the interstices. Extraction of the protein at 0.9% GdL did not result in increased Young's Modulus possibly because denaturation may have been slow to occur, that is, it may have taken longer than 20 h. It could also be that there is a critical concentration of soluble protein required to form a gel network, similar to the crosslinker theory of Flory (1941, 1953), and this concentration had not been attained.

In terms of WHC, Offer and Trinick (1983) found that studies of filament lattice dimensions as a function of pH approximately paralleled changes in WHC as a function of pH. Water uptake was minimal at the IEP of myofibrils and increased on either side. Hamm (1960) found that a majority of the water held within the myofibrils of meat was found in the spaces between thick and thin filaments. This interfilamental spacing was not constant and varied with pH, sarcomere length, ionic strength, osmotic pressure and whether the muscle was relaxed or in rigor (Rome 1967, 1968, 1972; Elliot, 1968; April *et al.*, 1972; Matsubara and Elliot, 1972; Goldman *et al.*, 1979; Millman and Nickel, 1980; Millman *et al.*, 1981; Millman, 1981). Three-fold changes in volume were reported by varying these parameters. It is suggested that the lowering of the pH below the IEP caused the myofibrillar proteins to swell in the present experiments. This could be so at conditions where there is partial extraction of the myosin, but the general myofibrillar structure is retained (at pH greater than 4.0).

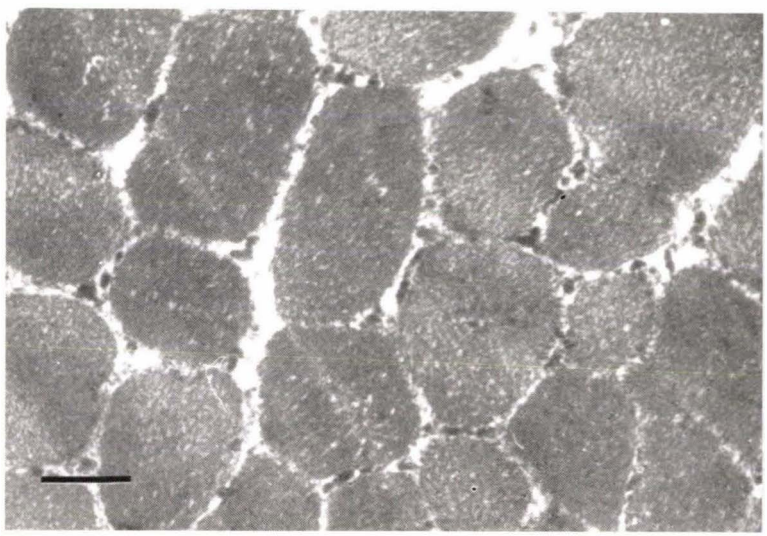
There was no indication of increased WHC or swelling at 0.9% GdL, but this may have been a result of not lowering the pH enough. This phenomenon is analogous to that of acid marinading, such as in the work of Rao *et al.* (1989a) who studied acetic acid marinading and found swelling increased along and across the muscle fibre axis between pH 5.1 and 4.4. Rao *et al.* (1989b) stated that the likely extent of swelling observed at pH 4.48 was related primarily to the osmotic pressure generated by the presence of a highly charged and concentrated dispersion of myofibrillar proteins.

Offer and Trinick (1983) concluded in general terms that water uptake by myofibrils at varying pH occurs by expansion of the filament lattice brought about by an increased electrostatic repulsive force and/or a diminution in one or more of the transverse structural constraints in the myofibril. Constraints of myofibrillar proteins to swelling include the sarcolemma around the fibre, Z- and M-lines (Matsubara and Elliot, 1972, Millman and Nickel, 1980) and the crossbridges of actin and myosin (Goldman *et al.*, 1979; Millman, 1981). Wilding *et al.* (1986) have shown that if the endomysial sheath surrounding a fibre is damaged at one point, much more swelling takes place there and they concluded that the endomysium acts as a mechanical restraint to swelling. In the present experiments, the use of myofibrillar proteins largely removes the constraints of connective tissue and therefore should allow for greater swelling.

Using the laser diffraction pattern to observe the presence of the entire sarcomere of myofibrils in a whole meat system it was observed that the diffraction pattern disappeared 2.5 h after addition of GdL, and in less than an hour with prior removal of the sarcoplasmic protein by washing with potassium chloride. The loss of the diffraction pattern could be a result of destruction of the myofibril structure or due to a lack of resolution of the structure as a result of swelling of the myofibrils as would be expected by incorporation of all solution into the structure as observed by Rao *et al.* (1989a,b) and Offer and Trinick (1983) using light and electron microscopy.

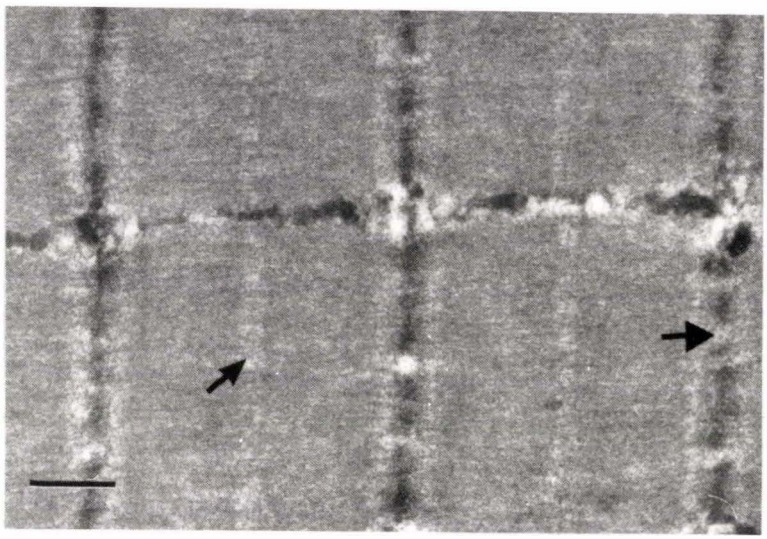
The laser diffraction experiments are a crude method, used in these experiments to determine the loss of sarcomere pattern. Analysis by TEM is better able to identify structural changes to the proteins. When gel samples of 3.6% GdL were viewed under TEM an amorphous

a

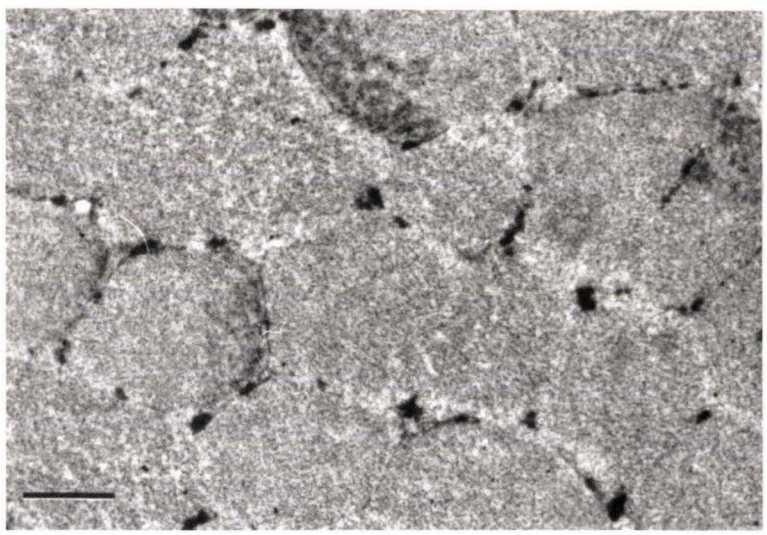


b

Figure 4.18 Transmission electron micrographs of raw muscle (pH 5.54). The letters on the micrographs signify, A: A-band; I: I-band; S: sarcomere length. The bars represent a) 0.5 μm, b) 1.0 μm. With permission from Rao *et al.* (1989b).

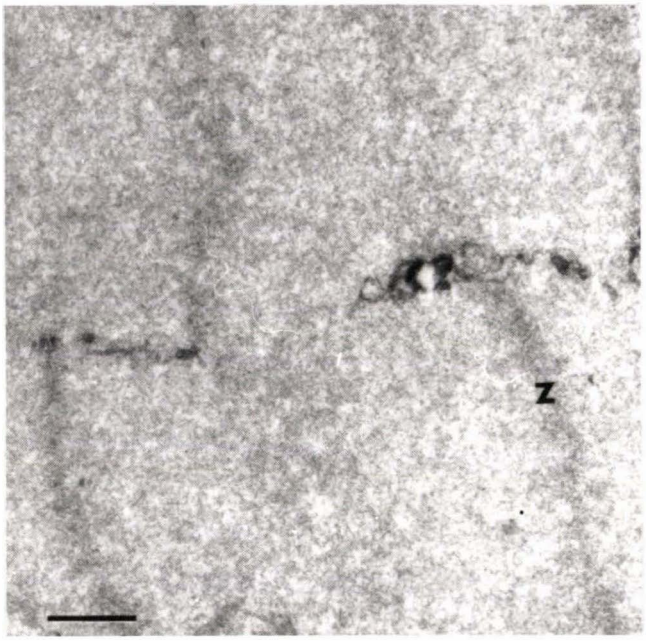


a

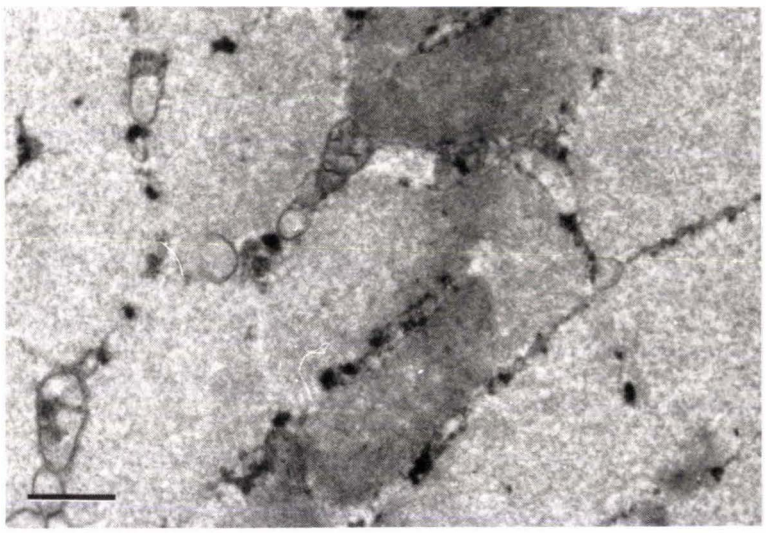


b

Figure 4.19 Transmission electron micrographs of acidified raw muscle (pH 4.48). The symbols on the micrographs signify, small arrow: H zones; big arrow: z lines. The bars represent a) 0.5 μm, b) 1.0 μm. With permission from Rao *et al.* (1989b).



a



b

Figure 4.20 Transmission electron micrographs of acidified raw muscle (pH 3.92). The symbols on the micrographs signify, z: z line. The bars represent a) 0.5 μm, b) 1.0 μm. With permission from Rao *et al.* (1989b).

structure was observed. This microscopy indicated either a total loss of regular structure of the myofibrils to a fine network or a mass of actomyosin, myosin, actin and their fragments, or extensive swelling of the myofibrillar proteins compared to the myofibrillar proteins without any additives (Figures 4.14a and 4.14b at pH 5.8, and 4.15a to 4.15d at pH 3.8-4.0). Comparison of these figures with the structures shown in the TEM studies of Rao *et al.* (1989b) on acetic acid marinading of beef muscle (Figures 4.18a and 4.18b of control, Figures 4.19a and 4.19b at pH 4.48, and Figures 4.20a and 4.20b at pH 3.92) suggests that the conditions produced by the addition of 3.6% GdL (pH 3.9) induced destruction of the myofibrillar structure similar to acid marinading. Similar to the observations in the present study, Rao *et al.* (1989b) noted that at pH 4.3, increased myofibrillar swelling led to adjacent myofibrils fusing together while A- and I-bands lost all structural detail and had a granular appearance, indicating complete depolymerisation of actomyosin and dispersion of myosin and actin filaments. Only Z-lines retained structural organisation. Similarly, Z-lines were also apparent in Figure 4.15a of the present study. Rao *et al.* (1989b) noted that the ultrastructural changes brought about by the acidification of these samples over the pH range 4.5 to 3.9 clearly indicated the severe disruptive nature of low pH on the myofibrillar components of meat giving an overall amorphous appearance.

The micrographs obtained in the present experiments using TEM must be viewed with caution. The use of phosphates in the preparative procedures could alter the conformation of the proteins and any network formed as well as the pH of the protein system and therefore the electrostatic charges which contribute to the structure of the gels formed. Any inadvertent change in tissue pH would undoubtedly alter the structure and behaviour of tissue proteins (Hayat, 1970). Hermansson *et al.* (1986) found that 2.5% gluteraldehyde distorted the structure of the gels. Also, subsequent slicing of the gels could distort their structure and it is possible that the sample is not representative of the overall gel. However, Clark and Ross Murphy (1987) stated that for denatured protein gels, a good case had been made for the reliability of TEM images, established by careful comparative studies (Clark and Lee-Tuffnell, 1986) involving the application of several different microscopy approaches, and other techniques, such as wide angle and small angle x-ray scattering.

4.3.3 Effects of NaCl and TSPP

4.3.3.1 The effect of NaCl

Generally, in the manufacture of meat products, 2% NaCl is used (Offer and Trinick, 1983) for the reasons of product binding, flavour and preservation (Sofos, 1986b). One of the functions of NaCl in meat products is to extract myofibrillar proteins. Extraction of rigor meat with salt in the absence of magnesium polyphosphate, is not selective for myosin and substantial amounts of actin and other myofibrillar proteins are also present in the extract (Grabowska and Hamm, 1978; Foegeding, 1987). Extraction and solubilization of these proteins contributes to meat particle binding, fat emulsification and WHC and thus reduces cook losses and improves quality and texture (Schmidt *et al.*, 1981; Acton *et al.*, 1983). Myofibrils are able to swell to at least twice their original volume in salt conditions widely used in the meat industry via capillarity (Offer and Trinick, 1983). Most of the water retained is held in the interfilamental spaces, but a substantial amount is held in the spaces between myofibrils. The concentration of NaCl required for maximum swelling is 0.8 M NaCl (4.3% NaCl), but is reduced to 0.4 M (2.2% NaCl) in the presence of pyrophosphate. At 0.4 M NaCl, only a small amount of swelling occurs. The maximum amount of swelling is not necessarily affected by the presence of phosphate and the maximum is attained when a substantial amount of the A-band is extracted. By comparison with water uptake at varying pH, Offer and Trinick (1983) concluded that water uptake by myofibrils at high salt concentration also occurs by expansion of the filament lattice as a result of increased electrostatic repulsive force and/or a diminution in one or more of the constraining forces, probably crossbridges, the M-line or the Z-line. Both effects required binding of ions to the myofibrillar protein. In the experiments presented here, 2.3% NaCl corresponds to approximately 0.43 M NaCl and would therefore not be expected to induce much swelling of the protein on its own (pH 5.6 to 5.8).

Hamm and Grau (1958) found that sodium acetate, the anion of which is hardly bound by muscle proteins, did not increase the hydration of muscle in the basic range of the IEP if the concentration was not too high. It was therefore assumed that the chloride ion and not the sodium ion of NaCl bound to myofibrils to cause swelling in the basic region of the IEP. If

a substantial number of chloride ions were bound to filaments, reinforcement of water holding properties resulted due to repulsion of filaments and subsequent swelling. Hermansson *et al.* (1986) noted that at a critical salt concentration, the myosin would be solubilised and new interactions could take place. This critical concentration would depend on other parameters, such as pH.

In the present experiments, the inclusion of 2.3% NaCl in the myofibrillar system increased the Young's Modulus at all four GdL concentrations (Figure 4.4) compared to the samples without NaCl (Figure 4.1). Similar to the results without added NaCl, at 0.9% GdL plus 2.3% NaCl the Young's Modulus could not be distinguished from the sample with NaCl, but no added GdL. However, at these lower GdL concentrations (0 and 0.9%) the Young's Modulus was 4 to 5 times greater in the presence of NaCl than in its absence. This may be explained by the suggestion that proteins extracted from the myofibrils in the presence of NaCl may be denatured at pH values of 5.5 and less and form a gelation network reinforcing the myofibrillar structure already present. At these lower GdL concentrations the Young's Modulus is comparable to the Young's Modulus with 1.8% GdL in the absence of NaCl (Figure 4.1) where similar gel formation was hypothesized. At 1.8 and 3.6% GdL, the Young's Modulus was further increased in the presence of NaCl. At pH values less than 4.5, the acidic conditions may have extracted protein additional to that extracted with NaCl and provided stronger denaturing conditions which induce faster and a greater amount of denaturation. Therefore, the conditions would have allowed more gelation to occur than at the higher pH values. The myofibrillar structure was retained at pH 4.0 in the presence of NaCl. This retention of structure is suggested to be a result of the lowered isoelectric point (IEP) of myofibrillar protein in the presence of NaCl which would require a lower pH to be attained in order to result in loss of the myofibrillar structure compared to in the absence of NaCl. The increased extracted protein could provide a stronger network further reinforcing this myofibrillar structure.

Binding of an anion to the protein reduces the IEP of the protein. At the molarity given (0.43 M NaCl), potassium chloride reduced the IEP of myosin to approximately 4.2 (Sarkar, 1950). Myofibrillar proteins may not be affected to the same extent, but a change as small as 0.3 pH units could be the difference between an impregnated composite system (where a myosin

network reinforces the myofibrillar structure), and a system of complete depolymerisation of actomyosin and dispersion of filaments. The reduced IEP could account for the retention of the myofibrillar structure at pH 3.8 (Figure 4.17a to 4.17c) compared to the loss of structure at pH 4.0 in the absence of NaCl (Figures 4.15a to 4.15d). The retention of the myofibrilllar structure suggests the constraints of the myofibrillar structure are largely intact at 2.3% NaCl in the absence and presence of GdL. The persistence of the myofibrillar structure as the pH is decreased and the corresponding increase in Young's Modulus at these pH values provides evidence for the formation of the impregnated composite system. The formation of a network reinforcing the already present myofibrillar structure would result in increased Young's Modulus while retaining the structure of the myofibrils.

Measures of springiness were undertaken with the Young's modulus measurements to further define the nature of the gels formed. The increased springiness at 0 and 0.9% GdL with the inclusion of NaCl (Figure 4.5) compared to springiness observed in the absence of NaCl (Figure 4.2) provides further support for network formation at these lower GdL concentrations. The decrease at 3.6% GdL compared to lower GdL values (Figure 4.5) could indicate a more tightly bound network. It is suggested that with the increasing interactions and crosslinks, the springiness increased. As more interactions and crosslinks occurred, the flexibility of the gel became restricted and the gel became less springy and more rigid or brittle. This transition to a more rigid gel is not reflected in the Young's Modulus which either increased or levelled with increasing rigidity or brittleness of the gel. This is an example of a situation where springiness and visual characteristics are required to more fully define the nature of the gel.

In the present work, the inclusion of 2.3% NaCl to the myofibrillar system resulted in changes to the visual appearance of the gels. In the presence of 2.3% NaCl, the changes ranged from a red glassy gel at 0 and 0.9% GdL to a pink crumbly mass, exuding much liquid upon compression, at 1.8 and 3.6% GdL within 2 h. Upon addition of NaCl and 1.8 or 3.6% GdL, the gels were initially red and glassy in appearance. Visually WHC showed trends as expected according to Hamm (1975) where NaCl increased the WHC at pH greater than the IEP, but decreased it at pH less than the IEP. These results were suggested to be due predominantly to the chloride ion of NaCl, which caused a weakening of the interaction between oppositely charged groups at greater pH and strengthening of interactions at lower

pH values. On the acidic side of the IEP of muscle, the electrostatic repulsion between positively charged groups of protein (in this case γ-globulin, but mentioned in general terms) is reduced by the binding of anions as a result of the decrease of protein net positive charge (Cann and Phelps, 1955). The result of this reaction is a tightening of the protein structure and a decrease in WHC. Decreased WHC was observed in the present experiments investigating soluble protein content, whereby solution was unable to be obtained by centrifugation at 1.8 and 3.6% GdL, but with the inclusion of salt, at these GdL concentrations liquid was obtained.

The immediate concentration of soluble protein in the presence of NaCl ranged from 22 to 24% of the total protein at 0 and 0.9% GdL, and 16% at 1.8 and 3.6% GdL. After 24 h no solution could be obtained by centrifugation in the presence of NaCl at 0% GdL. At 0.9% GdL, the protein content was 11% and decreased to 2% at 3.6% GdL. The addition of NaCl resulted in the immediate extraction and solubilisation of protein from the myofibrillar structure. It is proposed that with prolonged exposure to pH 5.5, the proteins denatured (Fretheim *et al.*, 1985) and gel formation occurred. As the pH was lowered and the denaturing conditions increased, the amount of protein denatured and the rate of denaturation were increased resulting in a greater amount of protein participating in network formation and therefore less soluble protein present. Solubilisation and dissociation of proteins increase the number of molecular interactions initiated by denaturation resulting in the formation of a firmer gel (Siegel and Schmidt, 1979b). The increased WHC near the IEP (Hamm, 1975) could account for the inability to obtain liquid upon centrifugation after 24 h of addition of NaCl with and without GdL.

Turning to the results of the experiments in the presence and absence of 1.8% GdL and increasing NaCl (Figures 4.6 and 4.7), it was noted that Offer and Trinick (1983) reported that high concentrations of NaCl often caused dissociation of an assembly of protein molecules because the ions effectively competed for electrostatic interactions. As stated earlier, solubilization and dissociation increase the number of interactions initiated by denaturation resulting in the formation of a firmer gel (Siegel and Schmidt, 1979b). The Young's Modulus levelled at 40 kPa at approximately 2% NaCl and with no added GdL. In the presence of 1.8% GdL (final pH of 4.5), the Young's Modulus increased up to a concentration of about

11% NaCl. The gel became noticeably crumbly and exuded liquid at 22% NaCl. Here is another instance where the caveat about interpreting Young's Modulus is evident. Note that while there was a reduction in the springiness and decrease in the WHC visually, the Young's Modulus began to plateau, providing no indication of a change in character of the gel. An increase in the interactions of protein would have effected the characteristics of the gel in terms of springiness. It is proposed that as the network became more tightly bound, the gel acquired more brittle or rigid characteristics and became progressively less springy. This occurred at about 11% NaCl. At the levels of NaCl of 22% and greater, the phenomenon of salting out or an excess of repulsive interactions could explain the deterioration of gel character and WHC. Salting out is defined as the mutual lowering of solubility which takes place when an electrolyte and an organic substance are dissolved together in water (Simpson and Weiner, 1989).

4.3.3.2. The effect of TSPP

Phosphates are generally used in meat products to enhance WHC and improve binding and therefore cook yield (Ellinger, 1972; Mahon *et al.*, 1971; Shimp, 1983b; Steinhauer, 1983). Polyphosphate can also prevent discolouration of meat, improve texture and prevent development of off-flavours and odours by inhibiting rancidity (Ellinger, 1972; Shimp, 1983a,b; Steinhauer, 1983), reduce refrigeration weep and increase protein extraction (Sofos, 1986b). These effects are often attributed to increased and controlled pH by the phosphates acting as buffers, and to increased ionic strength. Phosphate ions can act as polyanions in solution and in general, phosphates can interact with polyelectrolytic organic compounds such as proteins (Halliday, 1978). Phosphates increase the ionic strength of solutions and can bridge two or more positively charged sites, and thus, bind components or particles, which can lead to precipitation (Steinhauer, 1983). By attaching one end of their chain to a positively charged site and the other to water molecules, phosphates can also maintain particles in solution (Wagner, 1986). Potential drawbacks of phosphates on meat product texture (for example, tough, rubbery texture), flavour (such as metallic, astringent, soapy taste) and possible detrimental health effects (short term abdominal distress, bone calcium mobilization) can be avoided through optimization of levels and conditions of use (Karmas, 1970; Ellinger, 1972; Raines Bell *et al.*, 1977; Trout and Schmidt, 1983). Tripolyphosphate (TPP) is

commonly used because it is readily soluble in water. However, it is thought to hydrolyse to pyrophosphate in the meat and pyrophosphate is thought to be the effective agent (Hamm and Neraal, 1977).

The improvement on the binding property of sausages by various phosphates has been shown by Fukazawa *et al.* (1961c). The mechanisms by which phosphates bring about the improvement were assigned to:

1)the quantity of extracted protein by addition of the phosphate (in the case of the denatured state of the myofibrillar protein)

2)the increase in the concentration of "light" components rather than the quantity of extracted protein in the presence of 0.6 M NaCl (provided myofibrillar proteins are not denatured) and

3)the decrease in the viscosity due to the dissociation of soluble actomyosin.

Light components are taken as being myosin and the dissociable components of natural actomyosin.

In the present experiments the inclusion of TSPP resulted in no significant difference in Young's Modulus, except at 3.6% GdL where there was a noticeable increase in the Young's Modulus (Figure 4.8) compared to the Young's Modulus in the absence of TSPP (Figure 4.1). The immediate soluble protein content was similar to that in the absence of TSPP at 0.9 to 3.6 % GdL (Figures 4.12). In the absence of GdL, the added TSPP induced approximately 7% protein to solubilize immediately and this protein was still present with centrifugation after 24 h. (Figure 4.13). This increase could possibly be explained by the rapid increase in pH from 5.8 to 6.1 with TSPP addition. As the pH moves away from its IEP, myosin becomes more soluble. This could also explain the difference in soluble protein content after 24 h between 0.9% GdL at a final pH of 4.8 (25% soluble protein) and 0.9% GdL with added TSPP at a final pH of 5.1 (12% soluble protein content). At 1.8 and 3.6% GdL with TSPP added, no solution could be obtained by centrifugation, similar to the experiments in the absence of TSPP.

The concentration of TSPP (0.3%) was perhaps too low to be able to extract the myosin from the myofibrillar network, however, at 1.1% TSPP there was still no significant difference in

the results when compared to 0.3% TSPP at 0 and 1.8% GdL. The presence of magnesium is required for pyrophosphate to act as a dissociating agent of actomyosin (Samejima *et al.*, 1985). The ionic strength of the TSPP in solution, if it is assumed that all the TSPP is dissociated, is approximately 0.05 at 0.3% TSPP which would not have any significant effect on the myofibrillar structure. In light of these facts, it was therefore expected that TSPP alone would have no significant effect on the characteristics of the myofibrils in the absence of GdL. The increase in the Young's Modulus at pH 4.1 (3.6% GdL) could be a result of the depolymerisation of the actomyosin and dispersion of the filaments due to the lowering of the pH as in the absence of TSPP. This could enable the TSPP to interact with the myosin freed from actomyosin. The increased interactions at 3.6% GdL provided by the presence of TSPP, compared to GdL alone could account for the increased Young's Modulus at this pH.

4.3.3.3 The effect of TSPP and NaCl

Theno *et al.* (1978), Samejima *et al.* (1985) and Moore *et al.* (1976) among others, have reported a synergistic effect on binding strength and WHC in meat products with the addition of salt and phosphate. This synergistic effect was not apparent in these experiments. Instead, a decrease in Young's Modulus was observed with TSPP and NaCl alone (Figure 4.10) when compared to gels of NaCl addition (Figure 4.4). There were no significant differences in other curves. At 0 and 0.9% GdL the springiness was greater in the presence of TSPP and NaCl (Figure 4.11) compared to NaCl at these GdL concentrations (Figure 4.5). The similarity of Young's Modulus, springiness and visual characteristics between NaCl and NaCl plus TSPP at all four GdL concentrations suggests that the pH and NaCl effects dominate. Therefore, even at 3.6% GdL, where TSPP alone had a significant effect on the Young's Modulus, TSPP had no significant effect in the presence of NaCl. It should be noted that Young's Modulus was dependent on time and GdL concentration regardless of the addition of NaCl and/or TSPP.

4.3.4 A postulated sequence of events.

A series of events is postulated to describe the response of myofibrillar proteins to decreases in pH induced by the addition of GdL. As the pH decreased to 4.5, swelling of the protein

occured. Extraction of the A-band occurred at a pH between 4.8 and 4.5 and a gel network formed. At this pH the gel was an impregnated composite system of a myosin network formed about the residual myofibrillar structure. A critical amount of extracted protein was required to form this gel. Complete extraction of the A-band occurred as actomyosin was depolymerised and filaments were dispersed at a pH value between 4.5 and 3.9 and at values lower than this pH. The gel structure was no longer a reinforced composite system, but was of myosin which incorporated components, for example water and actin, into its structure. At a pH of 5.5 and less, extracted myosin was denatured with time allowing for new interactions to occur and gel formation to take place. Decreasing pH provided stronger denaturing conditions and therefore a faster rate of gel formation.

The addition of 2.3% NaCl to myofibrillar proteins plus GdL resulted in the formation of a composite system at pH values greater than 4.5 as a result of extraction of A-band proteins. The myofibril structure was retained to lower pH values than in the absence of salt because of the lowered IEP. The resultant gel had greater Young's Modulus at pH 4.0 due to the composite system in the NaCl containing gel compared to the myosin system formed at pH 4.0 with myofibrils and GdL only. With the inclusion of NaCl, swelling did occur. As the NaCl concentration increased, the number of interactions also increased with more extracted protein and stronger denaturing conditions. Therefore, the gelation network became tighter and at a critical level salting out occurred. With a tighter network, liquid was forced out of the system and therefore the WHC was decreased. The gel also became less springy and more rigid.

The inclusion of TSPP had no effect on the measured characteristics of the GdL-induced myofibrillar protein gels until the myofibrillar system had completely broken down. The pH and NaCl effects dominated the NaCl, GdL and TSPP system and no significant effect as a result of TSPP addition was observed.

This proposed series of events of the formation of the gel network of myofibrillar proteins is based upon the assumption that myosin is responsible for the network formation at these low pH values. It has been proposed that the myofibrillar structure contributes to the characteristics of the impregnated composite gel and that the myosin forms a gel about this

myofibrillar structure. Studies of the ability of myosin to gel at these low pH values could provide information pertaining to the assumption that myosin is largely responsible for the gel formation. It is possible that another protein, such as actomyosin, is acting as a crosslinker or perhaps it is the actomyosin that is forming gels. Investigations of myosin gel formation would also determine the importance of the myofibrillar structure in terms of the gel characteristics studied. Further evidence for myosin denaturation and gel formation would aid in an understanding of the mechanism of gel formation and therefore aid in formulation of a series of events to explain the results obtained.

CHAPTER 5

GdL-INDUCED GELATION OF MYOSIN

5.1 INTRODUCTION

In the previous chapter it was postulated that myofibrillar proteins formed gels when the pH was lowered slowly with the addition of gluconco-δ-lactone (GdL). Extraction of the A-band occurred at a pH between 4.8 and 4.5 and a gel network was suggested to have formed. It was proposed that the gel was an impregnated composite system of myosin reinforcing the already present myofibrillar structure. Since previous workers (for example, Fukazawa *et al.*, 1961a,b; Hashimoto *et al.*, 1959; Maesso *et al.*, 1970) have shown that myosin is the primary protein involved in heat-induced gelation of meat proteins, a study of myosin gelation could reveal a clear picture, eliminating the confounding effects of the other myofibrillar proteins.

The aims of this chapter are to:

- use rheological data to verify the assumption that myosin forms gels at these low pH values,
- investigate the conformational changes in myosin in order to provide evidence of protein denaturation and to study the effect of lowered pH on myosin gelation,
- determine if the solids formed are true gels or merely entanglement networks,
- investigate the effects of NaCl and TSPP on network formation of myosin, and
- investigate the types of bonds involved in the network formation and, therefore, the nature of the network.

Finally, a series of events is postulated to describe the results presented and relate these results to the events postulated to explain GdL-induced gelation of myofibrillar proteins.

5.2 RESULTS

5.2.1 Purity of the myosin preparation

The purity of the myosin gel preparations was determined by electrophoresis and densitometry to be 85 to 95% with an average of 91%. This purity assumes the correct designation of the light chain bands as was shown in Chapter Three (Figure 3.1).

5.2.2 The effect of addition of glucono-δ-lactone (GdL)

5.2.2.1 The rheological effect of addition of GdL to myosin

A summary of the results obtained from the investigation of gel formation as followed by the Bohlin rheometer is given in Table 5.1. An explanation of the parameters used is given in the discussion of this chapter (Section 5.3.2).

Reading from Table 5.1, addition of 0.30% GdL, for example, resulted in a maximum G' of 58 Pa at 14 ks, a relatively weak G'. After attaining this maximum, the G' slowly decreased. The phase angle, δ, ranged from 5 to 7° and G" ranged from 4 to 5 Pa. These results were obtained from a small amplitude oscillatory test which measured G', G", δ and dynamic viscosity with respect to time at a frequency, ω, of 1 Hz and a maximum strain amplitude of 2%. An oscillatory test at varied frequency resulted in a constant G' with frequency up to 1 Hz, but dropped off at 5 Hz indicating that at frequencies up to 1 Hz, the oscillation data was reliable in terms of this parameter. The strain sweep resulted in a gradual decline throughout, indicating that the myosin system was very liquid-like. The resultant gel was a white suspension similar to myosin alone. Further summaries are given for the addition of 0.70% GdL and 1.40% GdL addition to the myosin system and for myosin alone.

The G' results plotted against pH are shown in Figures 5.1 and 5.2. In Figure 5.1 the G' showed a maximum at pH 4.3 to 4.5 with an abrupt decline at lower pH values. There appeared to be two groups of results; one which increased to about 200 Pa, and the second

Table 5.1 Results obtained from the rheological study of GdL-induced myosin gels.

Gel	G' (Pa)	δ (°)	G" (Pa)	ω (Hz)	γ (m)	s	age (days)	Subjective Qualities
myosin	approx 13 plateau	3 to 25	1 to 3	-	smooth 0.0457	-		white, suspension
0.30% GdL	58 at 14 ks max, slow dec	5 to 7	4 to 5	1 lin. 5 drop	gradual decline	0.07		white, suspension
0.70% GdL	210 at 7.2 ks max, 49 at 48 ks dec	inc at 10 ks to 9	as for δ to 8	-	-	-	1	turbid, viscous
0.70% GdL	460 at 14 ks max, 195 at 46 ks dec	0 at 14 ks inc to 9	0.6 at 14 ks inc to 30	-	abrupt 0.0258	-	7	turbid, viscous
0.70% GdL	690 at 14 ks max, 214 at 48 ks dec	inc at 27 ks to 7	as for δ to 25	1 lin. 5 rise	abrupt 0.0252	0.14	8	turbid, viscous
0.70% GdL	227 at 3.6 ks max, 10 at 48 ks	4 at 5.4 ks inc to 27 at 48 ks	1.5 to 20	-	grad 0.0463	-	4	
1.40% GdL	173 at 1.8 ks max, then out of range of sensitivity	up to 35 min at 1.8 ks	-	-	-	-		clear, solution

Terms associated with Table 5.1:
0.30%, 0.70%, 1.40% GdL = concentrations of GdL used
plateau = curve reached a plateau at the specified time
grad = gradual incline/decline
min/max = curve reached a minimum/maximum at the specified time
inc/dec for G' curve = curve was increasing/decreasing at the final G' observed
inc/dec for δ and G" curves = increased or decreased at/to the specified time
lin. = linear
s = slope of a (log G') versus (log ω) plot
age = days of storage at 4° before myosin was used
clear = translucent

which had a maximum at about 460 to 700 Pa. The minimum G' of this second group of data was approximately 200 Pa.

An increase in GdL to 1.40% resulted in a curve similar to that of the lower group of data at 0.70% GdL (Figure 5.2). The pH at maximum G' was not significantly different to that at 0.70% GdL. With the increased concentration of GdL, the pH decreased to values below 4.0, where the system became liquid-like and translucent. At the final pH attained, the myosin behaved like a low viscosity liquid and the torque sensing element was unable to detect fluid

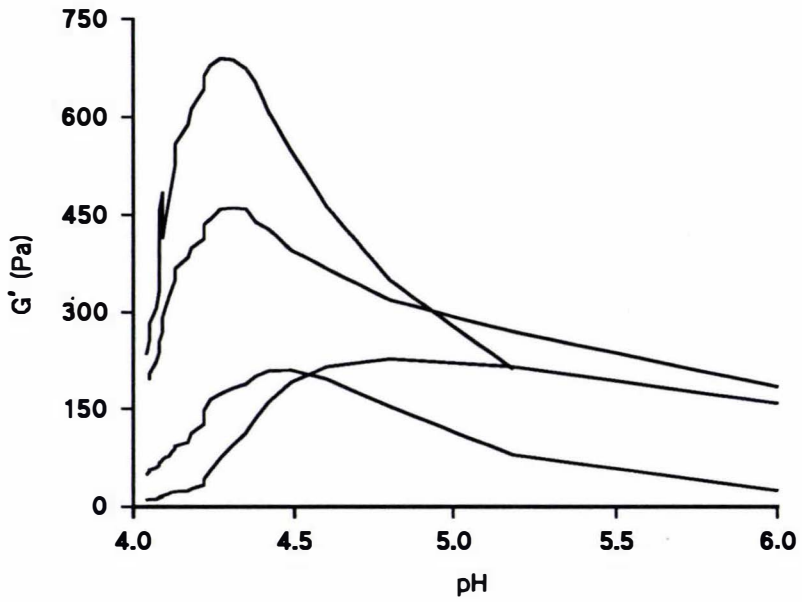


Figure 5.1. The development of the G' of myosin gels at 0.70% GdL.

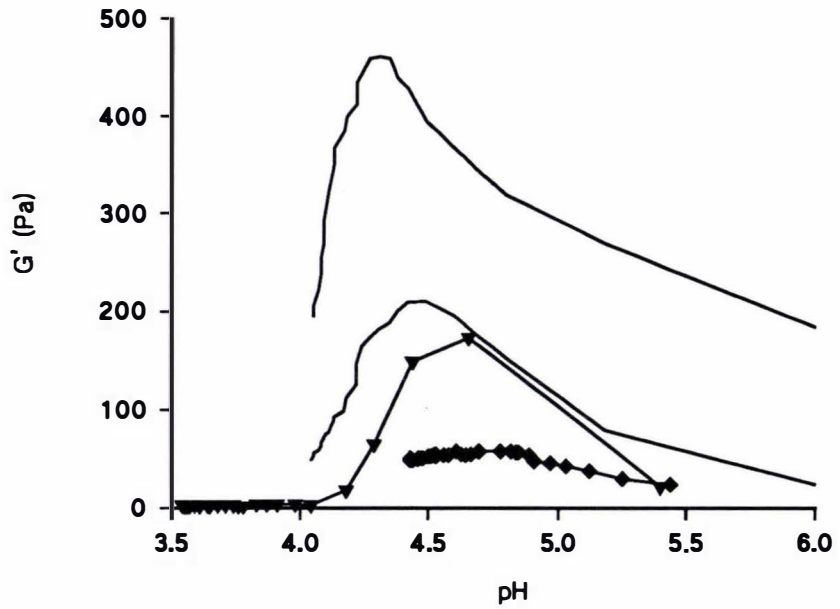


Figure 5.2. The development of G' of myosin gels at varied GdL concentrations. 0.30% GdL (◆), 0.70% GdL (–), 1.40% GdL (▼).

resistance to shear above the instrument noise. At 0.30% GdL, the G' was 54 Pa at its maximum between pH 4.5 and 4.8 (Figure 5.2). The pH did not decrease below 4.3. Although the maximum G' attained was less than that at the higher GdL concentrations, it was about four times greater than the G' of myosin alone. Corresponding minima in G" and δ values at G' maxima values were apparent at all GdL concentrations, followed by increases as the G' declined.

The strain results in Table 5.1 indicated that myosin alone and with 0.30% GdL had more liquid or viscous character than with the addition of 0.70% GdL, except for one of the sample repeats which showed a gradual G' decline with increasing strain, similar to myosin alone. The s result obtained indicated that myosin with 0.70% GdL addition had possibly formed an entanglement network. At the pH at which the s value was calculated, which is the final pH obtained, the myosin gel which had formed and reached a peak at about pH 4.3, had deteriorated in terms of G' (Figure 5.1) and was liquid-like. The strain sweep data and s values were a reflection of this liquid-like stage and not the more solid-like stage of maximum G' at the higher pH.

5.2.2.2 The effect of GdL addition on fluorescence

The addition of ANS to protein does not result in absolute fluorescence values. The fluorescence is a result of ANS interaction with hydrophobic sites on the protein molecules. There is no standard to compare the resultant data to and therefore data is compared from experiment to experiment. Hence, the data is not absolute data, but relative and the trends only can be noted, not the actual amount of denaturation.

The relative fluorescence of the myosin solution with 0.025% GdL added increased to pH 3.8 where it appeared to plateau to the minimum pH attained, 3.5 (Figure 5.3). Plotted against time, the fluorescence of myosin with 0.025% GdL increased parallel to the pH decrease of the sample (Figure 5.4). There was little variation between sample replicates. At concentrations varying from 0.013% to 0.125% GdL, fluorescence decreased with increasing GdL concentration at a given pH (Figure 5.5).

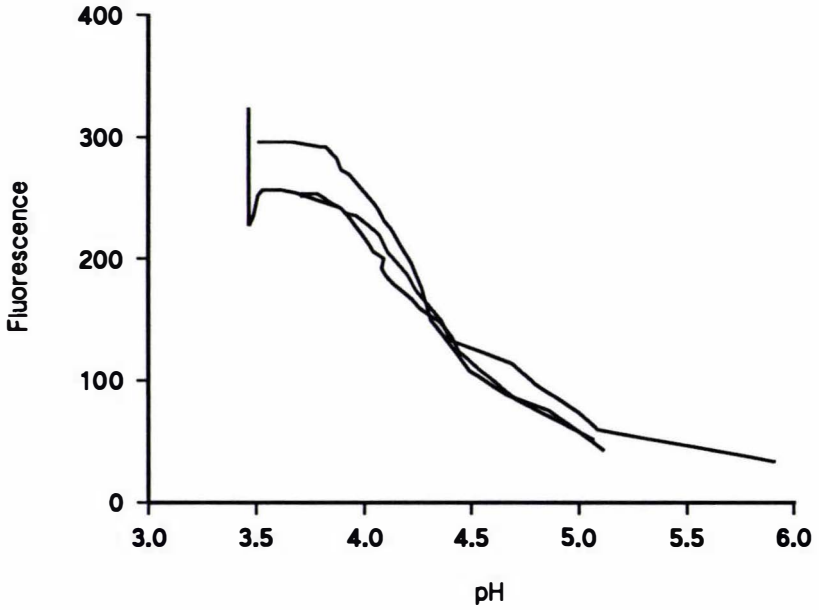


Figure 5.3. Fluorescence development of myosin solutions at 0.025% GdL.

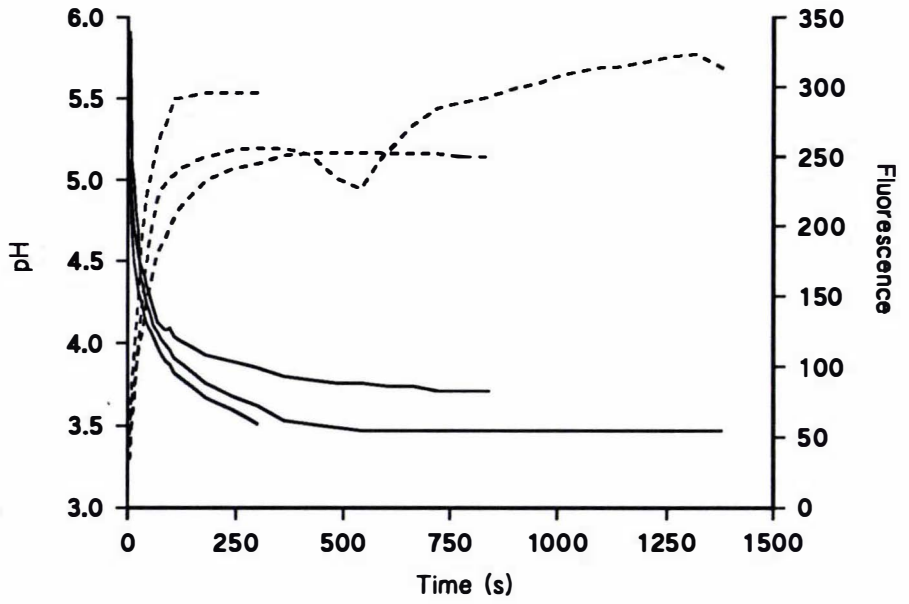


Figure 5.4. Fluorescence and pH development of myosin solutions at 0.025% GdL. pH (—), fluorescence (···).

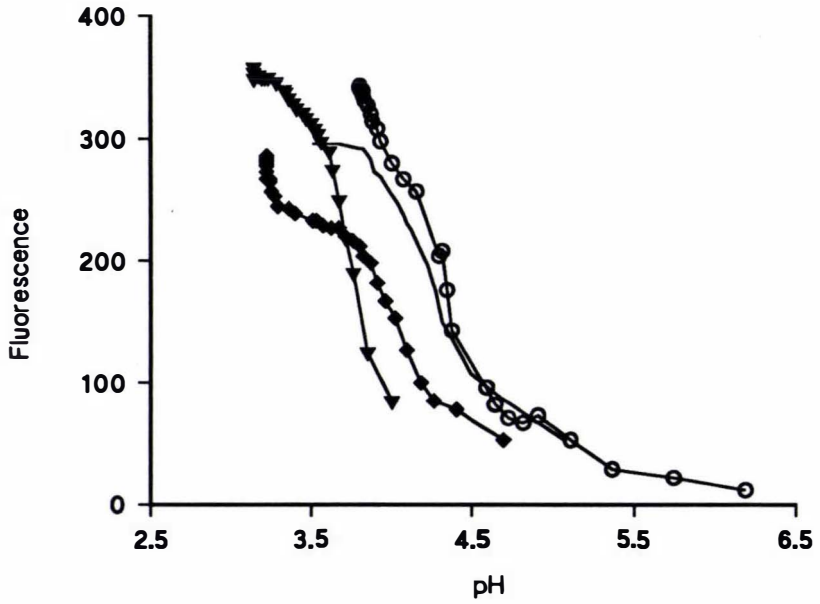


Figure 5.5. Fluorescence development of myosin solutions at varied GdL concentrations. 0.013% GdL (o), 0.025% GdL (–), 0.050% GdL (◆), 0.125% GdL (▼).

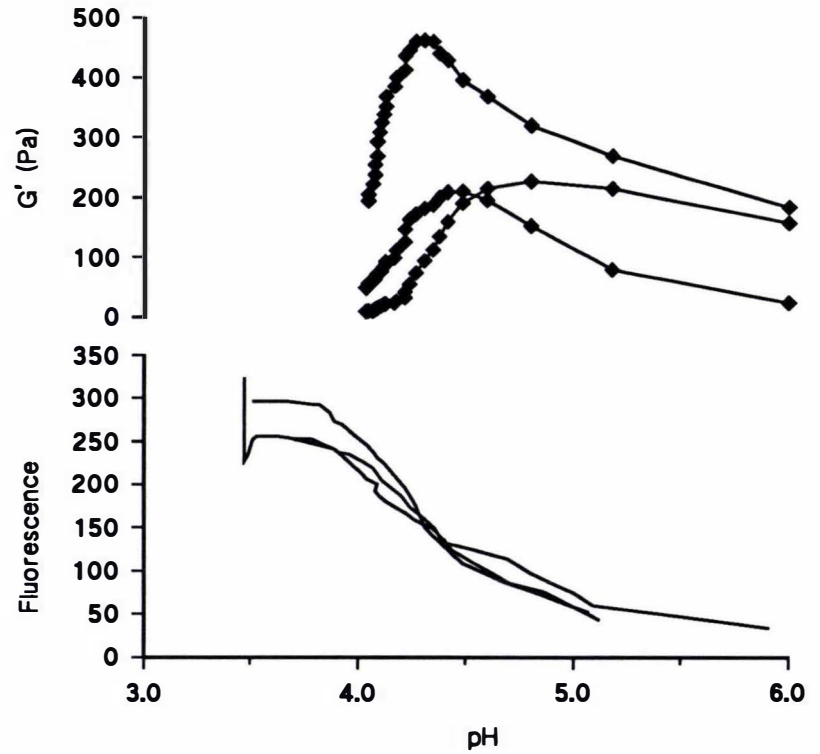


Figure 5.6. Comparison of rheological and fluorescence data. Fluorescence data of myosin solutions at 0.025% GdL (–), G' data at 0.70% GdL (◆).

When graphed together, the fluorescence at 0.025% GdL and the G' at 0.70% appeared to increase down to pH about 4.5. Thereafter, the fluorescence continued to increase, but the G' resulted in an abrupt decline down to a final pH of 4.0 (Figure 5.6).

5.2.2.3 Microscopy of GdL-induced myosin gels

Light microscopy was carried out using Nomarski differential interference contrast (NDIC). Nothing of significance could be drawn from this work. Micrographs of myosin at 100x magnification with and without added GdL are presented in Appendix One (Figures A1.1 and A1.2, respectively).

The TEM studies of myosin alone are shown in Figures 5.7a-d (pH 6.8). Similar micrographs of myosin with added GdL (pH 4.3) are given in Figures 5.8a-c and appeared to have a denser structure. It was difficult to obtain good definition at 15300x and 48000x magnification in the presence of GdL compared to myosin alone. The appearance of groups or aggregates of strands of myosin, perhaps filaments, in the myosin alone was not evident at pH 4.3, but may have been present in the densely packed structure observed.

5.2.3 The effect of addition of sodium chloride

5.2.3.1 The effect of sodium chloride on the rheological characteristics of myosin gels

A summary of the results obtained from the investigations of gel formation as monitored on a Bohlin rheometer is given in Table 5.2.

A concentration of 0.25% sodium chloride (NaCl) included in the myosin suspension showed characteristics of the resultant suspension similar to that of myosin alone (Table 5.1). Concentrations of 2.50 and 3.75% NaCl resulted in a translucent solution of myosin which behaved primarily as a low viscosity liquid and the torque sensing element was unable to detect fluid resistance to shear above instrument noise.

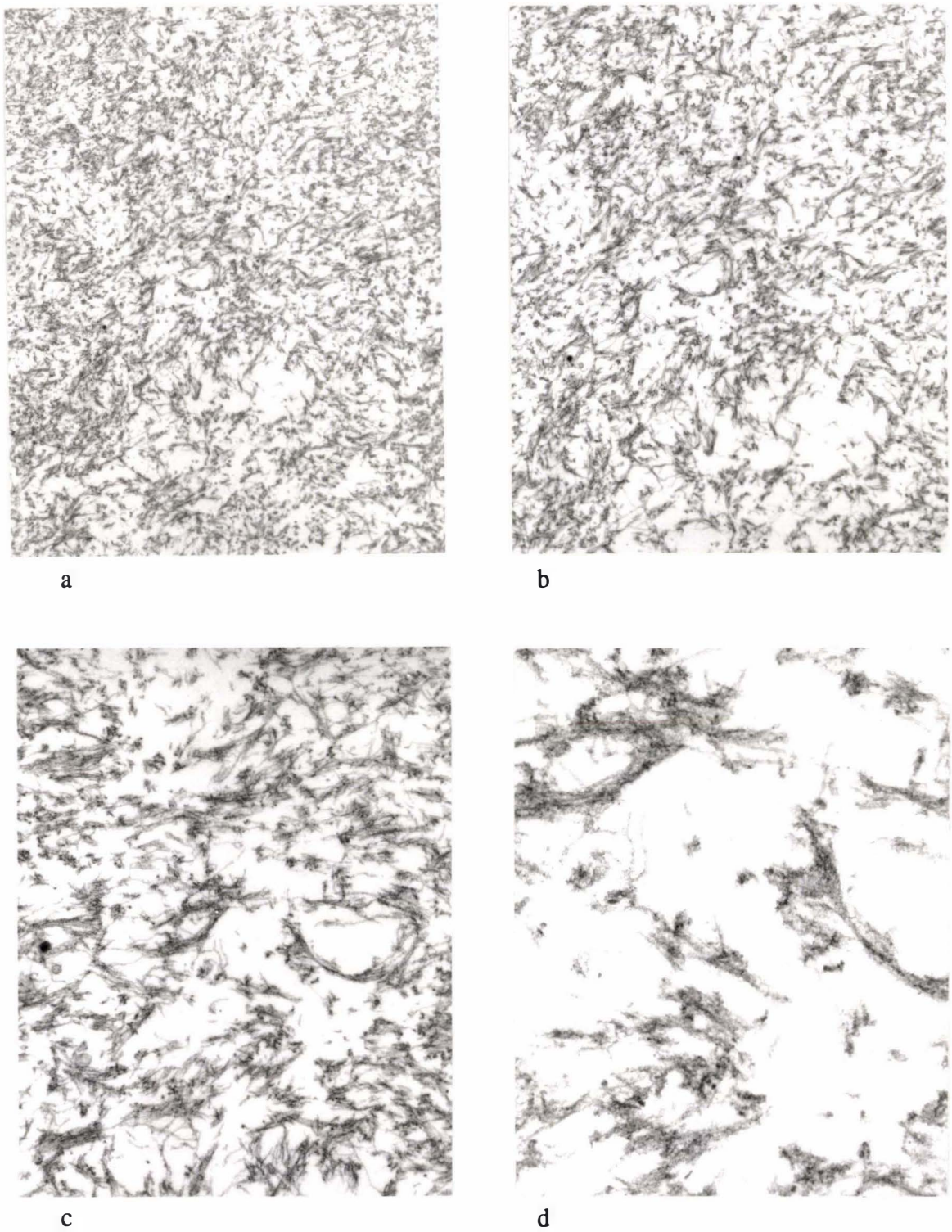
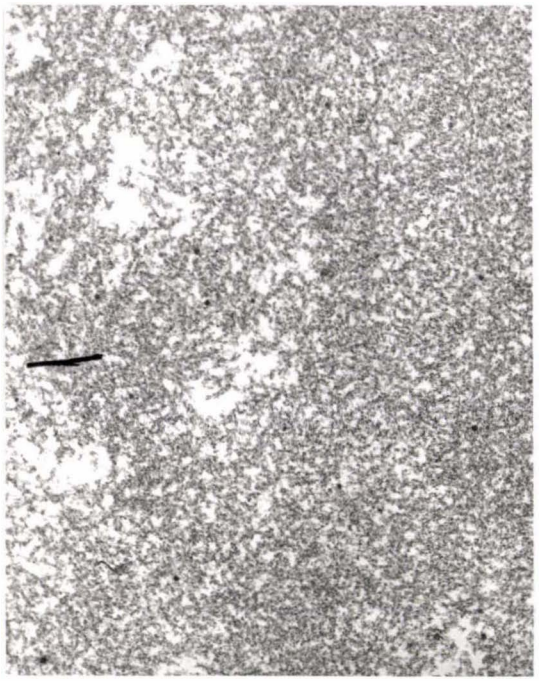
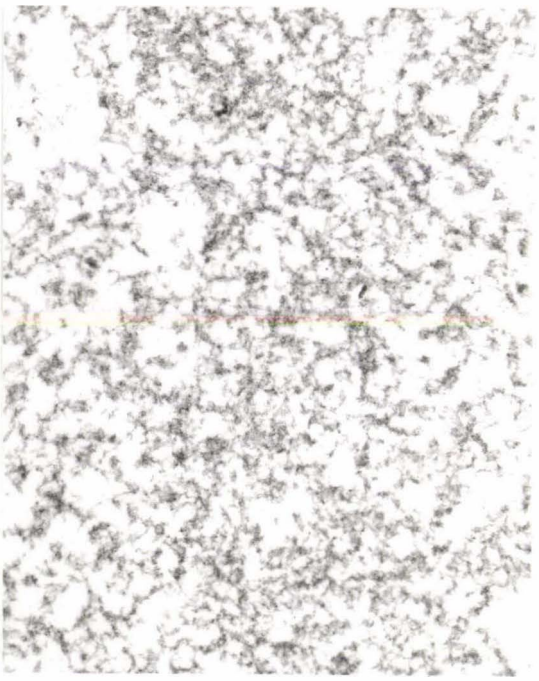


Figure 5.7 Transmission electron micrographs of myosin (pH 6.8).
The micrographs are at magnifications of a) 7800x, b) 11200x, c) 21200x, d) 48600x.



a



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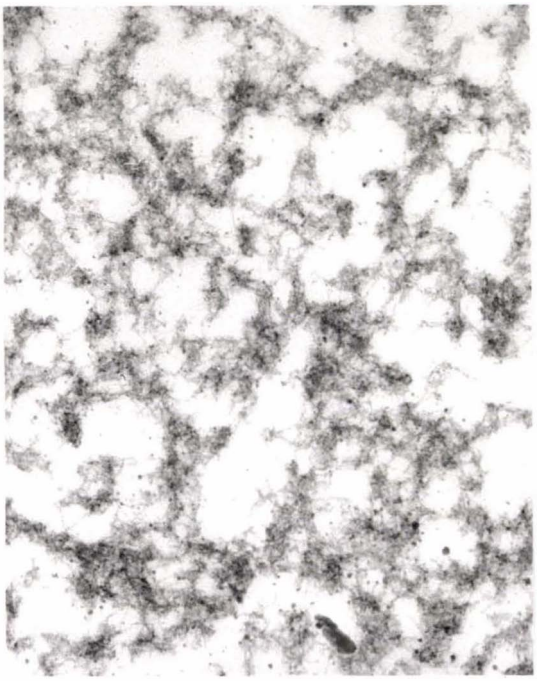


c

Figure 5.8 Transmission electron micrographs of myosin with GdL (pH 4.3).
The micrographs are at magnifications of a) 7800x, b) 15300x, c) 48600x.

Table 5.2 Results obtained from the rheological study of GdL-induced myosin gels in the presence of NaCl.

Gel	G' (Pa)	δ (°)	G" (Pa)	ω (Hz)	γ (m)	s	age (days)	Subjective Qualities
0.25% S	approx 20 plateau	2 to 10	1 to 3	-	smooth 0.0344	-		turbid, viscous
2.50% S	out of range of sensitivity							clear, solution
3.75% S	out of range of sensitivity							clear, solution
0.30% GdL 2.50% S	160 at 30 ks plateau grad	1 to 3	up to 9 mostly low	1 lin. 5 drop	smooth 0.0187	0.060		turbid, solution
0.70% GdL 0.25% S	97 at 14 ks max	14 ks min 5 to 16	7 to 10	1 lin. 5 rise	const.	0.143		turbid, viscous
0.70% GdL 2.50% S	550 at 48 ks inc grad	0 at 20 ks inc to 3	as for δ to 33	1 lin. 5 drop	abrupt 0.0050	0.050	1	turbid, viscous
0.70% GdL 2.50% S	863 at 48 ks	0 at 3.6 ks inc to 8	as for δ to 120	-	-	-	8	turbid, viscous
0.70% GdL 2.50% S	650 at 70 ks plateau grad	0 at 20 ks inc to 6	as for δ to 70	-	-	-	2	turbid, viscous
0.70% GdL 2.50% S	1090 at 48 ks grad	0 at 10 ks to 9	as for δ to 163	1 lin. 5 drop	abrupt 0.0021	0.007	5	turbid, viscous
0.70% GdL 2.50% S	771 at 52 ks inc	1 at 15 ks inc to 5	as for δ to 69	1 lin. 5 drop	abrupt 0.0033	0.037	5	turbid, viscous
0.70% GdL 2.50% S	521 at 48 ks 116 at 0 ks	0 to 2	2 to 25	-	-	-	7	turbid, viscous
0.70% GdL 3.75% S	1750 at 57 ks plateau grad	inc with G' to 17	as for δ to 560	1 lin. 5 drop	abrupt 0.0016	0.010		white, solid
1.30% GdL 2.50% S	530 at 21 ks plateau	1 to 2	5 to 15	1 lin. 5 drop	abrupt 0.0070	0.350		turbid, viscous

Terms associated with Table 5.2:
0.30%, 0.70%, 1.40% GdL = concentrations of GdL used
0.25%, 2.50%, 3.75% S = concentrations of NaCl used
plateau = curve reached a plateau at the specified time
grad = gradual incline/decline
min/max = curve reached a minimum/maximum at the specified time
inc/dec for G' curve = curve was increasing/decreasing at the final G' observed
inc/dec for δ and G" curves = increased or decreased at/to the specified time
lin. = linear
s = slope of a (log G') versus (log ω) plot
const. = constant
age = days of storage at 4°C before myosin was used
clear = translucent

The addition of 0.70% GdL and 2.50% NaCl to myosin produced a gel of 450 to 850 Pa at pH 3.8 (Figure 5.9). Ultimately the storage modulus was greater with 2.50% NaCl and 0.70% GdL (Table 5.2) than without added NaCl (Table 5.1). At similar pH, down to pH 4.3, the G' were similar with and without NaCl (Figures 5.9 and 5.1, respectively). Thereafter, the G' continued to increase in the presence of 2.50% NaCl with decreasing pH, but decreased in the absence of NaCl.

Decreased GdL concentration to 0.30% in the presence of 2.50% NaCl resulted in decreased G' compared to G' of myosin at 0.70% GdL and 2.50% NaCl (Figure 5.10). The G' levelled at 150 Pa at pH 4.5, three times the G' in the absence of NaCl (Figure 5.2). At 1.40% GdL, the final G' was approximately 550 at pH 3.3, with a levelling at this pH and a possible start of a decline in G' following this plateau (Figure 5.10).

Increased NaCl to 3.75%, in the presence of 0.70% GdL, resulted in significantly increased G' of approximately 1700 Pa at pH 4.0 compared to G' at 2.50% NaCl and 0.70% GdL addition (Figure 5.11). At 0.25% NaCl addition and 0.70% GdL the G' was approximately 100 Pa at its maximum at pH 4.0. The rate of increase of G' with respect to pH increased with increasing NaCl concentration.

The strain sweep results and s values (Table 5.2) indicated that at salt concentrations of 2.50 and 3.75% in the presence of 0.70 and 1.40% GdL, the network formed was a gel network and had more solid than liquid character. The sample with added 0.25% NaCl and 0.70% GdL had reached a peak G' and declined (Figure 5.11) as observed for myosin with 0.70% GdL (Figure 5.1). The s values and strain sweep data indicated a liquid-like sample as was observed. The addition of NaCl alone resulted in systems of more liquid than solid character, observed subjectively. These observations also explain the s values and the strain sweep data obtained which indicated a liquid-like sample.

The G" and δ values increased with decreasing pH while the G' developed at a high rate with respect to time. As this rate of G' development slowed, the G" and δ generally reached minima values and then increased with increasing G'. With the addition of both NaCl and

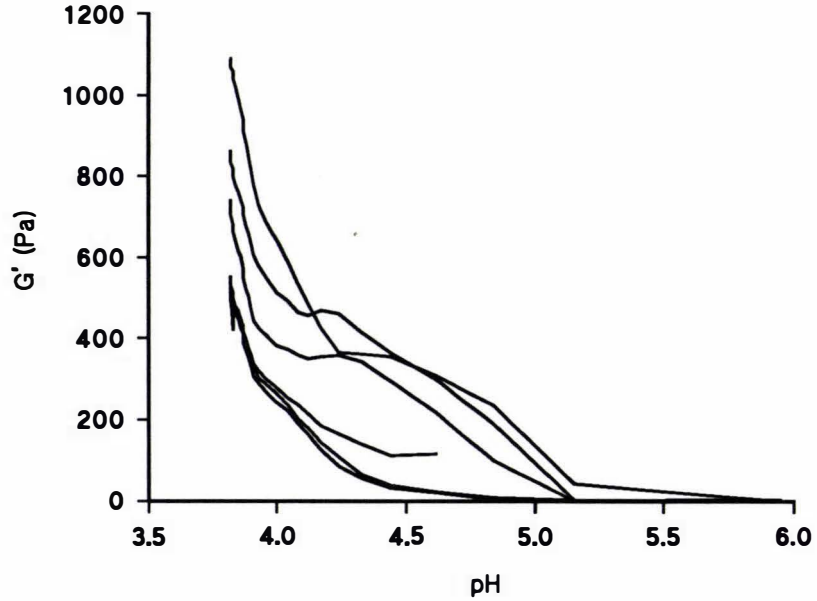


Figure 5.9. The development of G' of myosin gels at 0.70% GdL and 2.50% NaCl.

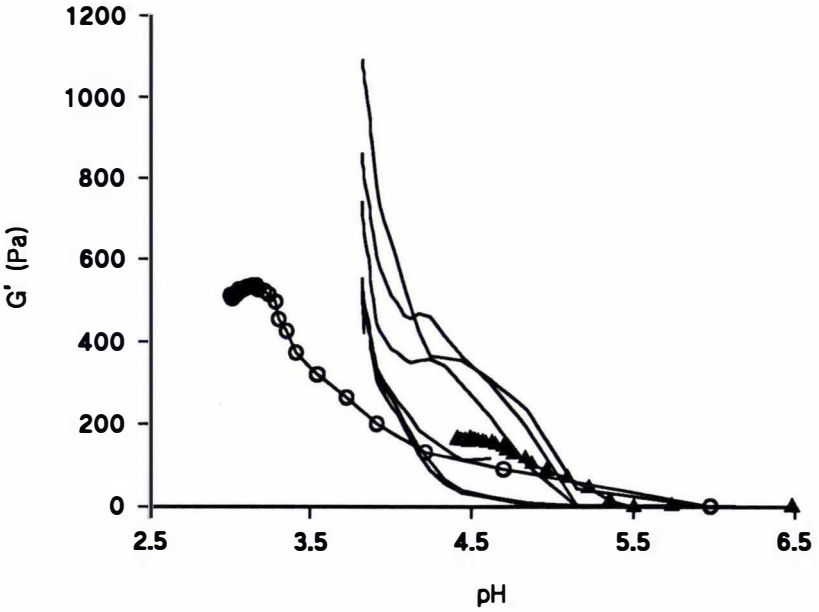


Figure 5.10. The development of G' of myosin gels at 2.50% NaCl and varied GdL concentrations. 0.30% GdL (▲), 0.70% GdL (–), 1.40% GdL (o).

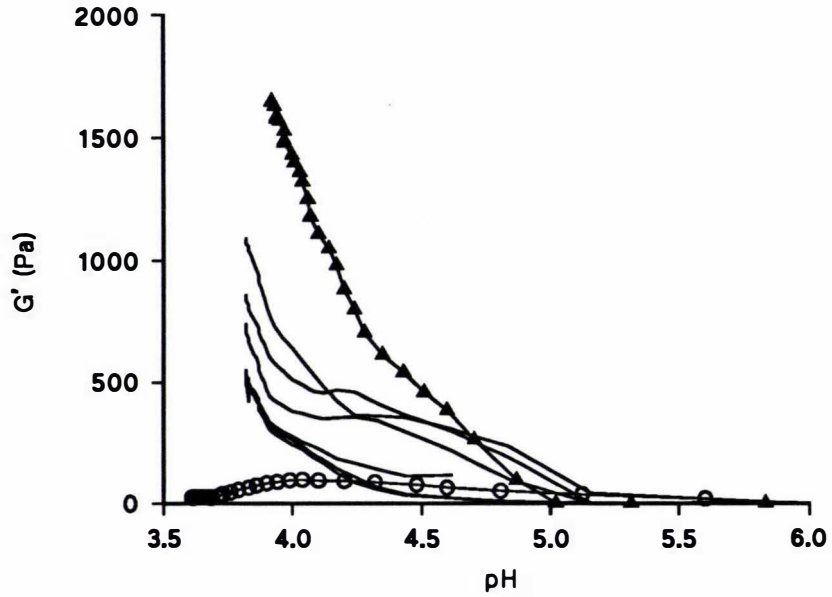


Figure 5.11. The development of G' of myosin gels at 0.70% GdL and varied NaCl. 0.25% NaCl (o), 2.50% NaCl (–), 3.75% NaCl (▲).

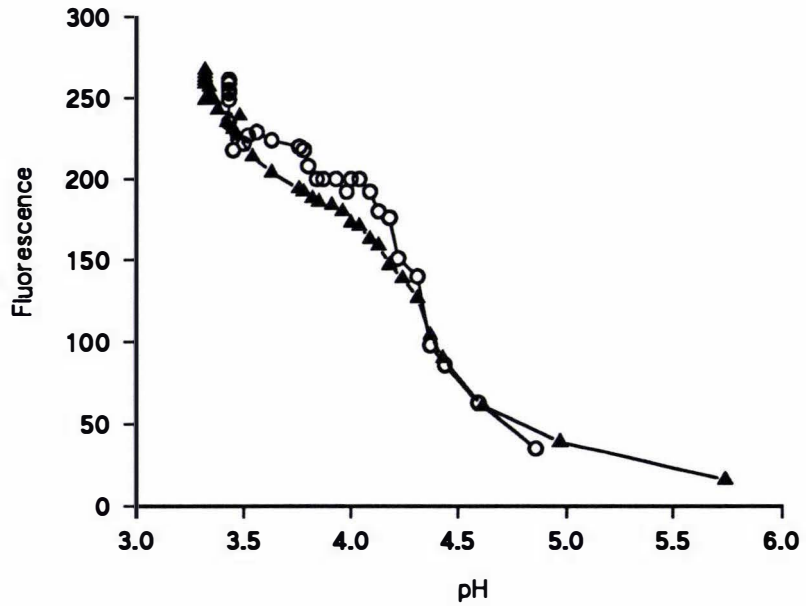


Figure 5.12. Fluorescence development of myosin solutions at 0.025% NaCl and varied GdL. 0.025% GdL (o), 0.050% GdL (▲).

GdL, the gels were turbid, opaque and viscous. As the G' increased, the appearance of the gels became whiter and more solid-like.

5.2.3.2 The effect of NaCl addition on fluorescence

The relative fluorescence of myosin solutions in the presence of 0.025% NaCl and 0.025 or 0.050% GdL increased from pH 6.0 to the minimum pH attained, pH 3.2 (Figure 5.12). Similar to the results for 0.025% GdL only, the fluorescence of myosin with 0.025% GdL and 0.025% NaCl increased parallel to the pH decrease with time (Figure 5.13). The fluorescence results at 0.025% NaCl and 0.025% GdL were similar to, but were less than the fluorescence of myosin in the absence of NaCl at a given pH (Figure 5.14).

The fluorescence decreased with increasing concentrations of NaCl from 0.013 to 0.125% at 0.025% GdL (Figure 5.15). The rate of development of fluorescence with respect to pH was similar at concentrations of 0.013 to 0.050% NaCl down to pH 3.5. At 0.125% NaCl the rate of development of fluorescence with respect to pH was less than at the lower NaCl concentrations. The final fluorescence values at NaCl concentrations of 0.013 to 0.050% with 0.025% GdL were similar.

Comparison of the results of fluorescence and G' data shows that the two techniques resulted in data of similar trends (Figure 5.16). The addition of 0.025% NaCl alone resulted in approximately zero fluorescence and did not increase with time.

5.2.3.3 The effect of NaCl on myosin gel structure

Light microscopy was undertaken at 100x magnification using NDIC on a solution of myosin with added 2.50% NaCl and a gel of myosin with 2.50% NaCl and 0.70% GdL added. Nothing of significance could be drawn from this work. The micrographs of myosin with added NaCl and with added NaCl plus GdL are in Appendix One (Figures A1.3 and A1.4, respectively).

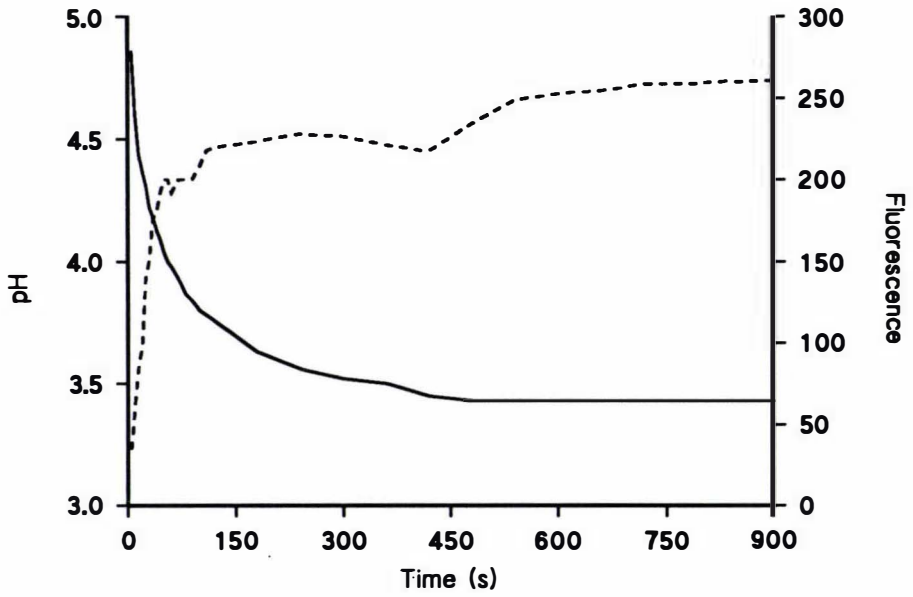


Figure 5.13. Fluorescence and pH development of myosin solutions at 0.025% GdL and 0.025% NaCl. pH (–), fluorescence (···).

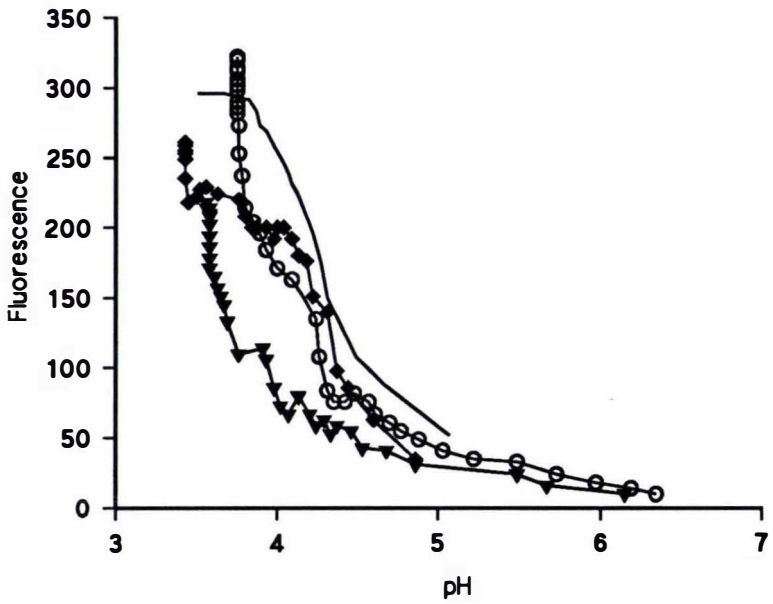


Figure 5.14. Fluorescence development of myosin solutions. 0.025% GdL (–), 0.025% GdL + 0.025% NaCl (◆), 0.025% GdL + 0.002% TSPP (○), 0.025% GdL + 0.025% NaCL + 0.002% TSPP (▼).

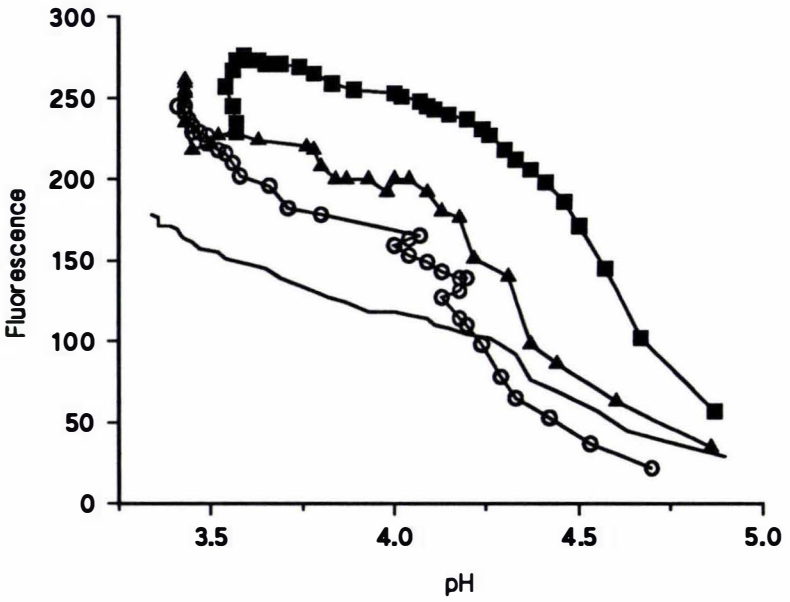


Figure 5.15. Fluorescence development of myosin solutions at 0.025% GdL and varied NaCl. 0.013% NaCl (■), 0.025% NaCl (▲), 0.050% NaCl (o), 0.125% NaCl (–).

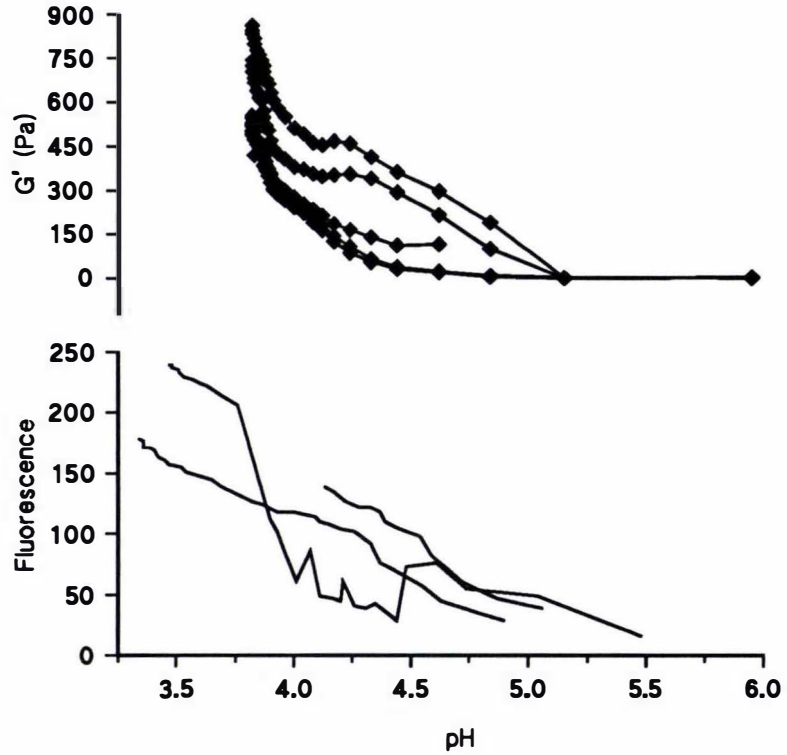
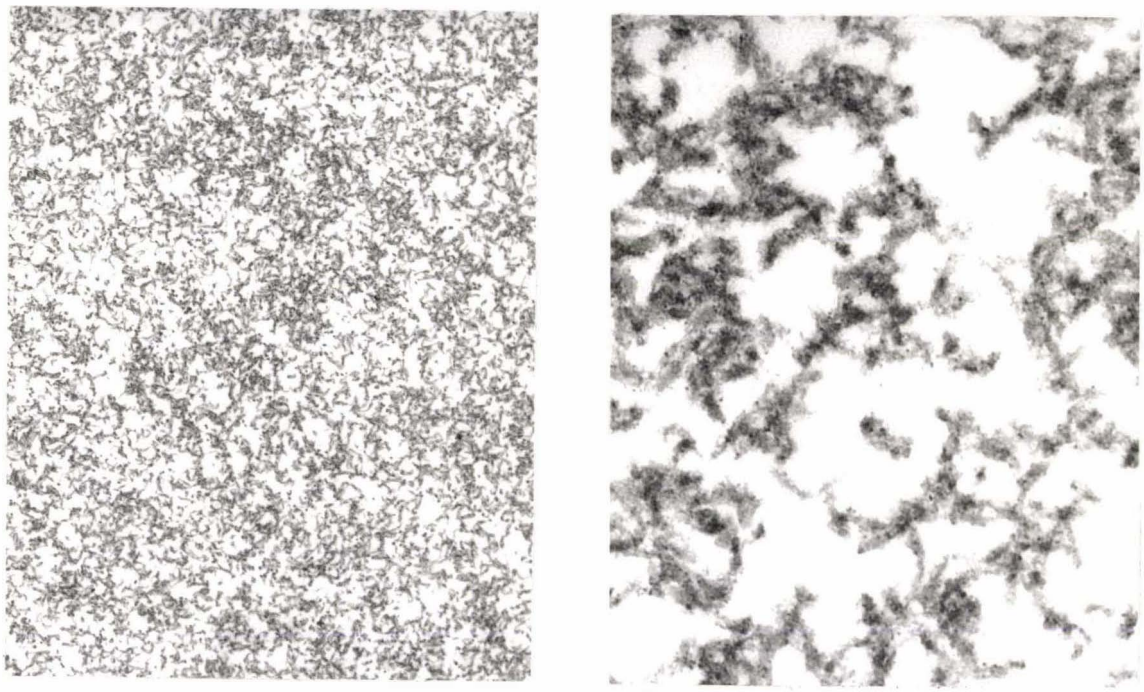


Figure 5.16. Comparison of rheological and fluorescence data. Fluorescence data of myosin solutions at 0.025% GdL and 0.025% NaCl (–), G' data of myosin at 0.70% GdL and 2.50% NaCl (◆).



a b

Figure 5.17 Transmission electron micrographs of myosin with 2.50% NaCl and 0.70% GdL added (pH 4.0). The micrographs are at magnifications of a) 7800x, b) 48600x.

Only two TEM micrographs of the structure of the gel containing 2.50% NaCl and 0.70% GdL were obtained (Figures 5.17a and b). The structure appeared midway between the samples of myosin alone (Figures 5.7a and d) and myosin at 0.70% GdL of pH 4.3 (Figures 5.8a and c). The presence of strands was evident, but these strands appeared shorter than with myosin alone and the gel appeared more dense.

5.2.4 The effect of addition of tetrasodium pyrophosphate (TSPP)

5.2.4.1 The effect of TSPP on the rheological characteristics of myosin gels

A summary of the rheological data obtained from studies undertaken on the Bohlin rheometer is given in Table 5.3.

The addition of 0.25% TSPP resulted in a gel of 350 Pa, approximately 27 times greater than that without TSPP. However, the pH ranged from 7.6 to 8.8 and was out of the acidic range of concern in the present experiments.

The inclusion of 0.25% TSPP in myosin suspensions prevented the pH from decreasing to values less than 4.0 at the GdL concentration of 0.70% (Figure 5.18). The G' obtained at 0.70% GdL and 0.25% TSPP with a final pH of 4.3 ranged from 810 to 1400 and the modulus was increasing at the final pH obtained. The range of G' values obtained with 0.25% TSPP plus 0.70% GdL was greater overall than G' obtained using 0.70% GdL (Figure 5.1) or 0.70% GdL and 2.50% NaCl (Figure 5.9) at a given pH.

At 0.30% GdL concentration with 0.25% TSPP, the pH reached a final value of 5.5 and G' was 580 Pa (Figure 5.19). Increased GdL concentration to 1.4% with 0.25% TSPP resulted in G' similar to that at 0.70% GdL with 0.25% TSPP to pH 4.5 where the higher GdL concentration resulted in a lag in G' development to pH 3.8 followed by an abrupt decline in G' at lower pH values.

Halving the TSPP concentration to 0.13% TSPP, resulted in a G' curve which resembled those at the bottom of the range of the myosin system with 0.25% TSPP and 0.70% GdL added

Table 5.3 Results obtained from the rheological study of GdL-induced myosin gels in the presence of TSPP.

Gel	G' (Pa)	δ (°)	G" (Pa)	ω (Hz)	γ (m)	s	age (days)	Subjective Qualities
0.25% T	300 at 3.6 ks 350 at 27 ks plateau	2 to 4	14 to 20	1 lin. 5 drop	abrupt 0.0094	0.200		turbid, viscous
0.25% T	350 at 1.8 ks 400 at 21 ks plateau	approx 0	approx 0	-	abrupt 0.0084	-		turbid, viscous
0.25% T	276 at 9 ks 340 at 50 ks grad	0 to 3	0 to 10	1 lin. 5 drop	abrupt 0.0098	0.110		white, solid
0.30% GdL 0.25% T	581 at 48 ks inc	2 to 3	18 to 25	1 lin. 5 drop	abrupt 0.0066	0.050		white, solid
0.70% GdL 0.13% T	427 at 43 ks max	0 to 1	0 to 10	1 lin. 5 drop	grad 0.0104	0.069		white, solid
0.70% GdL 0.25% T	550 at 3.6 ks 1380 at 48 ks inc	inc at 0 ks to 12	inc at 0 ks to 280	1 lin. 5 drop	abrupt 0.0016	0.008	7	white, solid
0.70% GdL 0.25% T	530 at 3.6 ks 870 at 48 ks inc	inc from 5 to 10	45 to 150	-	abrupt 0.0026	-	6	white, solid
0.70% GdL 0.25% T	146 at 0 s inc 875 at 48 ks	inc to 8	inc to 126	1 lin. 5 drop	abrupt 0.0022	0.008	6	white, solid
0.70% GdL 0.25% T	1250 at 48 ks	0 at 43 ks inc to 6	0 at 43 ks 8 to 40	1 lin. 5 drop	abrupt 0.0035	0.100	5	white, solid
0.70% GdL 0.25% T	785 at 52 ks plateau	0 at 10 ks 4 at 2 ks	1 at 10 ks 58 at 52 ks	1 lin. 5 drop	abrupt 0.0044	0.030	2	white, solid
0.70% GdL 0.25% T	810 at 48 ks plateau grad	inc at 20 ks to 5	as for δ to 70	1 lin. 5 drop	abrupt 0.0041	0.040	12	white, solid
0.70% GdL 0.38% T	420 at 3.6 ks 1120 at 41 ks inc	inc at 3.6 ks to 8	as for δ to 162	1 lin. 5 drop	abrupt 0.0020	0.026		white, solid
1.40% GdL 0.25% T	775 at 19 ks 187 at 48 ks	0 at 28 ks 8 at 48 ks	as for δ to 27 at 48 ks	1 lin. 5 drop	abrupt 0.0258	0.150		-

Terms associated with Table 5.3:
0.30%, 0.70%, 1.40% GdL = concentrations of GdL used
0.13%, 0.25%, 0.38% T = concentrations of TSPP used
plateau = curve reached a plateau at the specified time
grad = gradual incline/decline
min/max = curve reached a minimum/maximum at the specified time

inc/dec for G' curve = curve was increasing/decreasing at the final G' observed
inc/dec for δ and G" curves = increased or decreased at/to the specified time
lin. = linear
s = slope of a (log G') versus (log ω) plot
age = days of storage at 4°C before myosin was used

(Figure 5.20). The final pH was 4.3 at which the curve had reached a plateau, and was possibly beginning to decline. At a TSPP concentration of 0.38% a G' curve resulted which was similar to those at the top of the range of the 0.25% TSPP and 0.70% GdL myosin system. The final pH achieved with 0.38% TSPP added was 4.7 and the G' was still increasing at this pH.

The δ and G" generally increased with increasing G'. The gels formed were generally very viscous, turbid solutions or white solids. The s values and strain sweep data suggest gel networks of more solid than liquid character were formed and not entanglement networks with the addition of GdL and TSPP (Table 5.3). These results were evident at all levels of TSPP added in the presence of GdL at the three given levels; 0.30, 0.70 and 1.40% GdL. However, in the absence of GdL, the high s values and high strain data suggest that the gel formed may be an entanglement network, but the evidence is not conclusive and a gel may be formed.

5.2.4.2 The effect of TSPP addition on fluorescence

The addition of 0.002% TSPP to myosin solutions in the absence of GdL resulted in approximately zero fluorescence and showed no increase with time for 12 h. Fluorescence data obtained with the addition of 0.002% TSPP and 0.025% GdL was similar to that of 0.025% NaCl with 0.025% GdL and less than that of 0.025% GdL only (Figure 5.14). Increased GdL concentration did not result in significant change in the fluorescence development with pH (Figure 5.21).

Fluorescence of 0.025% GdL with 0.002% TSPP increased parallel to the decrease of pH with respect to time (Figure 5.22). Increasing the concentration of TSPP to 0.005% and 0.012% resulted in aggregates of myosin formed in the solution as the pH decreased. The presence of aggregates resulted in fluorescence data which was not reproducible. Reduction of the

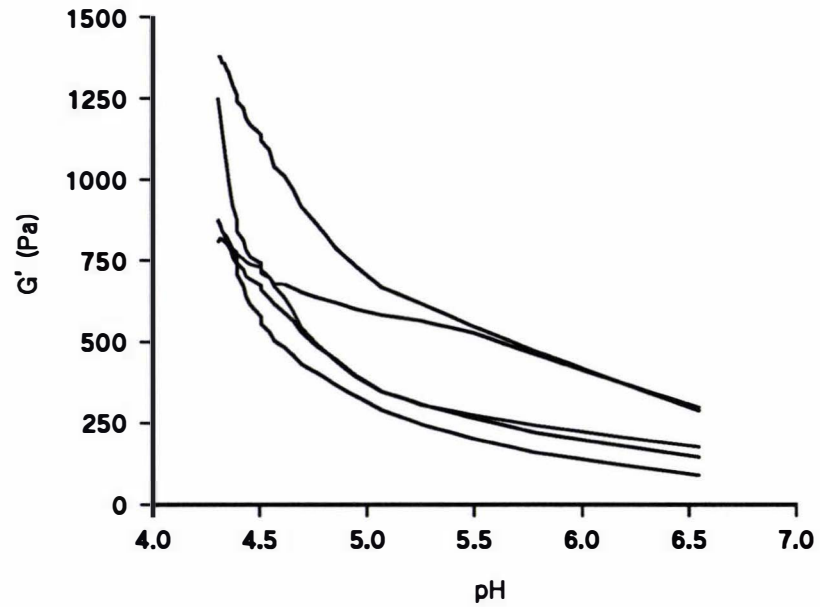


Figure 5.18. The development of G' of myosin gels at 0.70% GdL and 0.25% TSPP.

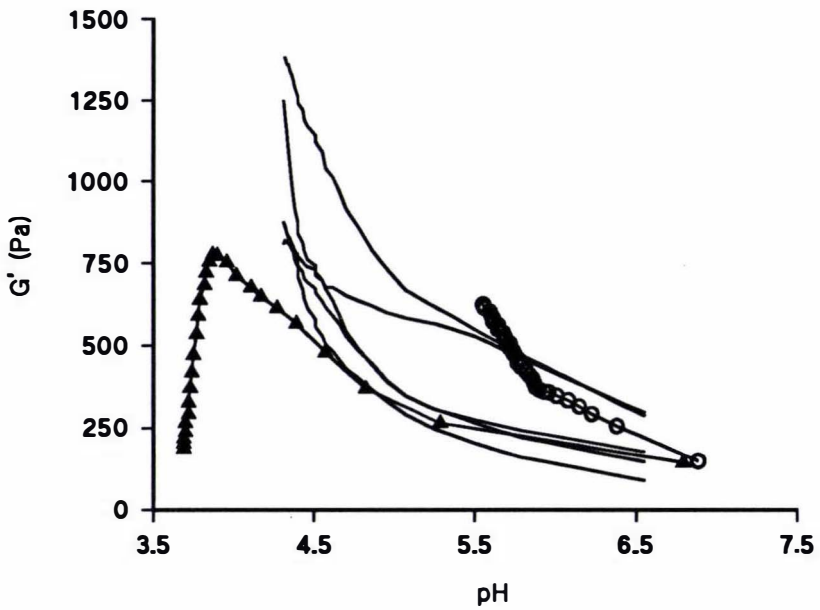


Figure 5.19. The development of G' of myosin gels at varied GdL concentrations and 0.25% TSPP. 0.30% GdL (o), 0.70% GdL (–), 1.40% GdL (▲).

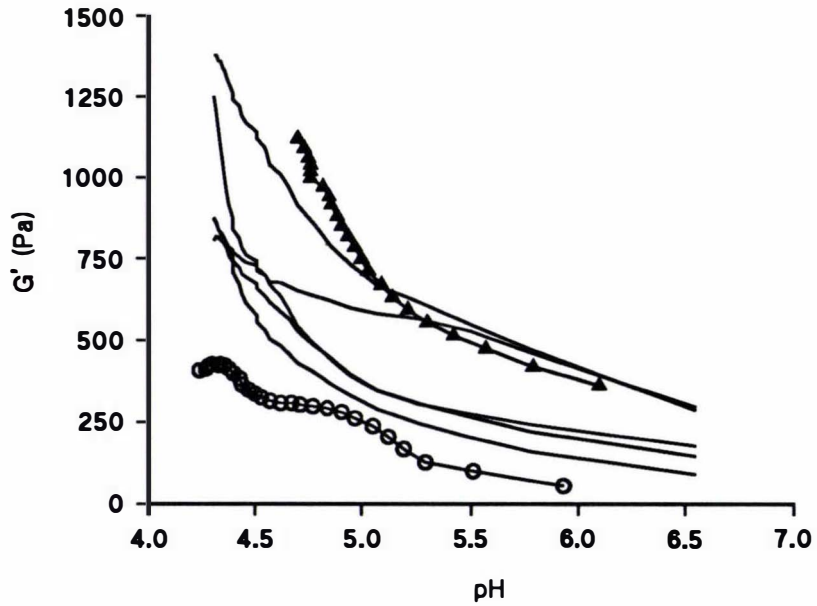


Figure 5.20. The development of G' of myosin gels at 0.70% GdL and varied TSPP concentrations. 0.13% TSPP (o), 0.25% TSPP (–), 0.38% TSPP (▲).

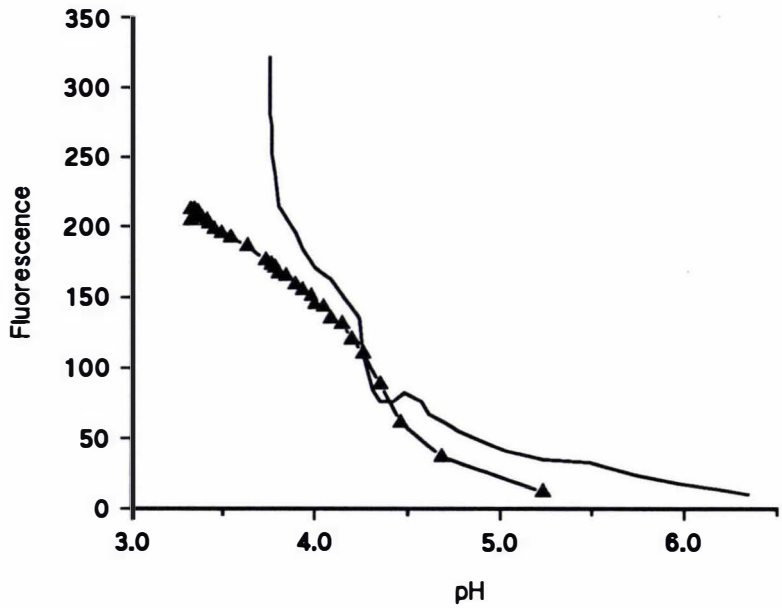


Figure 5.21. Fluorescence development of myosin solutions at 0.002% TSPP and varied GdL concentrations. 0.025% GdL (–), 0.050% GdL (▲).

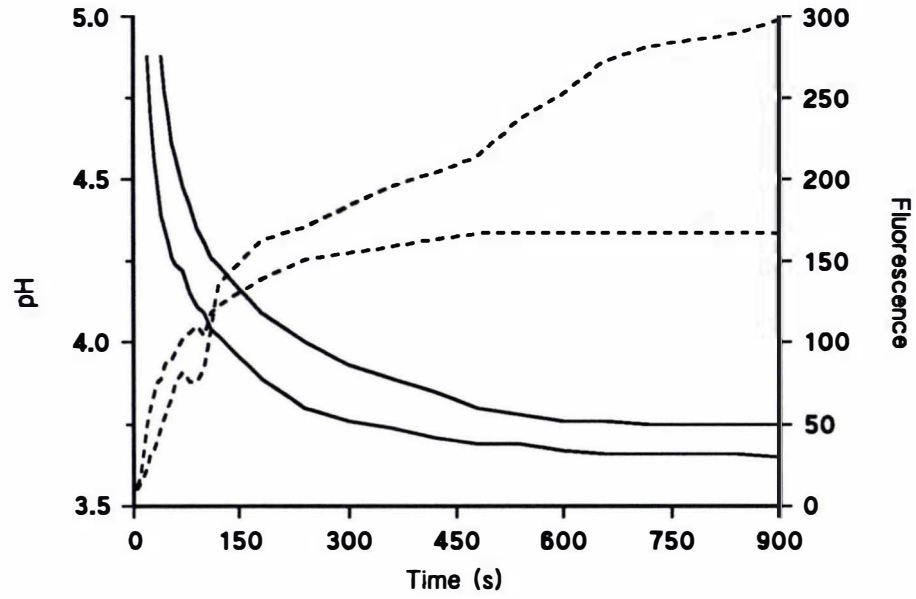


Figure 5.22. Fluorescence and pH development of myosin solutions at 0.025% GdL and 0.002% TSPP. pH (–), fluorescence (···).

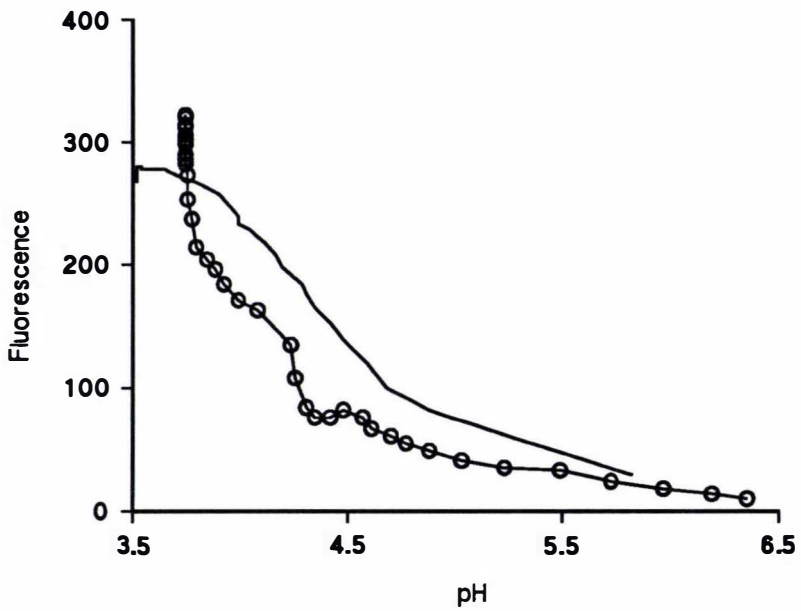


Figure 5.23. Fluorescence development of myosin solutions at 0.025% GdL and varied TSPP concentrations. 0.001% TSPP (–), 0.002% TSPP (o).

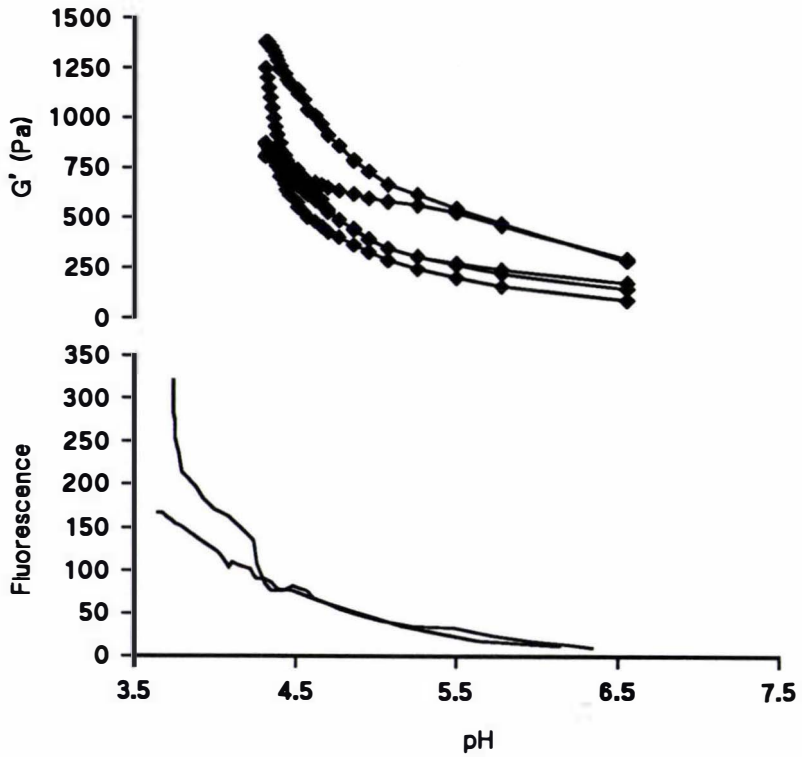


Figure 5.24. Comparison of rheological and fluorescence data. Fluorescence data of myosn solutions at 0.025% GdL and 0.002% TSPP (–), G' data of myosin at 0.70% GdL and 0.25% TSPP (◆).

TSPP concentration to 0.001% resulted in greater fluorescence at a given pH down to pH 3.7 (Figure 5.23). Comparison of fluorescence data at 0.002% TSPP and 0.025% GdL, with the G' values at 0.25% TSPP and 0.70% GdL, showed both followed similar trends with respect to pH (Figure 5.24). These results are similar to those observed for myosin in the presence of GdL (Figure 5.6), and GdL plus NaCl (Figure 5.16).

5.2.4.3 The effect of TSPP on gel structure

Light microscopy was undertaken on myosin plus 0.25% TSPP in the presence and absence of 0.70% GdL. The micrographs were taken at 100x magnification using NDIC. Nothing of significance could be drawn from this work. The micrographs of the TSPP sample and the TSPP plus GdL sample are given in Appendix One (Figures A1.5 and A1.6, respectively).

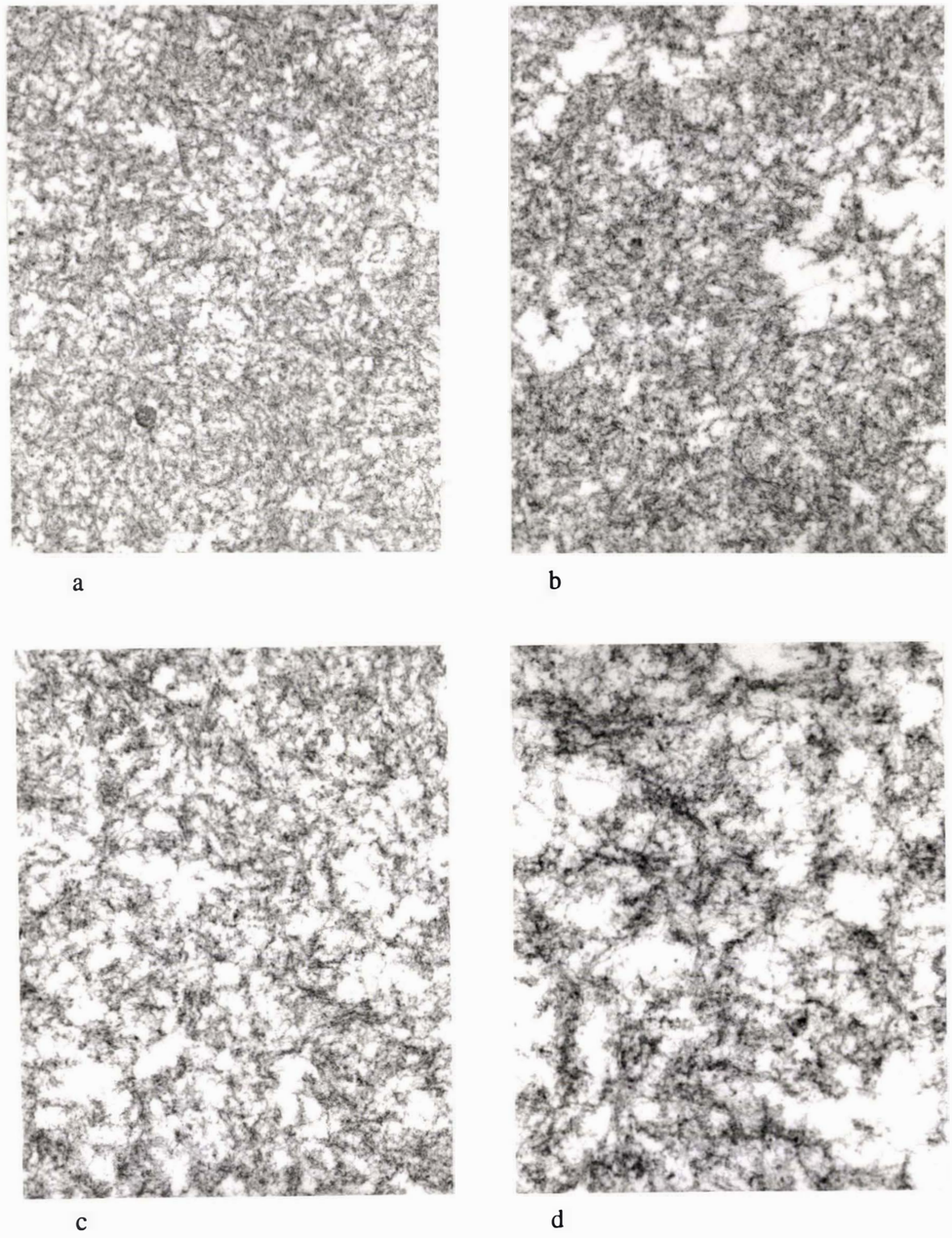
Micrographs of myosin with 0.25% TSPP added as observed under TEM (Figures 5.25a-d) showed a denser network than that of myosin alone (Figures 5.7a-d) or with 2.50% NaCl and 0.70% GdL (Figures 5.17a and b). There was no evidence of the strands observed in the micrographs of myosin alone and in the presence of NaCl and GdL combined. The myosin gel with 0.70% GdL only (Figures 5.8a-c) appeared to have similar structure but of less density than that of TSPP addition.

The inclusion of 0.25% TSPP in the 0.70% GdL system (Figures 5.26a-d) showed similar but slightly denser structure than that of myosin alone (Figures 5.7a-d) and 0.70% GdL with 2.50% NaCl (Figures 5.17a and b). Strands were evident in the TSPP system with GdL as with myosin alone and with GdL plus NaCl.

5.2.5 The effect of addition of TSPP and NaCl

5.2.5.1 The effect of TSPP and NaCl on the rheological characteristics of myosin gels

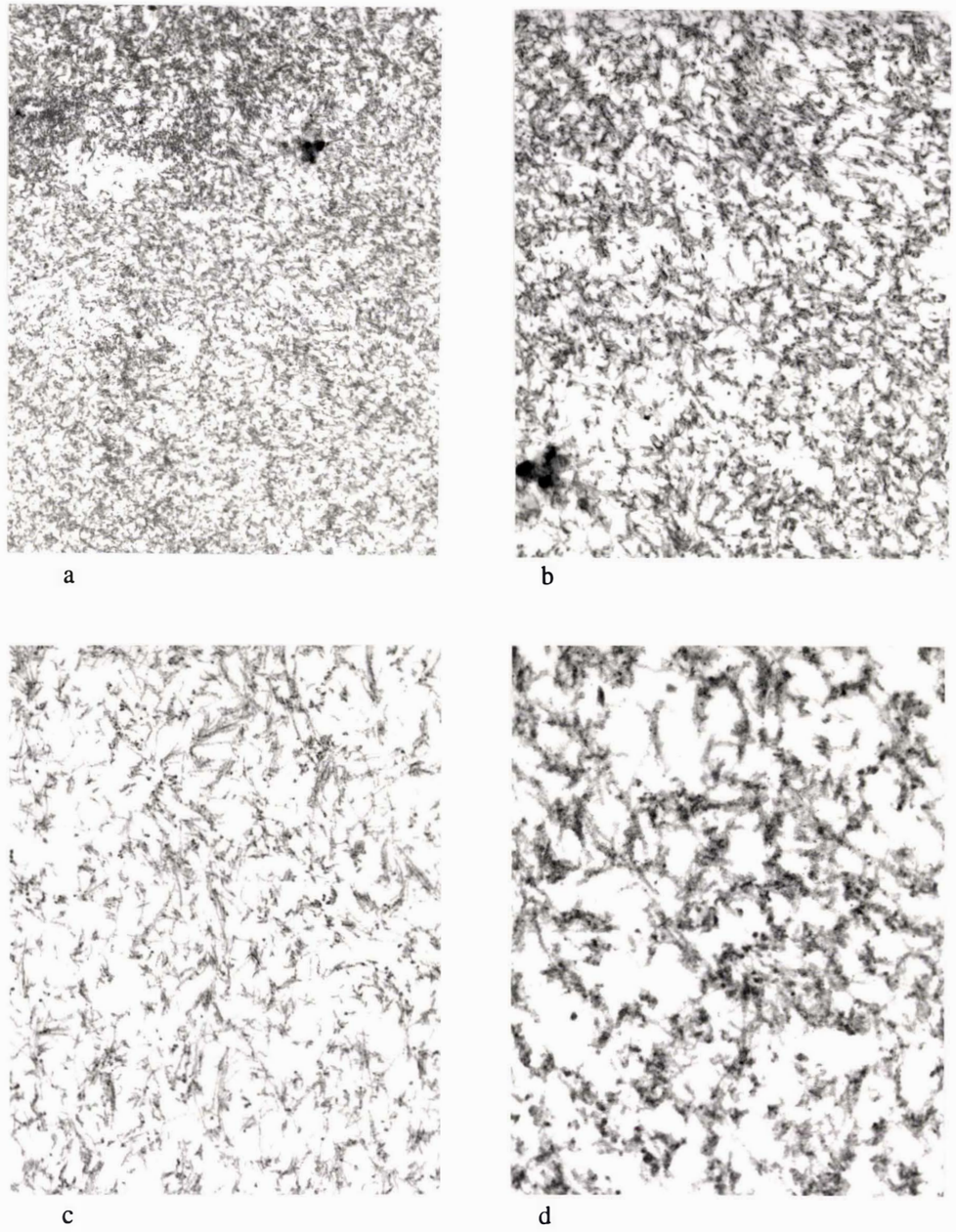
A summary of the rheological data obtained from the Bohlin rheometer used to measure the characteristics of myosin gels formed with added TSPP, NaCl and GdL, is given in Table 5.4.



a b

c d

Figure 5.25 Transmission electron micrographs of myosin with 0.25% TSPP (pH 7.8). The micrographs are at magnifications of a) 11200x, b) 15300x, c) 21200x, d)48600x.



a b

c d

Figure 5.26 Transmission electron micrographs of myosin with 0.25% TSPP and 0.70% GdL added (pH 4.3). The micrographs are at magnifications of a) 11200x, b) 21200x, c) 21200x, d)48600x.

Table 5.4. Results obtained from the rheological study of GdL-induced myosin gels in the presence of TSPP and NaCL.

Gel	G' (Pa)	δ (°)	G" (Pa)	ω (Hz)	γ (m)	s	Subjective Qualities
T + S	out of range of sensitivity						translucent solution
0.30% GdL T + S	250 at 48 ks inc grad from 27 ks	0 to 2 from 30 ks	approx 0	1 lin. 5 drop	abrupt 0.0103	0.060	white, solid
0.70% GdL T + S	1050 at 59 ks inc grad	inc at 25 ks to 9	inc at 25 ks to 17	1 lin. 5 drop	abrupt 0.0021	0.007	white, solid
1.40% GdL T + S	840 at 52 ks plateau grad	inc at 20 ks to 6	inc at 20 ks to 9	1 lin. 5 drop	abrupt 0.0031	0.048	white, solid

Terms associated with Table 5.4:
0.30%, 0.70%, 1.40% GdL = concentrations of GdL used
T + S = 0.24% TSPP and 2.50% NaCl
plateau = curve reached a plateau at the specified time
grad = gradual incline/decline
inc/dec for G' curve = curve was increasing/decreasing at the final G' observed
inc/dec for δ and G" curves = increased or decreased at/to the specified time
lin. = linear
s = slope of a (log G') versus (log ω) plot

The addition of 0.25% TSPP and 2.50% NaCl to myosin resulted in a clear solution. The myosin sample behaved like a low viscosity liquid and the torque sensing element was unable to detect fluid resistance to shear above the instrument noise. The addition of 0.30%, 0.70% and 1.40% GdL resulted in the formation of gel networks with solid character as evidenced by the G' data, the strain sweep results and the s values. These gels were white solids.

Similar to the G' development of 0.70% GdL and 2.50% NaCl (Figure 5.9) and 0.70% GdL plus 0.25% TSPP (Figure 5.18), the G' of the 0.70% GdL, 0.25% TSPP and 2.50% NaCl increased to 1050 Pa at a final pH of 3.8 (Figure 5.27). The addition of 0.30% GdL to myosin with 2.50% NaCl and 0.25% TSPP reached a final minimum pH of 5.5 and G' of 250 Pa, differing from the curves at higher GdL concentrations by a rapid rate of G' development at pH about 5.7. There was no difference in G' development at 0.70% GdL and 1.40% GdL. The inclusion of NaCl and TSPP in myosin systems at 0.70 and 1.40% GdL resulted in G' values intermediate between those of GdL with NaCl and GdL with TSPP (Figures 5.28 and 5.29).

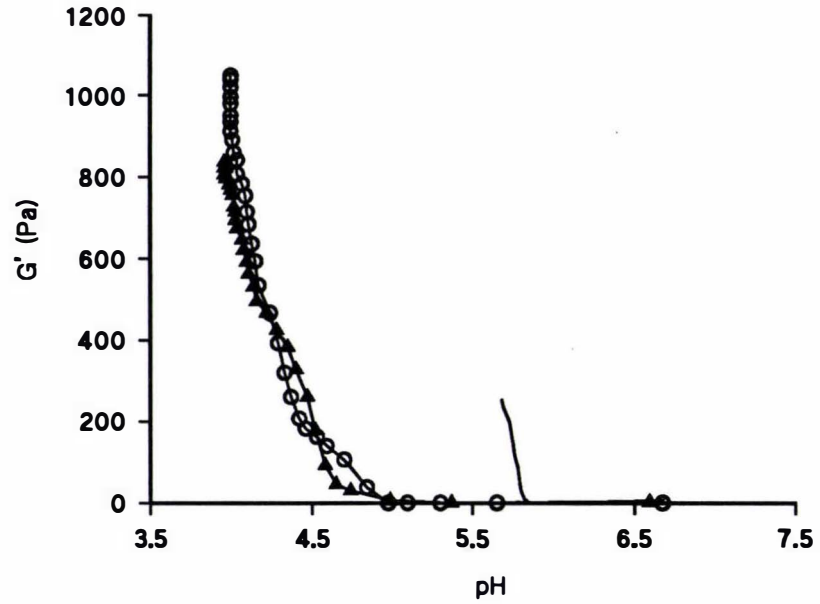


Figure 5.27. Development of G' of myosin gels at varied GdL concentrations with 0.25% TSPP and 2.50% NaCl. 0.30% GdL (−), 0.70% GdL (o), 1.40% GdL (▲).

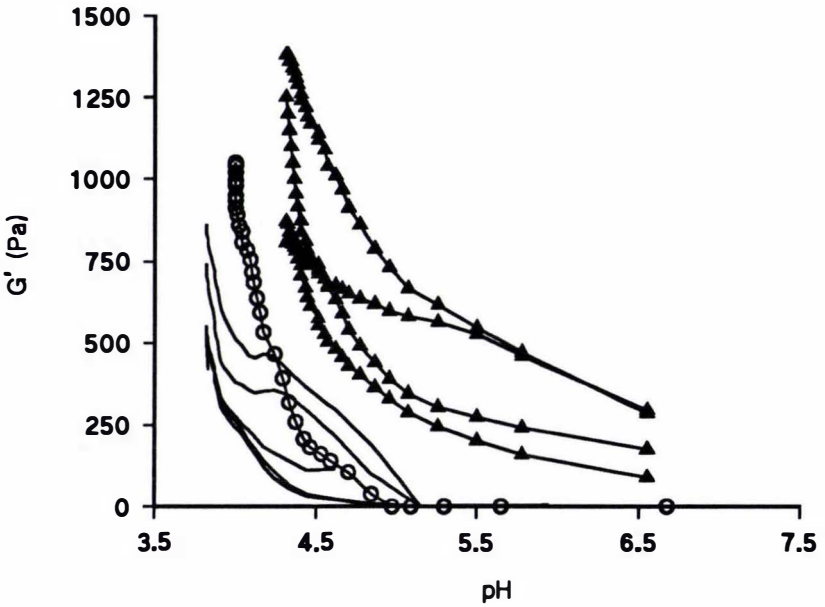


Figure 5.28. Development of G' of myosin gels at 0.70% GdL and 2.50% NaCl or 0.25% TSPP. GdL + NaCl (−), GdL + TSPP (▲), GdL + NaCl + TSPP (o).

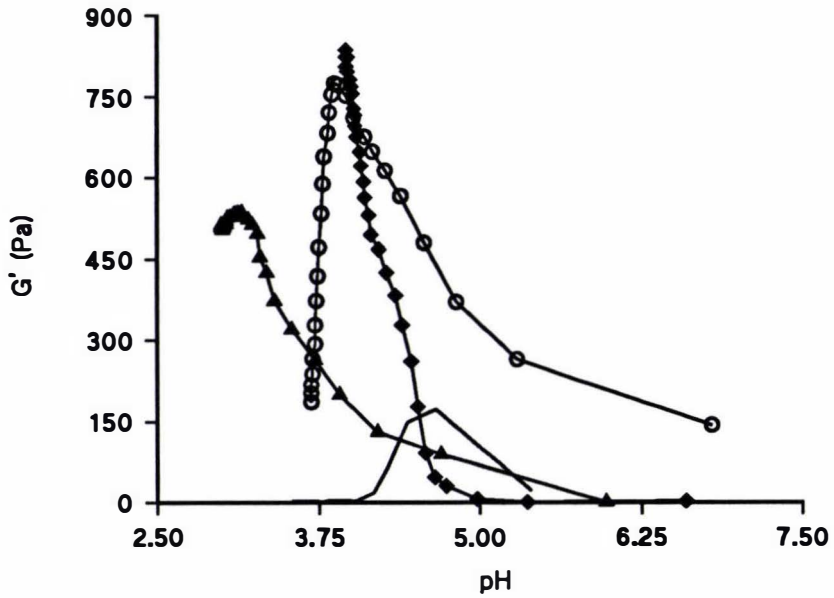


Figure 5.29. Development of G' of myosin gels at 1.40% GdL with 2.50% NaCl and 0.25% TSPP. GdL (–), GdL + NaCl (▲), GdL + TSPP (o), GdL + NaCl + TSPP (◆).

5.2.5.2. The effect of TSPP and NaCl on fluorescence

Fluorescence data obtained from myosin solutions containing 0.025% GdL, 0.002% TSPP and 0.025% NaCl was similar, but less than that obtained with GdL alone, with GdL and TSPP and with GdL and NaCl at a given pH (Figure 5.14). Similar to the results of these other combinations, the fluorescence of myosin with 0.025% GdL, 0.025% NaCl and 0.002% TSPP added increased parallel to the decrease of pH with time (Figure 5.30). Also similar to the results of these other combinations, the fluorescence data and G' results showed similar trends (Figure 5.31).

5.2.5.3 The effect of NaCl and TSPP on the structure of myosin gels

Light microscopy was undertaken on myosin with NaCl plus TSPP in the presence of GdL. The magnification at which the micrographs were taken was 100x and NDIC was used. Nothing of significance could be drawn from this work. The results are presented in Appendix One (Figure A1.7)

Micrographs using TEM (Figures 5.32a-c) showed a very dispersed or dilute system. There was evidence of the strands also observed in the samples of myosin alone (Figures 5.7a-d), GdL with NaCl (Figures 5.17a and b) and GdL with TSPP (Figures 5.26a-d).

5.2.6 The effect of sulfhydryl-blocking agents on the rheological characteristics of myosin gels.

Summaries of the results obtained from investigations of myosin gel formation with added sulfhydryl-blocking agents are given in Tables 5.5 and 5.6, for the agents PCMB and DTT respectively.

Only the samples with added 0.25% TSPP in the presence and absence of 0.70% GdL, 2.50% NaCl with 0.70% GdL, and 0.25% TSPP plus 2.50% NaCl in the presence of 0.70% GdL were investigated as these were the gels which remained solid to the final pH obtained in the absence of the sulfhydryl-blocking agents. The addition of 0.01% PCMB resulted in increased

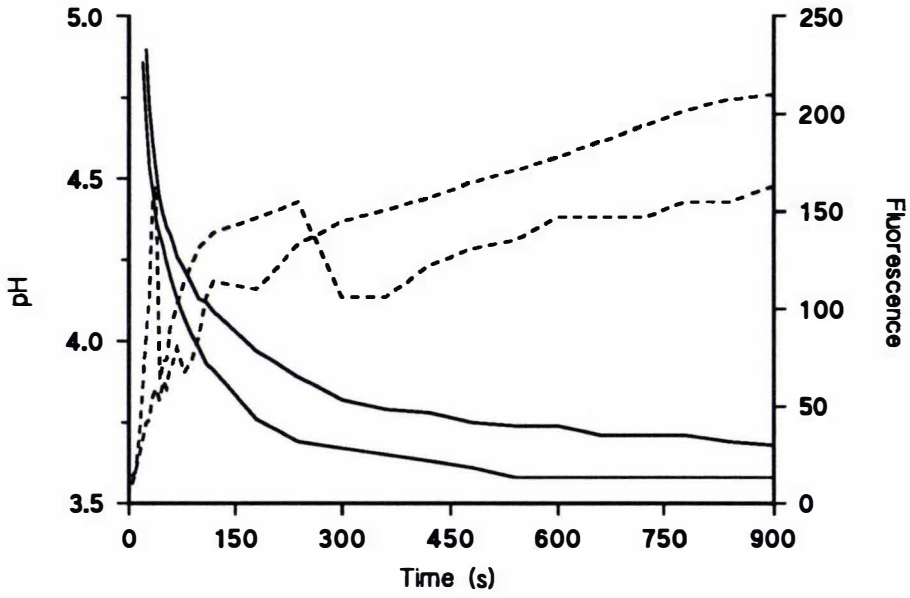


Figure 5.30. Fluorescence and pH development of myosin solutions at 0.025% GdL, 0.025% NaCl and 0.002% TSPP. pH (–), fluorescence (···).

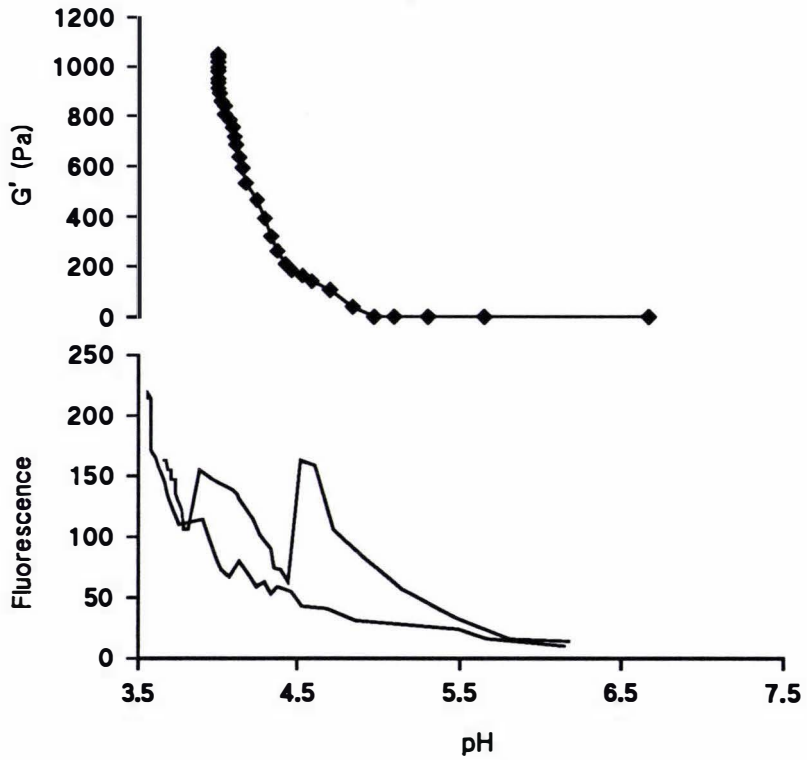


Figure 5.31. Comparison of fluorescence data of myosin solutions and G' of myosin gels. Fluorescence at 0.025% GdL, 0.025% NaCl and 0.002% TSPP (–), G' at 0.70% GdL, 2.50% NaCl and 0.25% TSPP (◆).

Figure 5.32 Transmission electron micrographs of myosin with 2.50% NaCl, 0.25% TSPP and 0.70% GdL added (pH 4.2). The micrographs are at magnifications of a) 11200x, b) 15300x, c) 48600x.

G' of myosin gels with 2.50% NaCl and 0.70% GdL added at pH less than 4.5 compared to myosin gels in the absence of PCMB (Figure 5.33). The addition of 0.005% and 0.011% DTT had no effect on the G'.

In the presence of 0.25% TSPP and 0.70% GdL, addition of 0.01 and 0.02% PCMB had no effect on G' compared to gels without the added PCMB (Figure 5.34). There was also no effect observed on the G' with the inclusion of 0.011 or 0.005% DTT. The addition of 0.02% PCMB had no effect on the myosin gel formed with 0.70% GdL, 2.50% NaCl and 0.25% TSPP (Figure 5.35).

The addition of 0.10% PCMB to myosin resulted in gel formation within 24 h storage prior to addition of GdL, TSPP or NaCl. When these additives were stirred into the myosin, the gel that had formed, was broken. The resultant gel was in the lower part of the range of gels formed without the addition of PCMB (Figure 5.36). The addition of 0.051% DTT did not have a similar effect on myosin during storage and was similar to gels formed without added DTT.

Table 5.5. Rheological data obtained from GdL-induced myosin gels with added PCMB.

Gel	S-Block	G' (Pa)	δ (°)	G" (Pa)	ω (Hz)	γ (m)	s
0.70% GdL S	0.01% PCMB	1380 at 36 ks plateau	inc at 10 ks to 12 plat	as for δ to 300	1 lin. 5 drop	-	0.003
0.70% GdL S	0.01% PCMB	898 at 54 ks plateau 327 at 7.2 ks	inc at 19 ks to 7	as for δ to 112	-	-	-
T	0.02% PCMB	280 at 18 ks plateau	constant 2 to 4	10 to 20	-	smooth 0.0105	-
0.70% GdL T	0.01% PCMB	973 at 52 ks plateau	inc at 0 ks to 10	as for δ to 167	-	-	-
0.70% GdL T	0.02% PCMB	1450 at 37 ks plateau	inc at 0 ks to 20	as for δ to 541	-	abrupt 0.0011	-
0.70% GdL T	0.10% PCMB	578 at 18 ks max 749 at 43 ks inc	inc at 21 ks to 5	as for δ to 66	1 lin. 5 drop	abrupt 0.0035	0.040
0.70% GdL T + S	0.02% PCMB	900 at 50 ks inc	inc at 2 ks to 9	as for δ to 144	1 lin. 5 drop	abrupt 0.0024	0.020

Table 5.6. Rheological data obtained from GdL-induced myosin gels with added DTT.

Gel	S-Block	G' (Pa)	δ (°)	G" (Pa)	ω (Hz)	γ (m)	s
0.70% GdL S	0.005% DTT	858 at 50 ks bump 320 at 9 ks	inc at 18 ks to 9	as for δ to 139	1 lin. 5 drop	abrupt 0.0027	0.014
0.70% GdL S	0.011% DTT	892 at 46 ks bump 267 at 7.2 ks	inc from 16 ks to 9	as for δ to 142	1 lin. 5 drop	abrupt 0.0024	0.011
T	0.011% DTT	671 at 48 ks plateau 500 at 1.8 ks	inc at 0 ks to 4	as for δ to 45	-	abrupt 0.0046	-
0.70% GdL T	0.011% DTT	1020 at 48 ks inc 646 at 5.4 ks	inc at 0 ks to 8	as for δ to 145	-	-	-
0.70% GdL T	0.051% DTT	801 at 52 ks inc 600 at 1.8 ks	inc from 0 ks to 6	as for δ to 8	-	-	-

Terms associated with Tables 5.5 and 5.6:
0.70% GdL = concentration of GdL used
T, S = 0.25% TSPP, 2.50% NaCl
0.01%, 0.02%, 0.10% PCMB = concentrations of PCMB used
0.005%, 0.011%, 0.051% DTT = concentrations of DTT used
plateau = curve reached a plateau at the specified time
min/max = curve reached a minimum/maximum at the specified time
grad = gradual incline/decline
inc/dec for G' curve = curve was increasing/decreasing at the final G' observed
inc/dec for δ and G" curves = increased or decreased at/to the specified time
lin. = linear
s = slope of a (log G') versus (log ω) plot

5.2.7. The effect of sugars on the rheological characteristics of GdL-induced myosin gels.

A summary of the rheological data obtained from GdL-induced myosin gels formed with the inclusion of sucrose, glucose or sorbitol is given in Table 5.7.

In the presence of 0.70% GdL, inclusion of 10.0% sucrose resulted in decreased G' of myosin gels compared to gels formed without the added sucrose (Figure 5.37). A similar decrease was observed when sucrose was included with 0.70% GdL and 0.25% TSPP (Figure 5.38). Strain sweep data and s values indicated that the gels formed were gel networks, not entanglement networks for all samples except that of myosin with 0.70% GdL and 10.0% sucrose added (Table 5.7). This sample had liquid-like characteristics.

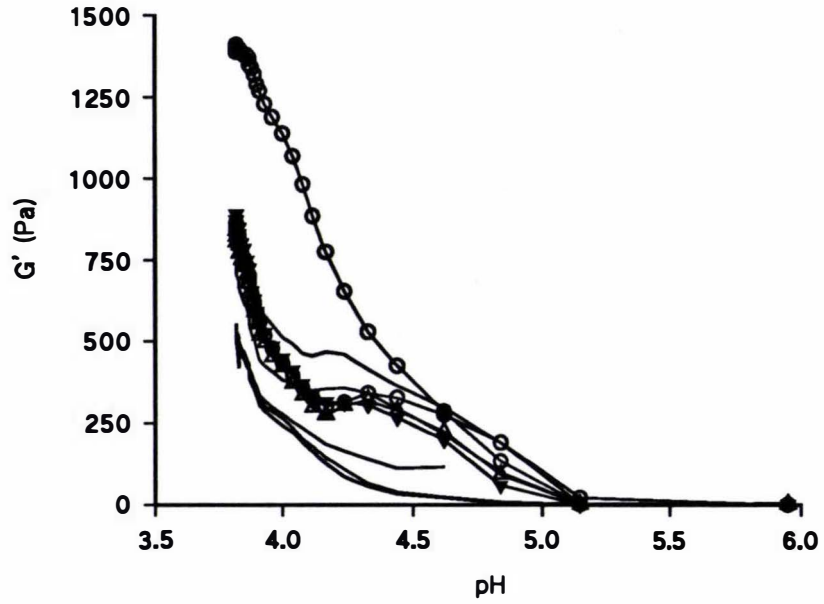


Figure 5.33. Development of G' of myosin gels at 0.70% GdL and 2.50% NaCl. No PCMB or DTT (–), 0.01% PCMB (o), 0.005% DTT (▵), 0.011% DTT (▾).

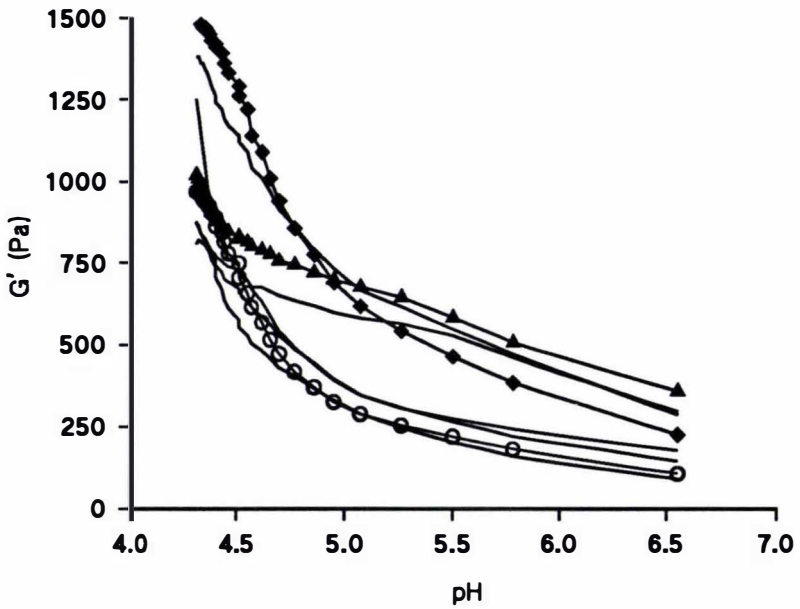


Figure 5.34. Development of G' of myosin gels at 0.70% GdL and 0.25% TSPP. No PCMB or DTT (–), 0.01% PCMB (o), 0.02% PCMB (◆), 0.011% DTT (▲).

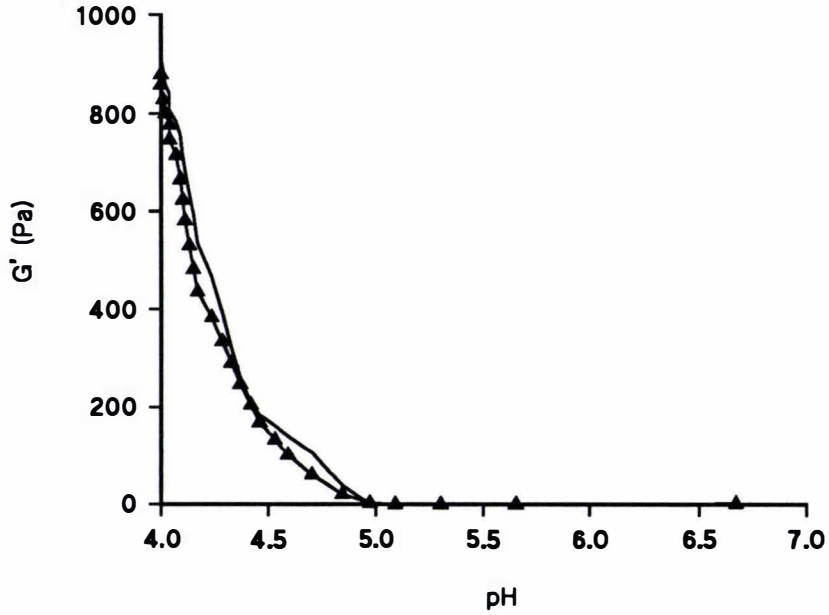


Figure 5.35. Development of G' of myosin gels at 0.70% GdL, 2.50% NaCl and 0.25% TSPP. No PCMB (–), 0.02% PCMB added (▲).

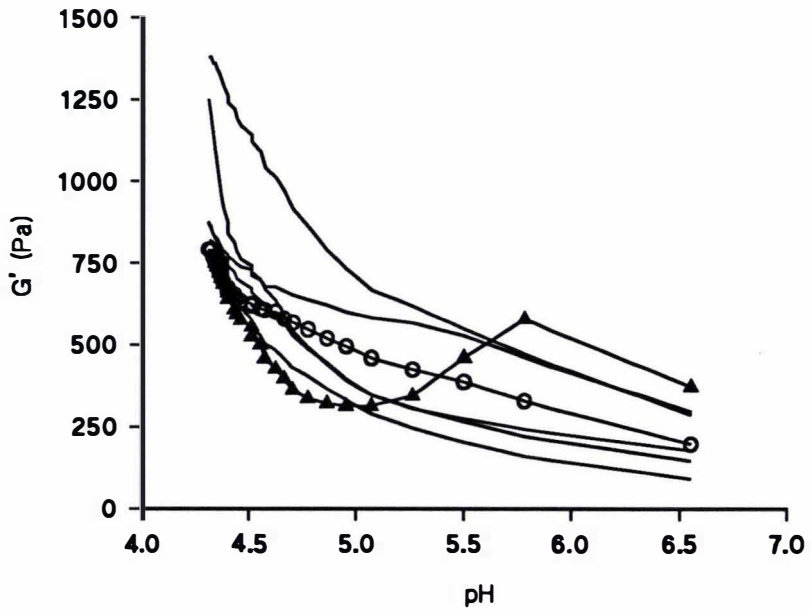


Figure 5.36. Development of G' of myosin gels at 0.70% GdL and 0.25% TSPP. No PCMB or DTT (–), 0.10% PCMB (▲), 0.051% DTT (o).

Table 5.7. Rheological data obtained from GdL-induced myosin gels with added sugars.

Gel	Sugar	G' (Pa)	δ (°)	G" (Pa)	ω (Hz)	γ (m)	s
myosin alone	sucrose	26 at 0 ks dec 17 at 48 ks	14 to 27	6 to 9	-	constant	-
0.70% GdL	sucrose	120 at 7.2 ks max 11 at 48 ks	5 at 1 ks to 26 at 45 ks	8 to 12	1 lin. 5 drop	grad 0.0973	0.178
0.70% GdL	sucrose	66 at 5.4 ks max 3 at 48 ks	6 to 40	2 to 11	-	linear	-
0.70% GdL S	sucrose	1430 at 48 ks plateau	2 at 5.4 ks 14 at 48 ks	as for δ to 341	1 lin. 5 drop	abrupt 0.0015	0.0
0.70% GdL S	sucrose	310 at 48 ks plateau	0 at 5.4 ks 16 at 52 ks	as for δ to 377	1 lin. 5 drop	abrupt 0.0027	0.0
0.70% GdL T	sucrose	360 at 23 ks plateau	1 to 4	6 to 17	1 lin. 5 drop	abrupt 0.0108	0.081
0.70% GdL S	glucose	1220 at 66 ks plateau	1 at 7.2 ks 13 at 66 ks	as for δ 4 to 280	1 lin. 5 drop	abrupt 0.0027	0.004
0.70% GdL S	sorbitol	1330 at 48 ks plateau	1 to 14 inc	inc to 340	1 lin. 5 drop	abrupt 0.0018	0.0

Terms associated with Tables 5.7:
0.70% GdL = concentration of GdL used
T = 0.25% TSPP
S = 2.50% NaCl
plateau = curve reached a plateau at the specified time
min/max = curve reached a minimum/maximum at the specified time
grad = gradual incline/decline
inc/dec for G' curve = curve was increasing/decreasing at the final G' observed
inc/dec for δ and G" curves = increased or decreased at/to the specified time
lin. = linear
s = slope of a (log G') versus (log ω) plot

In the presence of 2.50% NaCl and 0.70% GdL, the inclusion of sucrose resulted in myosin gels of increased G' compared to gels without the added sucrose (Figure 5.39). Similar curves to that with added sucrose were obtained with the inclusion of 10.0% sorbitol and 10.0% glucose in the presence of 2.50% NaCl and 0.70% GdL.

The addition of sucrose to myosin resulted in immediate G' values double that without added sucrose. However, the G' decreased to a similar value to that of myosin alone within 14 h.

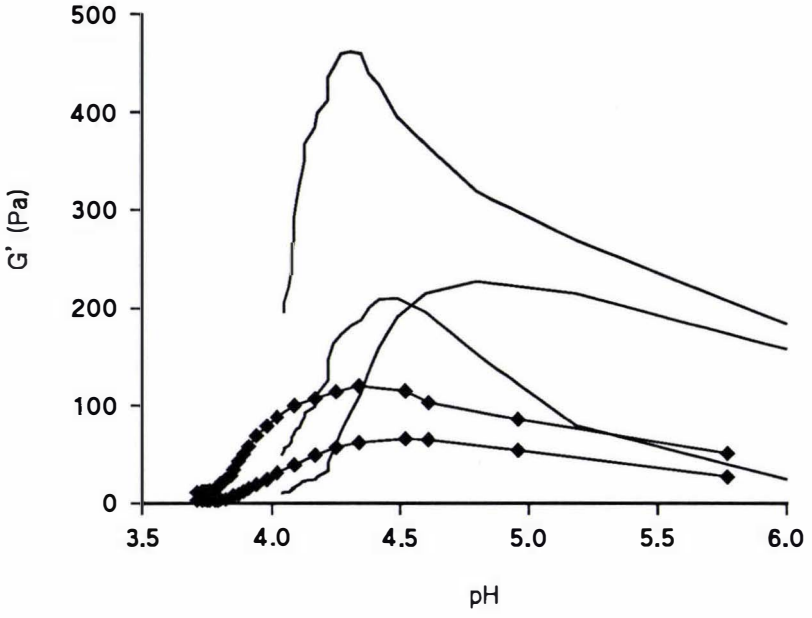


Figure 5.37. Development of G' of myosin gels at 0.70% GdL. No sucrose (–), 10.0% sucrose (◆).

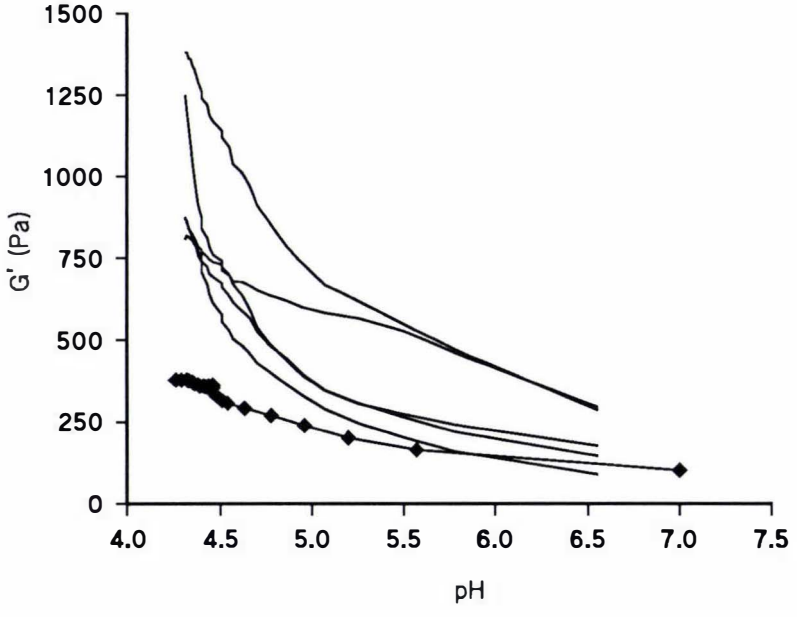


Figure 5.38. Development of G' of myosin gels at 0.70% GdL and 0.25% TSPP. No sucrose (–), 10.0% sucrose (◆).

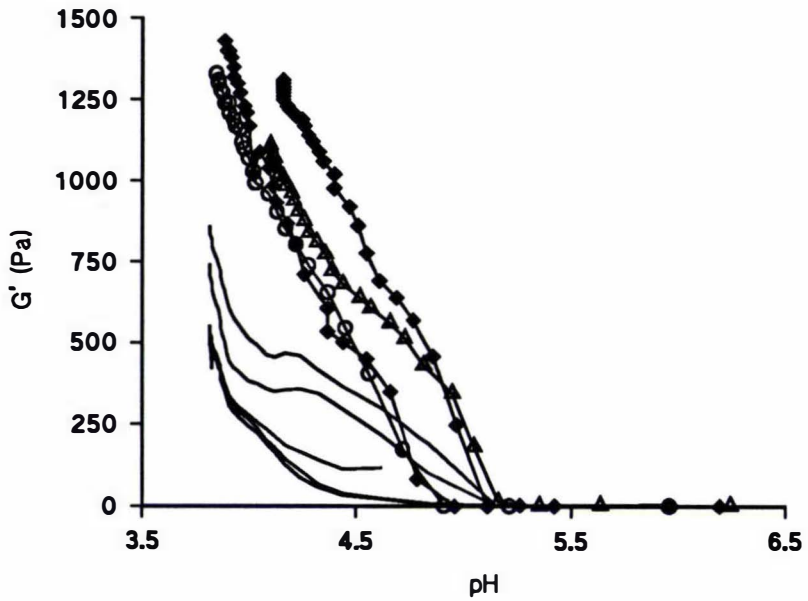


Figure 5.39. Development of G' of myosin gels at 0.70% GdL and 2.50% NaCl. No sugars added (–), 10.0% sucrose added (◆), 10.0% sorbitol added (○), 10.0% glucose added (△).

5.3 DISCUSSION

This discussion of results is divided into the following sections:

1. A description of the ANS fluorescence method,
2. A discussion of the rheological parameters measured,
3. The formation of true gels or entanglement networks,
4. The phenomenon of events over the pH course, namely,
    a) conformational changes as monitored by fluorescence probes,
    b) the development of G' and other rheological parameters,
    c) the effect of age of sample on rheological parameters,
    d) subjective changes during gel formation,
    e) changes in the ultrastructural characteristics of the gels as observed under transmission electron microscopy.

Section (4) is applicable to the addition of glucono-δ-lactone (GdL) only. This section is followed by:

5. The effects of sodium chloride (NaCl) and tetrasodium pyrophosphate (TSPP).

Section (5) is discussed with regard to the parameters used in section (4), that is, fluorescence, rheological effects, subjective characteristics, microscopy and sample age effects. The effect of TSPP is discussed briefly, a more detailed discussion being given in Chapter Six. The final sections are:

6. Bonding in the gelation of myosin,
7. A postulated series of events.

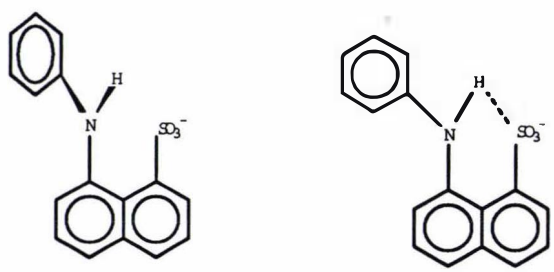
5.3.1 ANS fluorescence

The hydrophobic probe methods are the simplest type of method for measuring protein surface hydrophobicity (Nakai and Li Chan, 1988). The compound ANS probes an extremely hydrophobic site of myosin (Wicker *et al.*, 1989). The exposure of aromatic hydrophobic groups is targeted by ANS. Indirectly this measure of exposure of hydrophobic groups, especially an extremely hydrophobic site, can be used as an indication of denaturation of the protein molecule.

Denaturation has been defined as any modification of the secondary, tertiary or quaternary structure of the protein molecule, excluding any breaking of covalent bonds (Joly, 1965). That is, denaturation is a conformational change in the protein molecule. Any unfolding of the molecule would result in exposure of hydrophobic sites in an aqueous solution, and hence measurement of the exposure of these sites provides an indirect measure of protein denaturation.

Arntfield *et al.* (1989) demonstrated the use of ANS fluorescence as a measure of denaturation in ovalbumin and vicilin. While the fluorescence data did not provide any additional benefit in following protein unfolding, it provided a good complement or alternative to differential scanning calorimetry (DSC) analysis. The use of ANS fluorescence alone to monitor conformational changes during heating was successfully applied to the investigation of heat-induced myosin gelation by Wicker *et al.* (1986).

Direct linking of ANS fluorescence results with hydrophobicity requires great caution (Penzer, 1972). It was reported that ANS has two different conformations. The more unhindered shape was the one in which the planes of benzene and naphthalene rings were approximately perpendicular (I). The other shape had the two rings nearly coplanar because of hydrogen bonding between -NH and -SO_3 groups (II). Solvents which favoured intermolecular hydrogen bond formation, favoured formation of the more rigid conformation (II) which was fluorescent.



I II

Kato and Nakai (1980) and others from the same laboratory (Voutsinas *et al.*, 1983; Li Chan *et al.*, 1984) have questioned the reliability of ANS as a polarity probe, suggesting that *cis-*

parinaric acid (CPA) was a better probe for measuring hydrophobicity of proteins. However, later reports from the same laboratory noted the importance of considering aromatic (measured with ANS) as well as aliphatic (measured with CPA) hydrophobicity when studying protein structure-function (Hayakawa and Nakai, 1985; Li Chan *et al.*, 1985; Tsutsui *et al.*, 1986). The use of CPA in this laboratory was not possible due to the expense of the compound and the lack of necessary filters for the fluorescence measurement.

In the present experiments, the method of measurement of ANS fluorescence differed from other workers (Ma and Holme, 1982; Chan *et al.*, 1992; Wicker and Knopp, 1988; Arntfield *et al.*, 1989) in that the fluorescence was measured continuously. The addition of GdL induced a continual pH decrease and monitoring the fluorescence as pH changed detected unfolding as it occurred. The effect was therefore measured cumulatively. Measurement at a fixed pH was difficult, as even at the final pH obtained, the pH was slowly, but continuously, decreasing. At the initial stages of GdL addition, the pH decrease was rapid and change was difficult to measure reliably without continuous measurement.

5.3.2 Bohlin rheology

Hamann (1991) presented a summary of the physical meaning of development or theory behind rheological information. This summary is the basis of the present discussion of the rheological parameters which were used to characterise the myosin systems.

Small strain dynamic measurements were undertaken using a Bohlin rheometer on a myosin system ranging from liquid myosin to deformable gels. The instrument imposes the stresses and strains as sine functions. In principle the material is contained in an enclosure of simple geometry and the sample is stressed so that the stresses and strains can be calculated from force (or torque) and displacement respectively.

Shear strain can be written as

$$\gamma = \gamma_o \sin(\omega t) \qquad (5.1)$$

where γ_o is the strain amplitude, ω is the angular frequency in rad/s and t is the time. The shear rate will be the derivative of the equation (5.1) with respect to time

$$\dot{\gamma} = \gamma_o \omega \cos(\omega t) = \dot{\gamma}_o \cos(\omega t) \qquad (5.2)$$

The shear stress will, in general, be out of phase from the strain by an angle δ and can be written as

$$\tau = \tau_o \sin(\omega t + \delta) \qquad (5.3)$$

where τ_o is the stress amplitude. The ratio of shear stress divided by shear strain can be written as the sum of the two components, one in phase with the strain and the other 90° out of phase (Dealy, 1982). Using complex number notation in which the real term is the in-phase part and the imaginary term is the out of phase part

$$G^* = (\tau_o/\gamma_o)[\cos \delta + i \sin \delta] = G' + i\, G'' \qquad (5.4)$$

where i is the imaginary number $(-1)^{1/2}$, G' is the storage modulus, G" is the loss modulus and G* is the complex modulus. The ratio (τ_o/γ_o) is the absolute modulus, $|G^*|$. For a perfectly elastic material the stress and strain are in phase ($\delta = 0$) and the imaginary term is zero. In the case of a perfectly viscous material $\delta = 90° = \pi/2$ rad and the real part is zero. The ratio G"/G' is called the loss tangent and is equal to the tangent of the phase angle

$$\tan \delta = G''/G' \qquad (5.5)$$

This is proportional to the (energy dissipated)/(energy stored) per cycle. Specifically, the energy dissipated due to viscous behaviour per cycle divided by the maximum elastic energy stored is $2\pi \tan \delta$ (Whorlow, 1980). A perfectly elastic material would exhibit $\delta = 0$, whereas for a perfectly viscous material $\delta = \infty$. Protein gels are normally quite elastic and so values of δ are near 10°. A muscle sol prior to cooking exhibits some elasticity but is viscous, so a typical δ is 45°. The transition from sol to gel is evident from changes in δ.

For simplicity, experimental results from oscillatory shear are often presented in terms of G' and G" or tan δ as functions of temperature at a constant ω or as functions of ω at a constant temperature. Most food gelation studies have been done at a constant ω (often < 1 Hz) with temperature and/or time varying (Beveridge *et al.*, 1984; Bohlin *et al.*, 1984; Beveridge and Timbers, 1985; Samejima *et al.*, 1985; Noguchi, 1986). In the experiments presented here temperature and ω (except in the frequency curves) were constant and pH varied with time.

In Tables 5.1 to 5.7, the rheological data was presented in terms of various parameters measured using a Bohlin rheometer. Three different tests were undertaken, namely, small

strain oscillatory tests, frequency scans and strain sweep tests. The parameters used are described as follows.

The storage modulus, G', refers to the rigidity or the solid part of the sample. As G' increases, the sample has more elastic or solid character. Thus, G' is an indication of gelation.

The term storage modulus describes energy absorbed when a bond is subjected to an oscillation. The parameter, G" is the loss modulus and it follows that this is a measure of the amount of energy lost when a bond is subjected to an oscillation. This modulus refers to the liquid or viscous character of the sample. It is difficult to interpret G". Generally, both G" and G' increase with particulate matter. These parameters are bulk properties, that is they are the sum of interactions occurring. The phase angle, δ, gives an indication of the onset of gelation. Low δ values are indicative of good network formation, and good network formation gives high G'. Generally G" and δ followed similar trends in these experiments. These parameters, G', G" and δ, were measured using small strain oscillatory testing.

Frequency, as reported in the results, referred to the G' curve of an oscillation test in which the frequency of oscillations was the independent variable, that is, a frequency scan. For a viscoelastic solid the G' curve should smoothly increase with increasing frequency. The frequency used in the small strain oscillatory test should be in the linear region of the frequency scan for the data obtained to be considered reliable. At 5 Hz there was a decline in almost all of the frequency scans undertaken. The Bohlin rheometer used had a resonant frequency of 10 Hz which could account for the deviation from linearity as the frequency approached 10 Hz. While the chosen frequency of 1 Hz was in the 'linear' region of the curve, the resultant data should be valid in terms of this parameter. This data was obtained to ensure the use of an appropriate frequency.

Strain sweep is the application of increasing strain ultimately resulting in destruction of the system. The point at which destruction occurs is the failure strain. Generally, viscous samples will give a very smooth fall off at the point of destruction, if at all; a more elastic or solid-like sample will show an abrupt fall at the failure strain, but the strain may be high (for example, greater than one; Clark and Ross-Murphy, 1987). A comparison of the failure

strain measures provides an indication of the relative viscoelasticities of the samples; the greater the strain, the more elastic the sample.

5.3.3 True gels or entanglement networks?

It is difficult to distinguish between a solid and a very viscous solution. Results generated by the rheometer give an indication of the viscous or elastic characteristics, but do not differentiate between a solid and a liquid. It was stated in Chapter Four on work using myofibrillar proteins that there was no evidence presented to show formation of true gels in terms of the definition proposed by Glicksman (1982), but that the resultant 'solids' may have been very viscous solutions. Glicksman (1982) defined gelation as the association or cross-linking of randomly dispersed polymer chains in a solution to form a three dimensional network which immobilizes the liquid in the interstitial structures and which resists flow against pressure. In terms of rheology the G' values may describe an increase in viscosity or the formation of a solid network. The change from liquid to solid is continuous.

Stading (1991) reported that a gel could be defined rheologically through the dependence of G' and G" on the frequency which is usually known as the mechanical spectrum (Clark and Ross-Murphy, 1987; Ross-Murphy and McEvoy, 1986). When a polymer solution is gelled, G' increases until it reaches an apparent plateau. At this plateau, the mechanical spectrum may be obtained and the shape of it indicates if it is a gel or an entanglement structure (Figure 5.40).

A strong gel consists of a network formed by strong bonds, for example covalent bonds, while an entanglement network consists of long or branched polymer chains in high concentration which are nested into each other. A weak gel is here something intermediate between a strong gel and an entanglement network. Its network consists of chains bound by weak molecular interactions and no physical entanglements (Stading, 1991). The mechanical spectrum for the strong gel in Figure 5.40, consists of two lines which are nearly horizontal and parallel. The G" is often more straight than that shown. The most important feature is that the G' and G" are not frequency dependent. The spectrum for the weak gel is similar to the strong gel, but G' and G" increase with frequency in this case. At high frequency, the mechanical

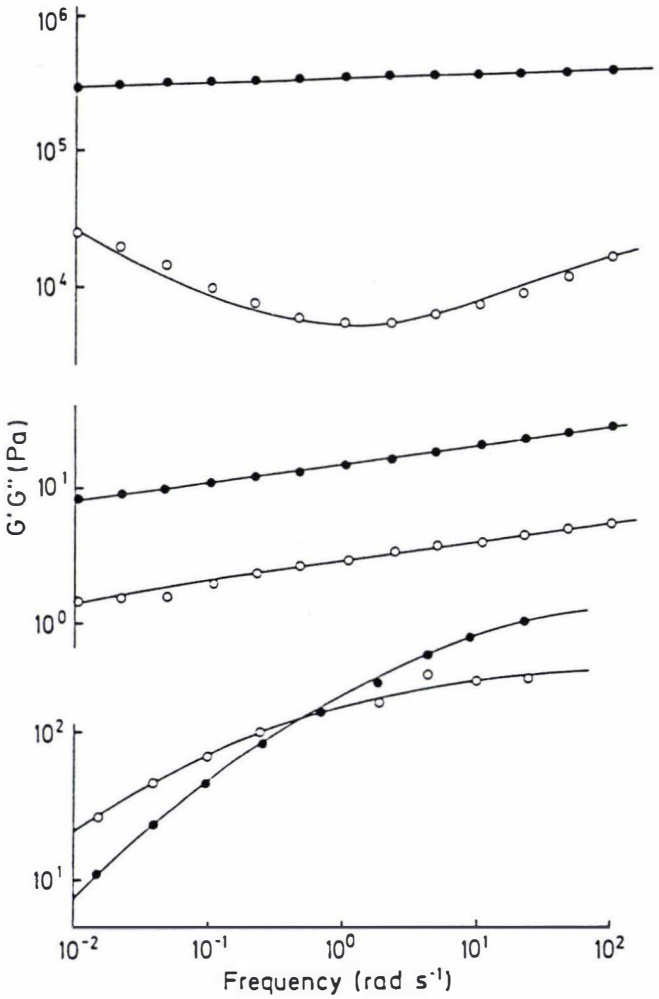


Figure 5.40. Mechanical spectra of a gel (top), a weak gel (middle) and an entanglement network (bottom). G' (●), G'' (○). From Clark and Ross-Murphy (1987).

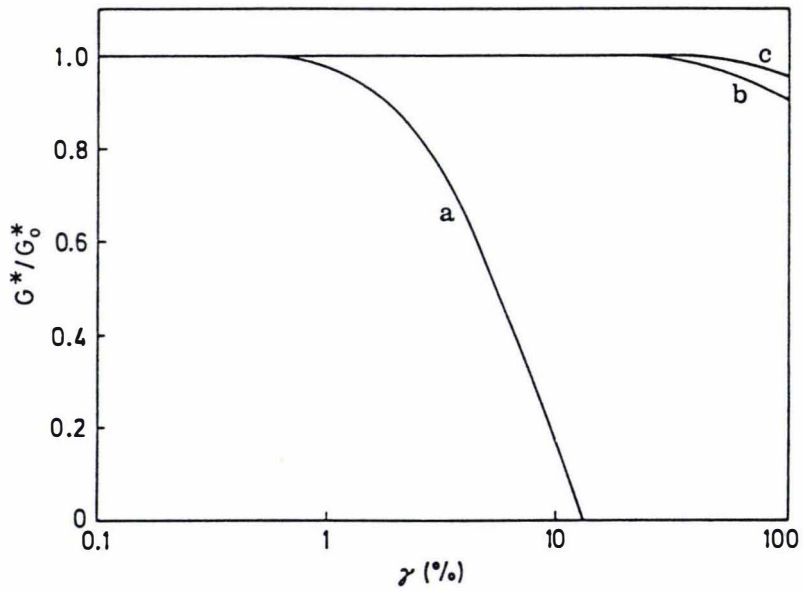


Figure 5.41 Strain dependence of the shear modulus for a) a weak gel, b) and entanglement network, and c) a strong gel. From Clark and Ross-Murphy (1987). The data were for a) 0.5% xanthan, b) 3% guar and c) 25% gelatin, respectively.

frequency of an entanglement gel could look like a weak gel or even a strong gel, but at low frequency it clearly shows that it flows. The s values (the slopes of the log of the G' versus frequency curves) used in the present experiments are ratios based on the graphs presented in Figure 5.40. A relatively high s value would indicate an entanglement network, compared to a low s value which would indicate a gel.

The strain dependence in Figure 5.41 also shows the differences between an entanglement network and a gel. A weak gel is linear only for very small strains, whereas an entanglement network and a strong gel are linear up to much higher strains. In the present experiments, comparison of these two methods provided an indication of the nature of the networks formed. Strain sweep data was measured in terms of G', however, G' should show similar trends to that of the shear modulus used in Figure 5.41. The weak systems (low G') obtained in the present system, compared to, for example, gelatin gels (Clark and Ross-Murphy, 1987), make it difficult to characterise the systems formed as true gels or entanglement networks. The characterisation as gel or entanglement network may result in data that is intermediate between the two categories. Comparison of strain sweep data and s values from different myosin systems resulted in the conclusion that most of the solids observed subjectively, were gels. The samples with TSPP added to myosin were the only samples which could have been entanglement networks. However, further characterisation of these networks was required to clearly define these gels. This may be achieved, for example, by obtaining more data for the present system and at different concentrations of myosin. Alternatively, Graesseley (1974) also reported that a weak gel could be differentiated from an entanglement network by a steady shear rate experiment. The terminal slope in a plot of log of viscosity versus the log of strain rate is then approximately 0.72 for an entanglement network and 0.9 for a weak gel.

It must be noted that Clarke and Ross-Murphy (1987) incorrectly reported the Graessley reference and stated that the calculation of terminal slope of a plot of log of viscosity versus log of strain (not strain rate) would result in data to differentiate between an entanglement network and a weak gel (Haisman, 1994). Stading (1991) has since referred to this quote by Clarke and Ross-Murphy (1987).

5.3.4 GdL addition - the phenomenon of events over the pH time course

In the previous chapter, Chapter Four, GdL was added to samples of myofibrillar proteins and gels were formed. It was postulated that the myofibrillar proteins formed gels when the pH was lowered slowly as with the addition of GdL. Extraction of the A-band occurred at a pH between 4.8 and 4.5 and at pH values below this pH, and a gel network was formed. The network was assumed to be an impregnated composite system of myosin reinforcing the already present myofibrillar structure. A minimum amount of extracted protein was required to form this gel. Complete extraction of the A-band occurred as the A-band of actomyosin was completely depolymerised and filaments were dispersed at a pH value between 4.5 and 3.9 and at pH values lower than this pH. The gel structure was suggested to no longer be an impregnated composite system, but a myosin network incorporating components, such as actin, into its structure. The loss of the myofibrillar structure reinforcing the myosin gel resulted in a decreased Young's Modulus.

In the experiments in the present chapter, GdL was added to myosin, eliminating the constraints of the myofibrillar system to gel formation. Addition of GdL to a myosin solution or suspension resulted in a slow lowering of the pH of the system. With this lowering of the pH, the fluorescence results indicated increased exposure of hydrophobic residues implying increased denaturation with decreasing pH. The fluorescence results were measured continuously and therefore were cumulative results. Hence, the plateau observed at pH 4.0 and less (Figure 5.3 and 5.4) indicated denaturation had stopped. Decreasing concentrations of GdL resulted in decreasing fluorescence curves at a given pH (Figure 5.5). Either the gluconic acid present at a given pH or the pH was responsible for the exposure of hydrophobic groups. Shielding of charges by gluconate anion could account for the small decreases observed. This would require that a lower pH be attained to achieve specified denaturation. The denaturation of myosin observed at low pH induced by the dialysis of citric acid into myosin suspensions (Fretheim *et al.*, 1985) would suggest that the denaturation is pH-induced and not a specific GdL or gluconic acid interaction.

The fluorescence experiments were undertaken on solutions of myosin instead of suspensions, because of the conditions required for measurement. It is assumed that similar trends in

results would be obtained with myosin suspensions. Rheological experiments were required to be undertaken on myosin suspensions in order that there was a sufficient concentration of myosin to allow gel formation to occur and G' to be measured. At 0.70% GdL, decreased pH resulted in increased G' of myosin suspensions down to pH 4.3 to 4.5 (Figure 5.1). At pH below 4.3 an abrupt decrease in G' was observed. Similar results were obtained at a given pH with 1.40% GdL indicating that gluconic acid concentrations at a given pH or lowering of the pH was responsible for the G' development. Similar to fluorescence, comparison with work by Fretheim *et al.* (1985) suggests that the changes in G' are largely a result of changes in pH.

The sample replicates at 0.70% GdL showed similar trends, but resulted in a wide range of data at a given pH. Ageing of myosin was reported to reduce the strength of heat-induced gels at ionic strengths of 0.1 to 0.3 (Ishioroshi *et al.*, 1979, 1983; Egelandsdal *et al.*, 1986). The loss of gel forming ability with time was suggested to be correlated with changes in the filament formability of myosin during storage. In the present work, azide was added to the myosin to slow microbial degradation of the protein and the sample was rarely kept for longer than 10 days. There was no indication of a weakening of G' due to increased sample age of the myosin used to form the gels at 0.70% GdL. In the four replicates used, the G' appeared to increase with increased age of myosin (Table 5.1). Ishioroshi *et al.* (1979) confirmed that aggregate formation occurred as the myosin solution aged.

The microstructure of myosin gels formed by dialysis to pH 4.0 at chilled temperatures was investigated by Hermansson *et al.* (1986). This study was undertaken using scanning electron microscopy (SEM) and at a concentration of potassium chloride of 0.6 M. It was observed that the network formed was fine and uniform. These results indicated that the nature of the gel was not a result of the ionic strength of the myosin system prior to denaturation. No explanation was given for this difference between thermally-induced and acid-induced gels.

In the present experiments, the gel structures observed using transmission electron microscopy (TEM) for myosin alone (Figure 5.7a-d) appeared to show aggregates of myosin strands. The size of the strands suggests that they are myosin filaments, or at least aggregates of myosin, and not myosin monomers. The apparent density of gels differed. The myosin alone appeared

to be a relatively disperse structure (Figures 5.6a-d) when compared to myosin plus 0.70% GdL at pH 4.3 (Figures 5.8a-c). The use of TEM did not produce micrographs in which the three dimensional image was as apparent as with SEM. Hence it was difficult to compare the present work with the SEM work of other researchers. Due to the limits on interpretation of the micrographs in terms of the apparent three dimensional image and the difficulty in comparison with other workers, little could be deduced from the electron micrographs.

In contrast, the s values and strain sweep data suggested that myosin alone did not form a gel. The s values and strain sweep data also did not indicate the formation of true gels of samples of myosin with 0.70% GdL added. The oscillation tests resulting in G' measurements with respect to pH (Figure 5.1) indicated that any gel that had formed, had deteriorated at the final pH and become subjectively liquid-like. This liquid-like system explains the strain sweep data and s values obtained for myosin with 0.70% GdL added.

Comparison of G' data with fluorescence results (Figure 5.6) showed that the increase of fluorescence with decreasing pH paralleled increased G'. It is suggested that as denaturation proceeded, gel formation of the denatured protein occurred down to pH 4.3. Thereafter, the gel that had formed, deteriorated with decreased pH. Ferry's theory of thermal gelation states that denaturation must precede aggregation in order for gel formation to occur (Ferry, 1948). Therefore, in order to have achieved true gels denaturation must have preceded gelation. However, if true gels formed, they were not characterised as such due to the latter gel deterioration, which preceded the strain sweep test.

At pH values less than 4.3, induced by the addition of 0.025% GdL to a myosin solution, an increase in fluorescence resulted as G' decreased (Figure 5.6). The cumulative nature of the fluorescence data suggests that as the fluorescence curve levelled with decreasing pH, denaturation stopped or fluorescence was quenched. Fretheim *et al.* (1985) observed that the gel strength decreased from 4.5 to 4.0. It was suggested that the gel network formed at pH greater than 4.5 remained dynamic. When the pH was decreased below 4.5, increased intermolecular repulsion had a deleterious effect on the strength of the gel network. These effects explain the decrease in G' evidenced at pH less than 4.3 and the deviation of the G' curve from the fluorescence curve.

5.3.5 Addition of NaCl and TSPP

5.3.5.1 The effect of addition of NaCl

In Chapter Four, the addition of 2.3% NaCl to myofibrillar protein resulted in an increase in Young's Modulus compared to myofibrillar protein alone. This was attributed to swelling of the myofibrillar structure. The addition of 2.3% NaCl and GdL to myofibrillar protein was suggested to result in an impregnated composite system at higher pH values than for GdL alone. This effect was explained by the enhanced extraction of the A-band and solubilization in the presence of NaCl. Greater extraction and solubilization of myosin allowed denaturation of more protein and a greater number of interactions to occur upon denaturation resulting in a greater Young's Modulus. This impregnated structure also appeared to remain intact to lower pH values than for GdL alone. This could be attributed to the lowering of the IEP induced by the presence of NaCl. Increased NaCl concentration resulted in increased Young's Modulus to about 22% NaCl where salting out occurred and the Young's Modulus declined.

In the present experiments, the effect of NaCl addition on myosin and its GdL-induced gels, was investigated. Using fluorescence it was observed that the denaturation of myosin in 2.50% NaCl and at two GdL concentrations was similar at a given pH (Figure 5.12). Denaturation of myosin with the inclusion of 2.50% NaCl and 0.70% GdL was less than at 0.70% GdL without the added NaCl (Figure 5.14). Increased NaCl concentration resulted in decreased denaturation at any given pH and at 0.025% GdL (Figure 5.12). These results can be explained by the effect of NaCl on the protein where the salt acts to stabilize the protein against acid-denaturation. The anions of the salt lessen the repulsive effect of the protons attached to the protein by shielding the charges. The effect of this shielding of the charges on the protein is a reduced IEP of the protein. The lower the IEP, the lower the expected pH required to result in a given amount of denatured protein. The ionic strength imparted by the concentrations of NaCl used ranged from approximately 0.022 to 0.215. However, the difference between the IEP at 0.025% GdL and 0.025% GdL with 0.025% NaCl would be small. The decreased fluorescence with added NaCl might have been the result of a quenching effect of NaCl on the fluorescent ANS compound formed. Any quenching effect of GdL would be carried through all the samples tested. However, without standards to

determine the effect of NaCl on fluorescence, a quenching effect of NaCl would result in differences between the NaCl plus GdL fluorescence data and the data for GdL alone.

A close reading of the methodology of investigations using ANS as a probe for denaturation of proteins in the presence of salts (for example, Wicker and Knopp, 1988; Wicker *et al.*, 1989; Arntfield *et al.*, 1989) showed no account of possible quenching effects of salts on fluorescence and hence one can infer that no quenching has been observed. With the elimination of the possible quenching effect it is suggested that the decrease in relative fluorescence could be a result of stabilization of the protein conformation by the salts. Increased NaCl would have an increased stabilizing effect on the protein resulting in a greater difference between the GdL and the GdL plus NaCl curves at greater NaCl concentration. This stabilizing effect, if present, like the ionic strength effect on the myosin IEP, could also explain the differences in fluorescence observed with varied NaCl concentrations (Figure 5.15).

Interpretation of the plots of the development of fluorescence with pH in Figure 5.14, suggests that the fluorescence was rapidly increasing at the final pH values attained, except for that of GdL alone. Replotting the fluorescence data of the GdL plus NaCl curve against time instead of pH (Figures 5.13) clarifies the effects of pH on fluorescence. The changes in fluorescence parallel the changes in pH for both systems. The fluorescence essentially does not increase for any significant time after the pH has essentially stopped decreasing. It is concluded from these results that the pH reduction is primarily responsible for the denaturation of the protein in these systems. Support for this suggestion is obtained from the investigation of acid-induced gelation of myosin undertaken by Fretheim *et al.* (1985) at 0.6 M potassium chloride. In these experiments, the heat absorbed to denature myosin was decreased at pH 5.5 over 40 h or more. This indicated that the denaturation was acid-induced.

Addition of 2.50% NaCl to myosin suspensions resulted in decreased G' so that the myosin behaved as a low viscosity liquid and the torque sensing element was unable to detect fluid resistance to shear above the instrument noise. Salt increases the heat-induced binding ability primarily by its ability to dissolve myosin by enhancing electrostatic repulsions (Hamm, 1960) and to dissociate myosin aggregates or filaments (Huxley, 1963). These effects increase the

number of interactions initiated by heating resulting in the formation of a firmer heat-induced gel (Siegel and Schmidt, 1979b). In the absence of heat, salt acts to dissolve myosin. This solubilization of myosin explains the solution obtained with salt addition in the absence of GdL, which resulted in G' values undetectable by the Bohlin rheometer.

Salt-induced increases in protein functionality have been clarified by more recent work. Most salt-induced increases in protein functionality were suggested to occur through changes in protein conformation (Damodaran and Kinsella, 1982). These results implicate salt-induced denaturation. It was stated that because of these changes, the proteins form a characteristic three-dimensional lattice structure when heated. Salts were suggested to produce these conformational changes by altering the hydrophobic and electrostatic interactions that stabilize the protein structure (Franks and Eagland, 1975). At high salt concentrations salts primarily affected the hydrophobic interactions, attributed to the observation that salts had little effect on electrostatic interactions at ionic strengths greater than 0.1 (Melander and Horvath, 1977). Above an ionic strength of 0.1, Von Hippel and Schleich (1969) stated that the high concentration of ions surrounding the charged protein residues shields the residues, preventing them from interacting with other charged particles. However, effects on hydrophobic interactions would suggest denaturation had occurred. Salts such as NaCl and potassium chloride have not been reported to induce denaturation. Addition of NaCl to myosin solutions did not result in fluorescence in the present experiments until the pH of the suspensions had been lowered. However, in the present experiments involving fluorescence the ionic strength was generally lower than 0.1, where it was suggested that electrostatic interactions were of greater importance.

Based on these results, it is suggested that at the ionic strength of NaCl used in the present rheological experiments (about 0.43), the increased G' compared to GdL alone, would partially be expected to be due to a shielding of charges, but largely due to increased hydrophobic interactions. However, the addition of GdL resulted in a continuously decreasing pH and therefore continuous production of positively charged sites on the protein molecules. These effects would have induced a greater electrostatic effect with added NaCl than at a fixed pH at this high ionic strength. However, the salt would also possibly also have affected hydrophobic interactions. As stated, the lack of increase of exposed hydrophobic residues is

explained by the fact that the ionic strength used in the fluorescence experiments was approximately 0.043 and therefore would affect electrostatic interactions, not hydrophobic interactions.

In the presence of 0.30, 0.70 or 1.40% GdL and 2.50% NaCl (Figures 5.9 and 5.10) the G' was similar at a given pH to myosin plus GdL without added NaCl (Figure 5.2). The G' differed in the presence of NaCl plus GdL in that it did not appear to decline or level at pH 4.3. However, in the 1.40% GdL plus 2.50% NaCl sample a levelling and possibly the start of a decline was evident at pH 3.1.

The reduction of the IEP of myosin as a result of shielded charges on the protein was studied by Sarkar (1950). It was observed that the IEP of myosin was less than pH 4.5 at 0.4 to 0.6 M potassium chloride, similar to the concentrations of NaCl used in the present experiments. The decrease in the IEP of myosin as a result of the shielded charges in the presence of NaCl could explain the continued increase in gel rigidity below pH 4.3 which was not seen in the presence of GdL without NaCl. A decline in G' appeared to be starting at pH 3.1 in the presence of 1.40% GdL and 2.50% NaCl. Perhaps a decline in G' would have been observed had the pH been decreased further, as expected with the decreased IEP. However, this would have entailed the use of greater concentrations of GdL to those used resulting in problems in achieving homogeneous addition of the GdL.

With increased concentrations of NaCl, the GdL-induced gels resulted in increased G' (Figure 5.11). The addition of GdL to myosin denatures the protein by lowering the pH as observed by fluorescence. Similar to the application of heat to a myosin suspension, pH decrease initiates interactions through denaturation resulting in gelation. Therefore, similar to heat-gelation, it is suggested that increased ionic strength or salt concentration results in a greater number of interactions initiated by lowering pH, resulting in the formation of a firmer gel as evidenced at increased NaCl concentrations (Figure 5.11).

The strain sweep data and s results in Table 5.2 indicated the formation of gel networks and not entanglements in systems of GdL and NaCl. This adds support to the suggestion that denaturation occurred and was followed by gelation. Figure 5.16 compares the denaturation

and gelation results. Similar to the results of GdL only (Figure 5.5) the two processes followed parallel trends. It appears that gelation occurred immediately upon denaturation.

Increased age of sample (Table 5.2) resulted in increased G' with increasing age with the exception of the seven day sample. This increase could be due to sample variation, but could also be a result of increased filament formation prior to sample preparation with increasing age (Ishioroshi *et al.*, 1979), as for the sample with 0.70% GdL added only.

Similar to the results of the myosin plus GdL samples, when the samples of GdL plus NaCl were too liquid-like to have G' measured accurately by the Bohlin rheometer, the visual characteristics of the gel were of a translucent liquid. As the G' developed the gels became turbid and viscous. The gels of greatest G' were white, and solid to the touch. As aggregation and gel formation occurred, the myosin became less translucent and more opaque. In the myosin alone, the ionic strength was close to zero and therefore it was expected that the myosin would be in an aggregated or filamentous form, hence the turbid appearance of myosin. As the ionic strength of the sample increased to 0.5 (Parsons and Knight, 1990) and greater, the myosin was solubilized to its essentially monomeric form and hence the solution became translucent and lost viscosity as the protein-protein interactions were reduced. Note that research has indicated that the monomeric form of myosin exists as a monomer-dimer equilibrium, in which rapid transformation can occur (Godfrey and Harrington, 1970) and hence the wording "essentially monomeric".

5.3.5.2 The effect of addition of TSPP

In Chapter Four, it was found that the addition of 0.3% TSPP to myofibrillar protein did not have any effect on the Young's Modulus of the GdL-induced gels until about pH 4.0 where the myofibrillar structure had broken down. At this pH the Young's Modulus was enhanced by the addition of TSPP.

The denaturation of myosin in the presence of 0.002% TSPP and 0.025% GdL was less than that of myosin with 0.025% GdL and similar to that with 0.025% NaCl and 0.025% GdL addition at a given pH (Figure 5.14). The small ionic strength imparted by this concentration

of TSPP was unlikely to significantly effect the IEP of myosin. The reduced fluorescence was possibly due to a stabilizing effect of the TSPP on the conformation of the protein. A similar stabilizing effect of NaCl was suggested to explain the reduced fluorescence with added 0.025% NaCl. The denaturation of 0.002% TSPP with 0.025% GdL showed opposite trends to the fall in pH both with respect to time (Figure 5.22), as was also observed for 0.025% GdL (Figure 5.4) and 0.025% GdL plus 0.025% NaCl (Figure 5.13). These results provided further support to the suggestion that denaturation is acid-induced. At higher concentrations of TSPP (0.005% and 0.012%) suspended aggregates of myosin resulted which, because of their opacity and inconsistency, resulted in inaccurate and unreliable data. There was no significant difference in the denaturation of the protein at the two GdL levels, 0.025 and 0.050%, in the presence of 0.002% TSPP at any specific pH (Figure 5.21).

The presence of 0.25% TSPP added to myosin suspensions resulted in gels of G' of 300 to 400 Pa at pH 7.6 to 8.8, approximately 27 times greater than the G' of myosin alone. However, this alkaline pH was out of the range of interest in the present work. The inclusion of 0.70% GdL in this system of myosin and 0.25% TSPP resulted in increased G' with decreased pH obtaining a range of values of final G' of 750 to 1400 Pa at pH 4.3 to 4.5 (Figure 5.18) compared to 200 to 600 Pa of myosin with GdL at pH 4.5 (Figure 5.1). There was no apparent levelling or decline in G' at the final pH of approximately 4.3. Increasing the GdL concentration to 1.40% at 0.25% TSPP resulted in a lower final pH obtained. The maximum G' attained was 750 Pa at pH 4.0, thereafter an abrupt decline was evident (Figure 5.19) similar to that at pH 4.3 to 4.5 with GdL alone (Figure 5.2). The G' at 0.30% GdL with 0.25% TSPP followed the upper curves in the range of curves at 0.70% GdL with 0.25% TSPP, and reached a minimum pH of only 5.5 (Figure 5.19).

A decreased TSPP concentration to 0.13% in the presence of 0.70% GdL resulted in a lower G' than that at 0.25% TSPP and a levelling and start of a decline at pH 4.3 and 450 Pa (Figure 5.20). The final pH attained, about pH 4.2, was less than that at the higher TSPP concentrations. Increased TSPP to 0.36% with 0.70% GdL resulted in a higher final pH of 4.7 and a G' curve which was similar to those in the upper part of the range of curves at 0.25% TSPP with 0.70% GdL.

Similar to the results of GdL (Figure 5.6) and GdL with NaCl (Figure 5.16), comparison of the denaturation data and the G' results (Figure 5.24) showed a strong correlation between the two curves. The s results and strain sweep data (Table 5.3) indicated that the myosin systems containing TSPP and GdL formed gels and not entanglement networks. However, the myosin system with only TSPP added indicated the formation of an entanglement network. But these results were not conclusive and could also have been indicative of a gel network. Similar to myosin with 0.70% GdL (Figure 5.1), the sample with 1.40% GdL and 0.25% TSPP added had reached its maximum G' and then deteriorated in terms of G' (Figure 5.19). The resultant strain sweep data and s values reflected this liquid-like state (Table 5.3).

Using TEM, myosin with added 0.25% TSPP only (Figures 5.25a-d) showed a gel similar to, but less dense than that of GdL alone (Figure 5.8a-c). There was no evidence of the strand-like appearance of myosin alone (Figure 5.7a-d) possibly due to the dense nature of the network, similar to myosin with 0.70% GdL added (Figures 5.8a-c). However, when the pH was reduced to 4.2, strands were evident and the system appeared similar to that at 0.70% GdL and 2.50% NaCl (Figures 5.17a and b).

A great deal of research has been undertaken on the role of phosphates in meat products (including, Hamm, 1971; Bendall, 1954; Trout and Schmidt, 1986a). However, little work has been undertaken on the effect of phosphates on isolated myosin. The effect of phosphates on muscle protein has been attributed to many processes. Hamm (1971) and Brotsky and Everson (1973) attributed the effect of phosphates on muscle protein to increased pH, increased ionic strength and a specific protein-polyphosphate interaction. In work on heat-induced myosin gels, Siegel and Schmidt (1979b) showed that the effect of pH was eliminated and the increase in binding ability was greater than expected by the ionic strength offered by 0.5% tripolyphosphate. The increased binding strength was therefore attributed primarily to the specific interaction between polyphosphate and the protein. This effect caused increased myosin solubility by dissociating the actomyosin contaminating the crude myosin samples. Hamm (1960, 1971) and Wierbicki *et al.* (1963) suggested that another potential mode of action of phosphates was through chelation of cations such as calcium, magnesium and zinc. Trout and Schmidt (1986a) in work using beef rolls proposed a theory to explain phosphates as meat binders in terms of pH, molar sodium chloride and molar phosphate concentration.

It was concluded that phosphates increased protein functionality at pH 5.50 to 6.35 mainly by altering hydrophobic interactions that stabilized the protein structure (Trout and Schmidt, 1986a).

Egelandsdal *et al.* (1986) stated that from research on heat-induced myosin gels, it appeared that pyrophosphate had only a simple ionic strength effect on the myosin system at 23°C. Egelandsdal *et al.* (1986) noted that pyrophosphate was a denaturant (Wright and Wilding, 1984; Tonomura *et al.*, 1963) and as such it apparently had an effect in the critical temperature range 54 to 58°C. However, the reference to Wright and Wilding is misleading (discussed in Section 6.3.1 of Chapter Six).

The large increase in G' as a result of TSPP addition to myosin in the present experiments indicated that TSPP produced an interaction not currently present or enhanced an interaction already occurring. The TSPP could act through electrostatic interactions and the little ionic strength imparted undoubtedly had an effect similar to that of NaCl, but smaller in scale due to the lower ionic strength. At the low ionic strength used, such a dramatic effect on G' as was observed was unlikely based on ionic strength effects when compared to the effect of addition of NaCl. The concept of pyrophosphate acting as a denaturant at the critical temperature could be paralleled to a critical pH. A greater amount of unfolding or denaturation of the protein would therefore be expected at a critical pH compared to samples in the absence of TSPP at similar pH. There was no observed increase in denaturation as indicated by fluorescence at any given pH.

In the absence of GdL a large G' formed immediately upon addition of TSPP to myosin. This suggested that a network had formed with the addition of TSPP to the myosin, and prior to lowering of the pH. Two possible types of network could have formed, a gel or an entanglement. Unlike the other samples investigated, the s values and the strain sweep data did not conclusively determine the nature of the network formed.

This large G' formed immediately on addition of TSPP was also evident in the presence of GdL. The strain sweep data and the s values indicated that a gel had formed in this sample,

but these measurements and calculations were made at the final pH obtained and did not necessarily relate to the network formed immediately upon TSPP addition.

An entanglement network consists of long or branched polymer chains in high concentration which are nested into each other (Stading, 1991). The rigid structure of myosin filaments is unlikely to permit the required flexibility to form an entanglement network. However, myosin monomers have a large molecular mass, approximately 500 kDa, and with the heads protruding from the coiled tail it is possible that in the essentially monomeric state, myosin could form an entanglement network. At the ionic strength of the myosin system with the added TSPP, it was expected that the myosin would be in a filamentous state. However, at the high pH induced by the addition of TSPP, the state of myosin is unsure. It is suggested that an entanglement network could occur through the addition of dissociated TSPP which would act as a concentrated negative charge, compared to, for example, NaCl addition. Addition of this charge to the largely negatively charged myosin could induce movement of the myosin molecules and result in the formation of an entanglement network.

The suggestion of entanglement network formation is based on the assumption that at the increased pH (for example, greater than pH 6.0) and low ionic strength (approximately 0.043) myosin filaments are dissociated to essentially monomeric myosin. The TEM micrographs of myosin with only TSPP added (Figures 5.25a-d) showed a lack of aggregates of strands observed, for example with myosin alone (Figure 5.7a-d). This data would support the presence of essentially monomeric myosin at the high pH values. However, this observation could have been a result of the apparent dense nature of the network with added TSPP, which may have masked any aggregation or strands. It is suggested that further TEM work might result in the determination of the state of the diluted, not dialysed myosin at different alkaline pH values at 4°C.

In support of the suggestion of entanglement network formation is the investigation of the effects of the addition of phosphates to beef rolls undertaken by Trout and Schmidt (1986a). It was concluded that at ionic strengths of salts of less than 0.15, electrostatic interactions were responsible for any effects observed.

In contrast to the suggested formation of an entanglement network, it is possible that the addition of TSPP to myosin may have induced gelation. According to Ferry's theory of gelation, denaturation must occur and precede aggregation to form a gel network (Ferry, 1948). Therefore, in order for the myosin sample to have formed a gel immediately upon addition of the TSPP, denaturation must have occurred.

Fluorescence at the initial measurement of the myosin sample suggests that no denaturation had occurred (Figure 5.14) supporting the formation of an entanglement network. However, if the denaturation occurred immediately upon addition of the TSPP and gelation occurred immediately upon denaturation, the hydrophobic sites exposed upon denaturation would no longer have been exposed at the time measurement of fluorescence began. Mixing and transference of the sample delayed the commencement of measurement of fluorescence to approximately 2.5 minutes after the addition of the TSPP and GdL. The possible denaturation and gelation prior to fluorescence measurement could explain the increased G' initially with TSPP and GdL, without any measure of denaturation. The solid formed in the absence of GdL had an almost immediate pH of 7.6 to 8.8 which is a range of pH not studied in these experiments. Perhaps, increasing the pH resulted in denaturation in a similar way to decreased pH as studied in the present experiments. Increasing the TSPP concentration, also resulted in increased initial G' (Figure 5.20) perhaps due to an increased concentration of denatured myosin.

Tonomura *et al.* (1963) observed that pyrophosphate in 0.6 M potassium chloride decreased the helical content of myosin and therefore acted as a denaturant. These findings were observed using optical rotary dispersion. Although at high ionic strength, the observations add support to the suggestion that myosin is denatured by pyrophosphate. The gel formed subsequent to denaturation, may involve electrostatic interactions. The ionic strength used, 0.6, is, however, in the range where Trout and Schmidt (1986a) stated that salts act through hydrophobic interactions. Therefore, according to the work of Trout and Schmidt (1986a) it is unlikely that TSPP, at the concentration used in the present experiments would have denatured myosin.

Trout and Schmidt (1986a) concluded that phosphates increased protein functionality at pH 5.50 to 6.35 mainly by altering hydrophobic interactions that stabilized the protein structure, supporting the suggestion that TSPP resulted in denaturation and subsequent gelation without lowering the pH. However, Trout and Schmidt (1986a) studied the effects of phosphates at ionic strengths of 0.15 to 0.43. At ionic strengths less than this, electrostatic interactions were thought to be responsible for any effects observed. Comparison of the results for added 0.25% TSPP with 2.50% NaCl in the present experiments suggests that electrostatic interactions were not responsible for the large initial G' observed in the TSPP system. Tonomura *et al.* (1963) suggested that pyrophosphate acted as a denaturant adding support to the suggestion that TSPP denatured myosin and formed a gel network through hydrophobic interactions at the low ionic strength used. However, electrostatic interactions may have participated in gel formation upon TSPP-induced denaturation of myosin.

Superimposing the 0.25% TSPP plus 0.70% GdL curve of G' values (Figure 5.18) onto the G' curves for 0.70% GdL (Figure 5.1) and 0.70% GdL plus 2.50% NaCl (Figure 5.9), it is observed that the shapes of the curves are similar down to pH 4.5 to 4.3. However, in the presence of TSPP the G' values became slightly greater than the values of the other samples as pH decreased. This could be explained by enhanced hydrogen bonding with the added TSPP. The actual TSPP plus GdL curve is greater at a given pH than the GdL or GdL plus NaCl curve. This difference is approximately the G' achieved immediately upon addition of TSPP and GdL. The G' data for the TSPP and GdL curve did not decrease at about pH 4.3 as for GdL alone. This could be due to the extra stability provided by the initial network formed with the addition of TSPP, and perhaps the enhanced intermolecular hydrogen bonding or electrostatic interactions of the acid-induced network.

Many of the theories proposed by other workers to explain the effects of phosphates on meat and myosin gelation and functionality have already been questioned or disputed. A more detailed discussion is given in Chapter Six.

In contrast to the samples with added GdL, and GdL with NaCl, increased sample age did not appear to result in increased G' with included TSPP and GdL (Table 5.3). This effect cannot be explained.

5.3.5.3 The effect of addition of NaCl and TSPP

In Chapter Six, it was found that the lack of effect of TSPP on the GdL-induced myofibrillar protein was observed when 0.3% TSPP and 2.3% NaCl were added together. The results resembled those of NaCl addition with and without GdL.

Fluorescence of myosin with added 0.025% GdL, 0.025% NaCl and 0.002% TSPP increased with decreasing pH (Figure 5.31). Similar to the samples with 0.025% GdL alone (Figure 5.4), 0.025% GdL with 0.025% NaCl (Figure 5.13) and 0.025% GdL with 0.002% TSPP (Figure 5.22), the fluorescence increased parallel to the decreasing pH with respect to time. The pH attained its minimum at 4.0. The fluorescence was less than that with 0.025% GdL, 0.025% GdL and 0.002% TSPP, or 0.025% GdL and 0.025% NaCl (Figure 5.13) possibly due to the combined stabilizing effects of the TSPP and NaCl and the lowered IEP with the increased ionic strength.

Addition of both 2.50% NaCl and 0.25% TSPP to myosin suspensions resulted in a clear solution similar to that obtained with the addition of NaCl alone. The solution was very liquid-like and below the sensitivity of the rheometer. With the addition of 0.70% GdL, 0.25% TSPP and 2.50% NaCl, a white solid had formed after 15 h. The solids formed were gels and not entanglement networks as evidenced by the calculated s values and the strain sweep data (Table 5.4).

The G' values of myosin with added GdL, 2.50% NaCl and 0.25% TSPP, were similar to GdL with 2.50% NaCl, at 0.70% GdL and 1.40% GdL (Figures 5.28 and 5.29). The minimum pH attained was 4.0. Similar to the G' curve observed with 2.50% NaCl and 0.70% GdL, the G' of 0.70% GdL, 2.50% NaCl and 0.25% TSPP was approximately zero at the initial pH. No decline was evident at the minimum pH values obtained, with the addition of 0.70% GdL, 2.50% NaCl and 0.25% TSPP. This can be explained by the decreased IEP and therefore the shielding of charges on the protein requiring that the pH must be lower for the repulsive charges to have a deleterious effect on gel structure as suggested to occur with the addition of NaCl plus GdL.

At 0.70% GdL, the curve of GdL with 2.50% NaCl and 0.25% TSPP was intermediate between GdL with TSPP and GdL with NaCl. It more closely resembled that of NaCl and GdL, but at a higher pH for a given G'. At 1.40% GdL the maximum G' was similar to that of GdL with TSPP, but the curve neither resembled that of GdL with TSPP nor GdL with NaCl.

A dispersed structure was observed using TEM (Figures 5.32a-c). Strands were evident as with myosin alone (Figures 5.7a-d), myosin with added NaCl and GdL (Figures 5.17a and b) and myosin with added TSPP and GdL (Figures 5.26a-d)

A synergistic effect has been noted on binding strength in meat products with the addition of salt and TSPP (for example, Theno *et al.*, 1978; Samejima *et al.*, 1985; Moore *et al.*, 1976). This effect was not evident in the present work. It would appear from the closer similarity of results of myosin with GdL, NaCl plus TSPP, and myosin samples with added GdL plus NaCl that the effects of NaCl on myosin dominated the effects of TSPP. However, TSPP appeared to play a role in that although the trends of the G' curves better resembled those of GdL with NaCl, the G' values were generally greater than with the added NaCl. It is suggested that there is a combined effect of the two additives, TSPP and NaCl, but this effect was not synergistic in terms of G'.

5.3.6 Bonding in gelation

Altering the pH using GdL undoubtedly resulted in electrostatic interactions. Electrostatic repulsive forces are coulombic repulsions between similarly charged groups and are more likely to occur than attractive forces with excess groups of the same charge. Ionic bonds are coulombic attractions between oppositely groups and have a bond enthalpy of 42-84 kJ/mol (Table 2.3). Ionic bonds have a higher bond enthalpy and are therefore less likely to form than electrostatic interactions in a low energy system. It is difficult to investigate electrostatic interactions without introduction of another system, such as the Hoffmeister series of salts. Electrostatic interactions are likely to play a role in the gelation process with the varying pH and added salt and phosphate. However, without further addition of salts or alteration of the pH of the system, the relative amount of this bonding is unable to be easily investigated.

Other bond types are also likely to have roles in the gelation process. Already it has been observed that hydrophobic sites were exposed with the lowering of the pH implying denaturation had occurred. Formation of a more stabilized molecule where the hydrophobic sites are no longer exposed would likely play a role in the gel formation. Hydrophobic interactions involve induction of dipole moments in apolar groups. The bond enthalpy associated with this interaction is relatively small at 4-13 kJ/mol (Table 2.3). The fluorescence measurements, taken on a cumulative basis due to the continuous nature of GdL hydrolysis, did not allow a decrease in hydrophobic site exposure to be observed should the molecules reform stable conformations. Heat gelation to specified temperatures has enabled these measurements to be followed (Ma and Holme, 1982; Wicker and Knopp, 1988; Arntfield *et al.*, 1989; Chan *et al.*, 1992). Use of similar techniques in the present experiments, where the pH of myosin was lowered to a specified pH and fluorescence measured, resulted in sample variance too large to enable any change in hydrophobic site exposure to be observed.

5.3.6.1 Hydrogen bonding

To investigate the role of hydrogen bonding in GdL-induced myosin gel formation, the use of a competitive source for hydrogen bonding was adopted. Hydrogen bonding is bonding in which a hydrogen atom serves as a bridge between two electronegative atoms, holding one by a covalent bond and the other by purely electrostatic forces (Morrison and Boyd, 1983). This bonding has an enthalpy of 4-21 kJ/mol (Table 2.3).

The large number of hydrogen and hydroxyl groups on carbohydrates and the relatively small size of these sugars, compared to myosin, makes sugars strong hydrogen bond competitors. A 10.0% sucrose solution included in a myosin sample with 0.70% GdL resulted in decreased G' (Figure 5.37), although the final sample consistencies were similar, being liquid-like with or without the added sucrose. The lowered G' with included sucrose indicated less gel formation and suggested that sucrose had inhibited intermolecular hydrogen bonding. However, the G' was only partially decreased compared to the G' attained in the absence of sucrose indicating that hydrogen bonding was only one of the types of bonds responsible for gelation.

Inclusion of 0.25% TSPP in the myosin, 0.70% GdL and 10.0% sucrose system also resulted in decreased G' (Figure 5.38). The initial G' of this curve was not decreased by the use of sucrose as a hydrogen bond competitor. Therefore, it is suggested that the initial network formation prior to acid-induced denaturation did not involve hydrogen bonding to any significant degree. Hydrophobic and electrostatic interactions may have been the dominant bond types. However, the large G' at pH 4.5 was significantly decreased with the inclusion of sucrose in this system. The resultant G' was similar to the G' of 0.70% GdL with added 10.0% sucrose, plus the added G' attained immediately upon the addition of 0.25% TSPP. These results suggested that the initial gel formed with TSPP addition did not involve hydrogen bonding and was retained throughout the subsequent acid-induced denaturation and following gelation. It is also suggested that the slightly enhanced G' at given pH compared to 0.70% GdL only or 0.70% GdL plus 2.50% NaCl to pH 4.5 was a result of enhanced hydrogen bonding. The hydroxyl groups of the undissociated TSPP at this pH would form hydrogen bonds with available sites on the protein molecules, for example carboxyl and amine groups. If this hydrogen bonding occurred across the TSPP and connected protein molecules, the G' of the resultant gel might be increased.

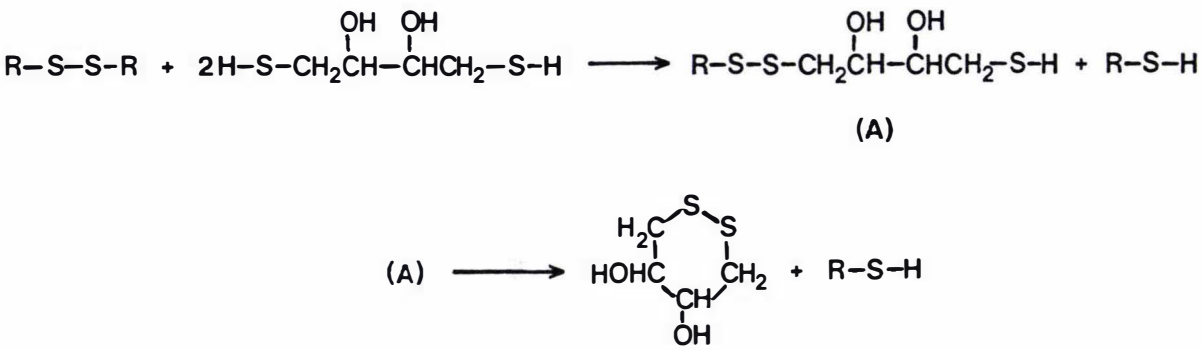
The myosin, GdL, and sucrose system resulted in enhanced G' in the presence of NaCl (Figure 5.39). Sorbitol and glucose were also investigated to determine if the effect was sucrose specific or a general carbohydrate effect and G' was increased in each study (Figure 5.39). The use of glucose also provided evidence that the effect was not limited to non-reducing sugars, but a general carbohydrate effect. With the limited data presented, this effect of increased G' could not be explained.

5.3.6.2 Sulfhydryl bond formation

In an investigation by Fretheim *et al.* (1985), addition of dithiothreitol (DTT) partially decreased the gel strength of myosin gels induced by acid-dialysis. The gels had a pH of 4.5 and potassium chloride concentration of 0.6 M. These results suggested that the formation of disulphide bonds was involved in network formation. Although a myosin molecule contains more than 40 cysteine residues (Buttkus, 1971; Hofmann and Hamm, 1978), only two thiol groups of cysteine residues, called SH-1 and SH-2, which are located on the myosin heavy

chain (MHC) in the S-1 domain (Balint *et al.*, 1978; Mornet *et al.*, 1984) have been shown to be reactive. It was suggested (Samejima *et al.*, 1988) that these groups may participate in generating disulphide cross-linkages during heat-induced gelation. With the parallel between acid-induced gelation and heat-induced gelation it is therefore possible that these thiol groups could also be participating in acid-induced gelation. Disulphide cross-linkages are covalently bound cross-linkages, that is, the linkages involve the sharing of electrons. Covalent linkages have relatively high bond enthalpies of 126-419 kJ/mol (Table 2.3). Two compounds, PCMB and DTT were used to block and reduce the sulfhydryl groups, respectively, preventing the groups from forming disulphide cross-linkages. PCMB is effective by the reaction adapted from the general transmetallation reaction of organomercury compounds described by Bloodworth (1977),

Bloodworth (1977) also stated that while hydrogen-ion concentration can affect formation of Hg-S complexes, the resulting complexes are usually stable over the entire pH range. The compound DTT reduces disulphide bonds and through a mass action effect keeps protein thiol groups in their reduced state. The reaction was described by Fontana and Toniolo (1974),



In the present experiments, there were no significant differences between the rigidities of the "blocked" and the control samples and therefore there was no evidence to support the participation of disulphide cross-linkages in the GdL-induced gelation of myosin (Figures 5.33 to 5.36). The addition of 80 mol PCMB/mol myosin (0.10%) resulted in a clear gel after 24 h. Upon addition of TSPP and GdL the gel was stirred and subsequently broken. The resulting gel formed with addition of TSPP and GdL was less rigid than that without the added PCMB. However, this difference in rigidity could not be taken as evidence of disulphide cross-linkages participating in gelation of myosin due to the unusual conditions of the formation and breakage of the gel formed prior to the addition of TSPP and GdL. Addition of 80 mol DTT/mol myosin (0.051%) did not result in the formation of a gel and the rigidity of the gel formed with the further addition of TSPP and GdL was not significantly different to that formed without the addition of DTT. These results are contrary to those of Fretheim *et al.* (1985) and suggested that the head to head interactions indicated by disulfide bonding were not involved in acid-induced gelation.

5.3.7 A postulated sequence of events

The events which occurred over the course of gelation resulted in data, much of which is contradictory to results presented by other workers. A brief summary of these contradictions follows.

Denaturation by lowering the pH implied the involvement of hydrophobic interactions in the present experiments. This type of bonding was not mentioned in the investigation of acid-induced myosin gelation undertaken by Fretheim *et al.* (1985). A slow rate of denaturation was measured at pH 5.5 in the investigation of Fretheim *et al.* (1985). Denaturation, in the present experiments, appeared rapid and began at pH 5.0 to 5.5. Sulfhydryl bonding was suggested to participate in acid-induced gelation of myosin in the investigation of Fretheim *et al.* (1985), but in the present experiments there was no evidence of this type of bonding involved in gelation. However, hydrogen bonding was shown to be involved in gel formation in the present experiments, but was not mentioned in the investigation of Fretheim *et al.* (1985). Fretheim *et al.* (1985) observed decreased gel strength at low ionic strength compared to high ionic strength and attributed this effect to lesser shielded charges of the protein at low

ionic strength. The ionic strength was lowered after gelation had occurred. The present experiments supported the suggestion that the effect of low ionic strength was related to the lesser amount of shielded charges. However, in the present experiments, the ionic strength was lowered prior to gelation and by using this method, the effects of lowered ionic strength on myosin could be interpreted in terms of the IEP of myosin.

Trout and Schmidt (1986a) suggested that in heat-induced gelation over the pH range 5.50-6.35 and at ionic strengths of greater than 0.1 M, hydrophobic interactions were primarily responsible for gelation. In support of Fretheim *et al.* (1985), the present experiments suggested that in acid-induced denaturation, electrostatic interactions played a large role in gelation. However, hydrophobic interactions were implicated. The inclusion of TSPP in the present experiments resulted in increased G', compared to in the absence of TSPP. It was suggested that denaturation could possibly have occurred supporting the suggestion of Tonomura *et al.* (1963) that TSPP is a denaturant. This gelation was suggested to involve hydrophobic and electrostatic interactions contradictory to the results of Trout and Schmidt (1986a) where electrostatic interactions only were considered important at ionic strengths of less than 0.1. However, in support of Trout and Schmidt (1986a) is the suggestion that an entanglement network may have formed due to movement of protein molecules induced by the charged phosphate molecules and therefore network formation could be a result of electrostatic interactions. Enhanced G' subsequent to this initial gel or entanglement network formation compared to GdL alone, was suggested to be achieved through enhanced hydrogen bonding.

The following discussion contrasts data from the present experiments with that from other workers in an effort to reconcile some of the observed discrepancies. Finally, a sequence of events is postulated, resulting in gelation.

5.3.7.1 Discussion of contradictory results

In the determination of the mechanism of GdL-induced gelation, the types of bonds and interactions involved have been investigated. The addition of GdL alone resulted in hydrophobic site exposure which would likely have produced hydrophobic interactions

involved in the subsequent formation of a three-dimensional network. Fretheim *et al.* (1985) and Hermansson *et al.* (1986) observed gelation of myosin at 4°C via dialysis. Fretheim *et al.* (1985) investigated denaturation of the myosin which should implicate hydrophobic interaction with the exposure of these sites as the molecules are unfolded. However, there was no mention of this type of interaction and its possible participation in gel formation. Fretheim *et al.* (1985) and Hermansson *et al.* (1986) studied myosin in salt concentrations of 0.6 M potassium chloride, introducing ionic strength effects to the system. The investigation of Hermansson *et al.* (1986) was of the different structures observed under SEM at varying conditions of pH, ionic strength and temperature, and did not discuss the bonding involved in gelation.

Similar to the work of Fretheim *et al.* (1985), denaturation of myosin was observed at pH 5.0 to 5.5. Denaturation at pH 5.0 to 5.5 in the experiments undertaken by Fretheim *et al.* (1985) proceeded slowly. After 40 h at pH 5.5, the transition of heat absorbed at denaturation had almost disappeared indicating acid-induced denaturation. This rate of increase of denaturation was not investigated at lower pH values. The rapid denaturation observed in the present experiments was generally at pH values lower than 5.5, but started to occur at this pH. With harsher pH conditions, as with increased temperature, increased denaturation could be expected.

In the present work, denaturation was measured by a hydrophobic probe method, whereas Fretheim *et al.* (1985) used differential scanning calorimetry, (DSC). The method of DSC was unable to be used in the present experiments due to the myosin concentration of the gels formed being too low for the sensitivity of the available differential scanning calorimeter. Fretheim *et al.* (1985) concentrated gels of myosin by centrifugation to enable DSC analysis. In heat-induced gelation, several transition temperatures have been observed (Chapter Two, Section 2.4.2.4) indicating denaturation of different regions of the myosin molecule. Fretheim *et al.* (1985) only investigated the disappearance of one denaturation transition at approximately 50°C.

The lowering of the pH would change the charges on the myosin molecules affecting electrostatic interactions. The extent of these interactions is difficult to determine without the

addition of salts or other acids, which would add extra parameters to the system and change the IEP of myosin. Fretheim *et al.* (1985) used electrostatic interactions to explain the effects of lowered pH on myosin. The present results support the suggestion that the lowering of the pH from the IEP of myosin (pH 5.4) increased the positive charges on the myosin molecules and thereby the repulsion between them. It was also presumed in the present experiments as in the investigation of Fretheim *et al.* (1985), that the gel strength decreased below pH 4.3-4.5 because the network formed initially remained dynamic and allowed increased intermolecular repulsion to have a deleterious effect on the strength of the network. Increased intermolecular repulsion was introduced through further decrease in pH. Fretheim *et al.* (1985) observed these effects at a salt concentration of 0.6 M potassium chloride. Lowering of the salt concentration resulted in decreased gel strength at pH 4.5. The loss of gel strength was attributed to loss of shielded charges. However, gelation was not instigated at this salt concentration as in the present experiments, and the salt concentration was lowered subsequent to gel formation, by diaysis. Therefore, the effects of, for example lowered IEP, on the gelation process were not observed in the investigation of Fretheim *et al.* (1985). Fretheim *et al.* (1985) could have implied that the lesser shielding of charges meant the pH at which maximum gel strength occurred was higher. However, this implication was not stated and this assumption is difficult to draw from the limited experimental work presented.

The use of a low concentration of myosin in the study by Fretheim *et al.* (1985) of 10 mg/ml compared to 20 mg/ml in the present experiments could explain the decline in gel rigidity with decreasing pH from pH 4.5 compared to 4.0 in the present experiments at similar salt concentration. A lower concentration of myosin would have less protein in the network structure, therefore a lesser number of possible interactions, such as hydrophobic and electrostatic interactions. The lesser number of interactions would make the structure weaker at the lower protein concentration. A lesser excess of repulsive interaction might result in deterioration of the gel structure at 10 mg/ml compared to 20 mg/ml myosin. Experiments using 10 mg/ml myosin on the Bohlin rheometer might resolve this discrepancy.

The addition of NaCl to myosin plus GdL would be expected to result in increased interaction when the protein is denatured. The increased number of interactions would result through solubilization of myosin due to enhanced electrostatic repulsions (Hamm, 1960) and

dissociation of the myosin filaments (Huxley, 1963). Salts have been suggested to produce conformational changes by altering the hydrophobic and electrostatic interactions that stabilize the protein structure (Frank and Eagland, 1975). Trout and Schmidt (1986a) suggested that at high ionic strengths (>0.1), salts primarily effect the hydrophobic interactions, since salts have little effect on electrostatic effects at ionic strengths greater than 0.1 (Melander and Horvath, 1977). Above an ionic strength of 0.1 the high concentration of ions surrounding the charged protein residues shields the residues, preventing them from interacting with other charged particles (Von Hippel and Schleich, 1969). However, electrostatic charges are effected by the charges on the protein. Continuous lowering of the pH of the solution results in a continuous change in the charges on the protein. Therefore, electrostatic interactions would likely play a greater role than at constant pH.

Introduction of TSPP to the myosin system resulted in immediate formation of a solid-like system. Strain sweep data and s values indicated the gel was possibly an entanglement network, but could not conclusively be differentiated from a gel. This immediate network formation was also observed for the myosin, TSPP and GdL system. According to Ferry's theory of gelation, denaturation must precede aggregation for gel formation to occur (Ferry, 1948). Similar to work of Tonomura *et al* (1963), if a gel had formed it appeared that phosphate must have induced denaturation of the myosin and subsequently gel formation had occurred. In the presence of GdL this was observed as a high G' immediately upon the commencement of measurement of G'. Denaturation or unfolding of the myosin would likely result in hydrophobic bond formation. According to Trout and Schmidt (1986a), salt addition, such as phosphate addition at pH 5.50 to 6.35, effected electrostatic interactions at ionic strength of less than 0.1, not hydrophobic interactions. However, unfolding of the molecules in the present experiments, would have resulted in the exposure of hydrophobic sites. The exposure of these sites suggests involvement of hydrophobic interactions in gel formation, but it is also possible that electrostatic interactions were involved.

Trout and Schmidt (1986a) suggested that at low ionic strengths phosphates, like other salts, acted through electrostatic interactions. This investigation was undertaken at pH 5.50 to 6.35. In the present experiments, electrostatic interactions could be responsible for network formation at the high pH obtained initially with the addition of TSPP. At this pH the TSPP

would have a greater percentage of dissociated hydroxyl groups than at pH about 4.5. Dissociated TSPP, as a polyanion, is able to interact with the cationic groups of the myosin molecules forming intermolecular crossbridges which could have participated in gel formation after denaturation if a gel formed. However, if an entanglement network formed, the introduction of dissociated TSPP molecules and therefore concentrated anionic charge could have resulted from the movement of the myosin through repulsion. This suggestion of entanglement network formation supports the work of Trout and Schmidt (1986a) where the effects of phosphates at low ionic strengths were attributed to electrostatic effects. In contrast to the smaller ions of NaCl, the ionic charges concentrated on the TSPP molecule could induce an effect greater than and different to that observed with the addition of NaCl.

The inclusion of sucrose in the myosin plus GdL system resulted in decreased G' compared to in its absence. The large number of hydroxyl groups on the sucrose molecule makes it a strong competitor for hydrogen bond sites. A reduction in the G' with the addition of sucrose indicates reduced intermolecular hydrogen bonding. As for hydrophobic interactions, this type of bonding was not mentioned in the formation of myosin gels at 4°C in the work of Fretheim *et al.* (1985).

The inclusion of sucrose in the myosin, GdL and TSPP system resulted in decreased G' compared to the sample without the added sucrose. The shape of the G' curve of the TSPP plus GdL sample was similar to, but increased at a slightly higher rate with respect to pH than the sample with GdL alone down to pH 4.5. Addition of sucrose resulted in a G' similar to that of the combined G' of the GdL sample with added sucrose, plus the gel formed immediately upon TSPP addition. From these results it was suggested that at this low concentration of TSPP, 0.25%, the phosphate effect on myosin was not due to the added ionic strength or electrostatic interactions. Instead hydrogen bonding appeared responsible for any added gel rigidity formed through acid-induced gelation compared to G' in the absence of TSPP. The initial network formed was not affected by the inclusion of sucrose and it is suggested that this network was a result of electrostatic and hydrophobic interactions. This network formed initially appeared to remain throughout the acid-induced gelation. Trout and Schmidt (1986a) did not mention hydrogen bond involvement in gelation of myosin.

No sulfhydryl bonding was observed to be involved in gelation in the present experiments using DTT and PCMB as sulhydryl-blocking agents. Fretheim *et al.* (1985) using DTT observed that sulfhydryl bonding was involved in gelation. There was no mention of the use of DTT in the materials and methods section of the paper. Data in the results section indicated one measurement at pH 4.5 and 0.6 M potassium chloride. Data for gel strength was collected using a plunger test on an Instron Universal Testing Machine and the initial slope was normalised and recorded as percent gel strength. While error bars were provided for other gel strength measurements and the error was small, there was no indication of the reproducibility of this sample using this relatively crude method of measurement of gel strength, compared to measurement of G' using Bohlin rheology. Normalizing the sample could also have affected the placement of the data obtained in the DTT experiment of Fretheim *et al.* (1985).

5.3.7.2 A postulated sequence of events

A sequence of events is postulated to describe the response of myosin to decreases in pH induced by the addition of GdL.

As the pH of myosin solution with added GdL was decreased, exposure of hydrophobic sites resulted indicating denaturation of the protein had occurred. Parallel to denaturation was the process of gel formation involving hydrogen bonding, and hydrophobic and electrostatic interactions. Eventually denaturation stopped (pH 4.3 to 4.5), but the pH continued to decrease and the gel formed became liquid-like due to an excess of repulsive electrostatic forces.

The inclusion of 0.025% NaCl in the myosin plus 0.025% GdL system resulted in less denaturation at a given pH. The amount of denaturation increased with decreasing pH. The addition of NaCl lowers the IEP of myosin and stabilizes protein molecules by shielding the charges of the protein as the pH is reduced. Therefore, with increased NaCl a lower pH was required to result in a given amount of denaturation compared to in the absence of NaCl. A stabilizing effect of NaCl on the protein conformation could also have decreased denaturation.

The addition of 2.50% NaCl to a myosin suspension resulted in solubilization of the myosin and a translucent solution was obtained. With added GdL, a gel formed and the G' of the gel was essentially the same at pH above 4.5, with or without the addition of 2.50% NaCl. However, as the pH decreased below 4.5 there was no observable decline in the G' until pH 3.1 in the presence of NaCl. This was attributed to the lowering of the IEP of myosin by NaCl, where shielding of charges lessens repulsion between molecules and therefore the deleterious effect of excess repulsive forces would not have been achieved until this lower pH value. Increasing the concentration of NaCl increases the degree of dissociation of myosin aggregates or of myosin filaments and solubilizes the protein. These effects resulted in increased interactions initiated by denaturation. These increased interactions resulted in more bonding involved in the three-dimensional matrix formed and increased G'. The bonds involved in gel formation with NaCl plus GdL were electrostatic and hydrophobic interactions. There was no evidence to support or oppose hydrogen bond involvement.

The addition of 0.25% TSPP to myosin resulted in formation of a gel or entanglement network at pH 7.6 to 8.8. If a gel had formed, the addition of TSPP to myosin may have induced denaturation immediately upon addition, possibly by increasing the pH. Gelation could have occurred immediately upon denaturation and involved hydrophobic and electrostatic interactions. These effects would have resulted in gel formation prior to acid-induced denaturation. However, if an entanglement network had formed and not a gel, this could be explained by the introduction of dissociated TSPP molecules. These concentrated ionic charges could induce movement of the protein molecules and result in an entanglement network.

Addition of GdL plus TSPP produced decreased pH and acid-induced denaturation resulted after initial gel formation. The initial network formed remained throughout the acid-induced gelation. Hydrogen bonding played a role in the secondary gelation enhancing the G' to values greater than that resulting from acid-induced gelation in the absence of TSPP. Hydrophobic and electrostatic interactions were also likely to be involved.

The combination of GdL, TSPP and NaCl addition to myosin resulted in G' values intermediate between GdL plus TSPP and GdL plus NaCl. Denaturation was decreased

compared to GdL with TSPP or GdL and NaCl, possibly a result of the additive stabilizing effects of these salts on the protein conformation and ionic strength effects on the myosin IEP due to the addition of NaCl and TSPP. The G' effects of addition of both NaCl and TSPP appeared to be dominated by the NaCl effects and therefore the effects of ionic strength. However, TSPP influenced the resultant G' and denaturation. There was no evidence of sulfhydryl bond formation in any of the gels formed.

It is difficult to compare the composite myofibrillar system with data from a crude myosin system, essentially a single myofibrillar protein, without knowledge of the interactions among protein or the dissociation state of the myofibrillar protein. However, the following trends were observed.

The G' of the myosin and GdL sample followed similar trends to the Young's Modulus of the myofibril and GdL system, increasing to a maximum at pH about 4.3 to 4.5 then abruptly decreasing. The inclusion of NaCl with GdL resulted in increased G' or Young's Modulus in myosin or myofibrillar protein, respectively. Inclusion of TSPP in the myofibrillar system had no effect on gelation until the myofibrillar structure had been completely broken down. In the myosin system, the restrictions of the myofibrillar structure were removed and TSPP formed gels with myosin alone and increased the G' of myosin and GdL gels compared to gels formed in the absence of TSPP. Combinations of TSPP and NaCl did not result in a synergistic effect on the gel characteristics and the resultant data was dominated by the effects of NaCl addition or was intermediate between the effects of NaCl and TSPP addition for both the myofibrillar protein and myosin.

In Chapter Six, the effects of TSPP on myosin are further investigated. The effect of phosphate on myosin has had little previous investigation, although there is much work on meat products or myofibrillar proteins. Many theories to explain the effects of phosphate on heat-induced gelation, in terms of the greatly increased gel strength (or similar parameters), have been proposed and many disputed. Trout and Schmidt (1986a, 1987) undertook a series of experiments to investigate hydrophobic and electrostatic interactions induced by the presence of phosphates. The experiments involved the use of phosphates of varying chain lengths at similar molar strengths. Similar investigations using phosphates such as

tripolyphosphate and hexametaphospate would add further understanding to the process of gel formation of myosin.

In addition to studies of gel formation induced by heating myosin, the reversibility of these gels has often been investigated and reversibility of acid-induced myosin and myofibrillar protein gelation is the subject of study in Chapter Seven. This characteristic provides an indication of the extent and nature of denaturation and the stability of the gel formed. In heat-induced gelation, reversibility is studied merely by cooling the protein system. However, in the acid-induced gel situation, reversibility is more difficult to achieve as the acid must be removed or the pH of the sample raised. In the present situation, the pH was lowered slowly by using GdL. The rate of removal of the acid or increase of pH may be important in terms of reversibility. Investigations of the reversibility of the gels formed would provide further understanding of the nature of the gel formed.

CHAPTER 6

THE EFFECT OF PHOSPHATES ON GdL-INDUCED MYOSIN GELATION

6.1 INTRODUCTION

Many meat products today incorporate salt (sodium chloride) to aid in product binding, flavour and presentation (Schmidt *et al.*, 1981; Acton *et al.*, 1983). The possible involvement of sodium consumption in the development of hypertension has prompted public health and regulatory authorities to recommend a reduction in the dietary intake of sodium chloride (Sofos, 1989). As sodium chloride levels are reduced in meat products however, meat cohesion, water retention, product quality and shelf-life may be compromised (Sofos, 1983, 1985a, b, 1986b). The meat industry has therefore turned to the use of various phosphates, which can partially replace sodium chloride and restore product quality and identity (Keeton, 1983; Madril and Sofos, 1985; Puolanne and Terrell, 1983a, b; Seman *et al.*, 1980; Sofos, 1985a,b; Trout and Schmidt, 1984, 1986a; Whiting, 1984).

Phosphate ions have more than one negative charge and can act as polyanions in solution. In general, phosphates can interact with polyelectrolytic organic compounds, such as proteins (Halliday, 1978). Phosphates increase the ionic strength of solutions and can bridge two or more positively charged sites, and thus bind components or particles, which can lead to precipitation (Steinhauer, 1983). By attaching one end of their chain to a positively charged site and the other to water molecules, phosphates can also maintain particles in solution (Wagner, 1986).

Although a great deal of research has been undertaken on the role of phosphates in meat products (including Bendall, 1954; Fukazawa *et al.*, 1961c; Trout and Schmidt, 1986a), little work has been undertaken on the effect of phosphates on isolated myosin. This has not,

however, prevented a number of hypotheses being suggested to explain the effect of phosphates on heat-induced gelation of myosin or meat proteins, largely based on studies using meat or myofibrillar proteins.

These hypotheses have suggested phosphate action via ionic strength effects (Brotsky and Everson, 1973; Hamm, 1971), pH increase (Brotsky and Everson, 1973; Hamm, 1971), chelation of cations, such as calcium, zinc and magnesium (Hamm, 1960; 1971; Wierbicki *et al.*, 1963), specific protein-phosphate interaction (Brotsky and Everson, 1973; Hamm, 1971; Halliday, 1978; Steinhauer, 1983; Siegel and Schmidt, 1979b), and hydrophobic interactions (Trout and Schmidt, 1986a). Tonomura *et al.* (1963) noted that pyrophosphate was a denaturant and as such apparently had an effect in the critical temperature range of heat-induced gelation, 54 to 58°C.

In the previous experiments using myofibrillar proteins, it was found that pyrophosphate had no effect on Young's Modulus until the myofibrillar structure had apparently completely disintegrated. With the loss of the myofibrillar structure, the tetrasodium pyrophosphate (TSPP) enhanced the Young's Modulus of the gel formed. No synergistic effect was apparent with sodium chloride (NaCl) and TSPP at the concentrations studied. With the removal of the myofibrillar structure by using a crude myosin preparation instead of myofibrillar protein, the effect of TSPP was greater than the effect of NaCl. The added TSPP produced an increased G' immediately upon addition compared to the G' observed in the absence of TSPP. Two possible types of network formation were proposed because the strain sweep data and s values could not distinguish between the two possibilities; true gel formation or entanglement network formation.

If an entanglement was formed, it was suggested that at the pH induced by the addition of TSPP to the myosin system, the TSPP molecule would be largely dissociated. The addition of this concentrated anionic charge, compared to, for example sodium chloride, could result in the movement of the negatively charged myosin. This movement could then result in entanglement of the myosin. The high molecular weight of the myosin and the heads protruding from the coiled tail would enable the myosin to form an entanglement. However, the myosin would only be likely to form an entanglement in the essentially monomeric form;

the rigid structure of the rods could prevent the flexibility required for this network formation. It was therefore suggested that the alkaline conditions must also induce the essentially monomeric state of myosin.

In contrast to this entanglement network formation, gelation was also suggested to explain the effect of TSPP on myosin. The suggestion that pyrophosphate acts as a denaturant (Tonomura *et al.*, 1963) could explain the initial gel formed with the addition of TSPP. It was suggested that this TSPP-induced denaturation could perhaps be due to increased pH, similar to denaturation induced by decreased pH. Immediate TSPP-induced denaturation followed by rapid gelation was suggested.

The lack of effect of a hydrogen bond competitor, sucrose, on the initial gel formed suggested that the gelation process involved hydrophobic and electrostatic interaction. The inclusion of GdL resulted in acid-induced gelation as the protein was denatured with decreasing pH. This secondary gelation was similar to that without the added TSPP. In the presence of TSPP, the G' of the acid-induced gel was enhanced, and this effect was suggested to be a result of added intermolecular hydrogen bonding. The inclusion of sucrose in the GdL plus TSPP system resulted in G' data which was similar to the combined G' of the GdL with sucrose sample plus the gel formed initially upon addition of the TSPP, down to pH 4.3. The increased G' at pH values less than pH 4.3, compared to GdL alone, was attributed to intermolecular hydrogen bonding, electrostatic and hydrophobic interactions and stability provided by the network obtained immediately upon TSPP addition. No involvement of sulfhydryl bonding was observed.

The aim of this investigation was to study the effects of addition of phosphates to myosin. It was hoped that this would enable a better understanding of the mode of action of TSPP on myosin, in particular, under the conditions of meat processing used today.

6.2 RESULTS

6.2.1 The effect of addition of TSPP on myosin gelation

The results of the effect of addition of tetrasodium pyrophosphate (TSPP) on myosin, alone and with GdL, are presented in the previous chapter (Chapter 5, Section 5.2.3). The range of G' values obtained with 0.7% GdL and 0.25% TSPP (Figure 5.18) was greater overall than G' obtained using 0.70% GdL only (Figure 5.1) or 0.70% GdL with 2.50% sodium chloride (NaCl) (Figure 5.9) at any given pH. A large initial G' was observed compared with samples without added TSPP (Figures 5.1 and 5.9). Increased GdL concentration to 1.40% with 0.25% TSPP resulted in G' similar to that at 0.70% GdL with 0.25% TSPP down to pH 4.5 (Figure 5.19). At pH values less than 4.5, the higher concentration of GdL used resulted in a lag in G' development compared to 0.70% GdL down to pH 3.8. At pH values below 3.8 an abrupt decline resulted.

White solids were generally formed with the addition of TSPP to myosin in the presence and absence of GdL. The s results and strain sweep data indicated gel networks of more solid than liquid character were formed for the addition of TSPP plus GdL (Table 6.1). The results of the sample with added TSPP only were not conclusive and could have indicated the formation of either a gel or an entanglement network.

The addition of 0.002% TSPP to myosin solutions in the absence of GdL resulted in approximately zero fluorescence and showed no increase with time for at least 12 h. Fluorescence data obtained with the addition of 0.002% TSPP and 0.025% GdL was similar to that of 0.025% NaCl with 0.025% GdL, but less than that of 0.025% GdL only (Figure 5.14). Increased GdL concentration did not result in significant changes in the fluorescence development with pH (Figure 5.21).

Reduction of the TSPP concentration to 0.001% resulted in greater fluorescence at given pH down to pH 3.7, thereafter, the pH at the lower TSPP concentration continued to decrease and the corresponding fluorescence levelled (Figure 5.23). Sample replicates were not taken and the differences observed could have been due to sample variation.

Micrographs of myosin with 0.25% TSPP and 0.70% GdL added (Figures 5.26a-d) showed a similar but denser structure than that of myosin alone (Figures 5.7a-d) and 0.70% GdL with 2.50% NaCl (Figures 5.17a and b). Strands were evident in the TSPP system with GdL, as in the samples of myosin alone and with GdL plus NaCl.

In Sections 5.2.5 and 5.2.6 of Chapter Five it was observed that no sulfhydryl bonding was involved in the gelation of myosin at 0.25% TSPP with 0.70% GdL. There was some hydrogen bonding, but this was only partially responsible for the G' obtained.

6.2.2 The effect of addition of different phosphates on myosin gelation

Table 6.1 presents a summary of the rheological data obtained from the addition of the phosphates; 0.09% sodium dihydrogen orthophosphate (OP), 0.25% TSPP, 0.21% tripolyphosphate (TPP) and 0.34% hexametaphosphate (HMP). The molar concentrations of the phosphates used were all approximately 5.5×10^{-3} M.

The results of addition of the four phosphates, each with 0.70% GdL are presented graphically in Figures 6.1 and 6.2. Orthophosphate with 0.70% GdL added produced G' values similar to, but slightly less than, the range of curves obtained for 0.70% GdL alone (Figure 5.1). The trend of a decrease in G' at pH 4.3 to 4.5 observed in the 0.70% GdL sample was also observed in this data set of OP and 0.70% GdL. The remaining phosphates, TPP and HMP with 0.70% GdL, produced similar G' curves with pH to those obtained with TSPP and 0.70% GdL. However, the G' curve of TPP with 0.70% GdL was greater than those of TSPP at a given pH, started at a higher pH and had a greater initial G'. The HMP curve showed a lag to pH about 5.0 and then an increase in G' to values of G' similar to TSPP plus GdL, but without the lag period. This G' curve for HMP addition with GdL started at a lower pH and had a lower initial G' than the G' curve of TSPP plus GdL. In the presence of HMP and GdL, the rate of increase of G' after pH about 5.0, when observed with respect to pH, appeared to be greater than that observed for TSPP or TPP, both with GdL (Figure 6.1). When observed with respect to time, it was apparent that the rate of formation of G' was similar to that observed for GdL with either TSPP or TPP (Figure 6.2).

Table 6.1 A summary of rheological data of myosin gels formed with added phosphates

Gel	Phosphate	G' (Pa)	δ (°)	G'' (Pa)	ω (Hz)	s	γ (m)
		approx 13 plateau	3 to 25	1 to 3	-	-	smooth 0.0457
0.70% GdL		210 at 7.2 ks max, 49 at 48 ks dec	inc at 10 ks to 9	as for δ to 8	-	-	-
0.70% GdL		460 at 14 ks max, 195 at 46 ks dec	0 at 14 ks inc to 9	0.6 at 14 ks inc to 30	-	-	abrupt 0.0258
0.70% GdL		690 at 14 ks max, 214 at 48 ks dec	inc at 27 ks to 7	as for δ to 25	1 lin. 5 rise	0.140	abrupt 0.0252
0.70% GdL		227 at 3.6 ks max, 10 at 48 ks	4 at 5.4 ks inc to 27 at 48 ks	1.5 to 20	-	-	grad 0.0463
	OP	1010 at 59 ks plateau	5 to 18	6 to 17	1 lin. 5 drop	0.048	smooth 0.1200
0.70% GdL	OP	max 115 at 9 ks dec	3 to 8	5 to 10	1 lin. 5 drop	0.165	smooth 0.0149
	TSPP	300 at 3.6 ks 350 at 27 ks plateau	2 to 4	14 to 20	1 lin. 5 drop	0.20	abrupt 0.0094
	TSPP	350 at 1.8 ks 400 at 21 ks plateau	approx 0	approx 0	-	-	abrupt 0.0084
	TSPP	276 at 9 ks 340 at 50 ks grad	0 to 3	0 to 3	1 lin. 5 drop	0.11	abrupt 0.0098
0.70% GdL	TSPP	550 at 3.6 ks 1380 at 48 ks inc	inc at 0 ks to 12	inc at 0 ks to 280	1 lin. 5 drop	0.008	abrupt 0.0016
0.70% GdL	TSPP	530 at 3.6 ks 870 at 48 ks inc	inc from 5 to 10	45 to 150	-	-	abrupt 0.0026
0.70% GdL	TSPP	146 at 0 s inc 875 at 48 ks	inc to 8	inc to 126	1 lin. 5 drop	0.008	abrupt 0.0022
0.70% GdL	TSPP	1250 at 48 ks	0 at 43 ks inc to 6	0 at 43 ks, 8 to 40	1 lin. 5 drop	0.100	abrupt 0.0035
0.70% GdL	TSPP	785 at 52 ks plateau	0 at 10 ks 4 at 2 ks	1 at 10 ks 58 at 52 ks	1 lin. 5 drop	0.030	abrupt 0.0044
0.70% GdL	TSPP	810 at 48 ks plateau grad	inc at 20 ks to 5	as for δ to 70	1 lin. 5 drop	0.040	abrupt 0.0041

Table 6.1 (cont.)

Gel	Phosphate	G' (Pa)	δ (°)	G" (Pa)	ω (Hz)	s	γ (m)
	TPP	525 at 61 ks grad	0 at 23 ks to 3 at 16 ks	as for δ to 31	1 lin. 5 drop	0.072	abrupt 0.0056
0.70% GdL	TPP	1220 at 19 ks plateau	inc 0 to 17	inc to 417	1 lin. 5 drop	0.0	abrupt 0.0026
	HMP	116 at 0 ks 354 at 59 ks	0 to 3	0 to 13	-	-	-
0.70% GdL	HMP	61 to 1210 at 37 ks grad	inc at 9 ks to 11 at 37 ks	as for δ to 230	1 lin. 5 drop	0.0	abrupt 0.0026

Terms associated with Table 6.1:
0.70% GdL = concentration of GdL used
OP, TPP, HMP = sodium dihydrogen orthophosphate, tripolyphosphate, hexametaphosphate
plateau = curve reached a plateau at the specified time
grad = gradual incline/decline
min/max = curve reached a minimum/maximum at the specified time
inc/dec for G' curve = curve was increasing/decreasing at the final G' observed
inc/dec for δ and G" curves = increased or decreased at/to the specified time
lin. = linear
s = slope of a (log G') versus (log ω) plot

Gels were not obtained at the final pH values with the addition of OP to myosin in the presence and absence of GdL. The addition of TPP, TPP plus GdL and HMP plus GdL all produced gels as indicated by strain data and s values (Table 6.1).

The samples without added GdL, increased the G' with the addition of phosphates compared to myosin alone (Table 6.1). The pH also increased with the addition of phosphates resulting in pH values of 6.0 to 8.8.

6.2.3 The effect of addition of nitrite on myosin gelation

Unfortunately, all attempts to deaminate the lysine and arginine residues of myosin were inadequate due to insufficient acid concentration in the protein gels. The deamination of these amino acids was to be used to investigate electrostatic interactions between the anionic TSPP and the cationic amine groups.

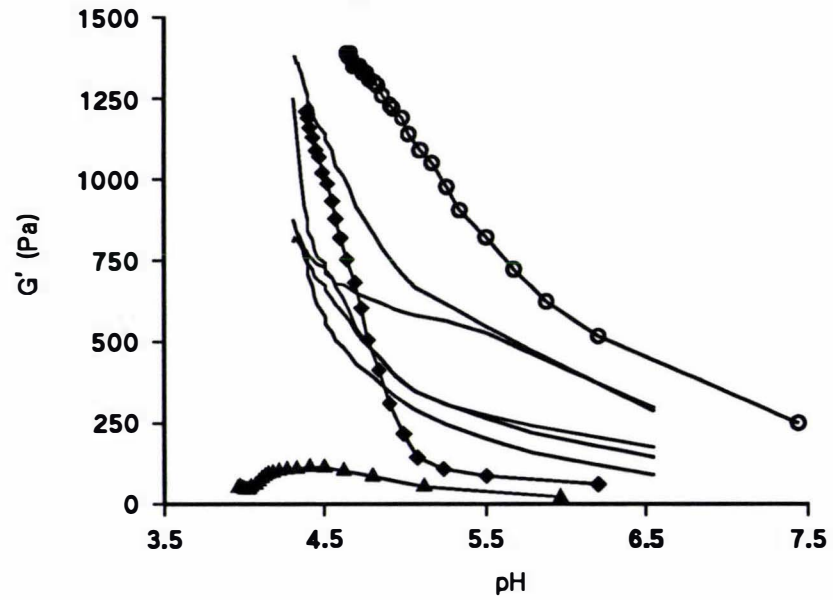


Figure 6.1 The development of G' of myosin gels with added phosphates. Orthophosphate (▲), TSPP (–), TPP (o), HMP (◆).

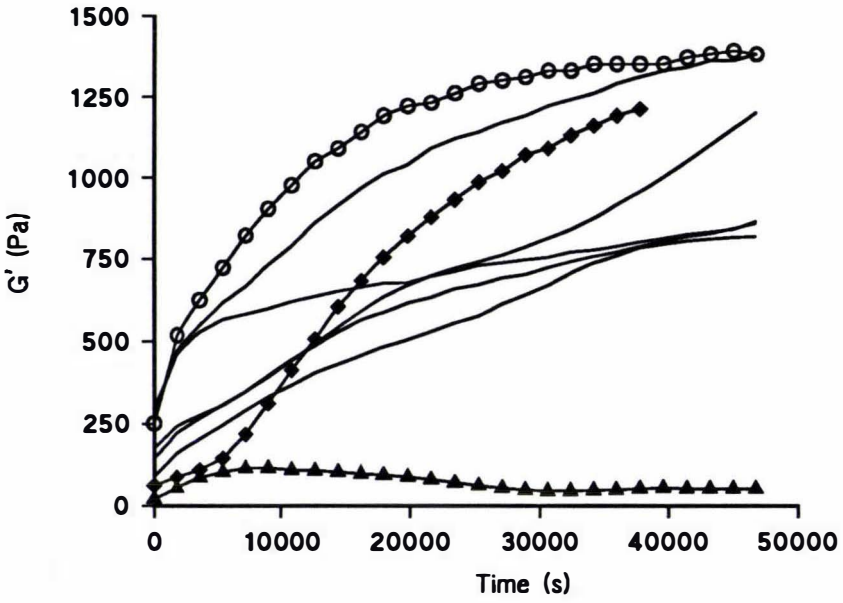


Figure 6.2 Time development of G' of myosin gels with added phosphates. Orthophosphate (▲), TSPP (–), TPP (o), HMP (◆).

6.3 DISCUSSION

This discussion is divided into two sections. The first is a general discussion of theories presented by other workers to explain the effect of addition of phosphates to meat proteins. Many of these theories are relevant to the addition of phosphates to myosin. The second section discusses the results obtained in the present experiments.

6.3.1 Theories to explain the effect of phosphates on the functionality of meat proteins

There are several theories explaining the efects of phosphates on meat proteins, however, many hve been questioned or disputed. The addition of phosphates, such as orthophosphate (OP), tripolyphosphate (TPP) and tetrasodium pyrophosphate (TSPP) to myosin increases the ionic strength and the pH of the system. These effects and specific polyphosphate interactions, such as with the actin binding site and electrostatic interactions, have been the basis of many theories (Hamm, 1971).

Increases in pH have been considered important in increasing water retention in meat since the early years of phosphate usage (Sherman, 1961; Shults *et al.*, 1972; Swift and Ellis, 1956, 1957). However, theories suggesting that pH increase was responsible for the phosphate effect on meat protein functionality were disputed when the addition of sodium hydroxide to meat protein did not increase water binding (Knipe *et al.*, 1985). Orthophosphate also increased the pH of meat proteins, but its influence on meat binding and water retention was negligible (Shimp, 1981; Trout and Schmidt, 1986a). In addition, Shimp *et al.* (1983b) found that TPP and pyrophosphate mixtures improved the water retention without changing the pH of buffered samples of meat protein.

Similar to increases in pH, increases in ionic strength of the meat have also been suggested to explain the increases in water retention. Egelandsdal *et al.* (1986) found that pyrophosphate had a simple ionic strength effect on the myosin system at 23°C. The effect of individual phosphates on ionic strength is variable (Trout and Schmidt, 1986c) and increases in ionic strength are difficult to determine due to variations in their dissociation. Increasing chain length of phosphates causes a decrease in dissociation which results in lower ionic strength

values with longer chain polyphosphates (Trout and Schmidt, 1986c). At ionic strength greater than 0.15 and pH 6.0, polyphosphates increased the functional properties of meat more than other salts causing comparable changes in pH and ionic strength (Shimp, 1983b; Puolanne and Ruusunen, 1980; Trout and Schmidt, 1986a). The extent of the synergistic effect however, decreased linearly as the chain length of phosphates increased (Trout and Schmidt, 1986a). Reduced phosphate dissociation and therefore ionic strength, at longer phosphate chain lengths was suggested to be a partial reason at least, for the reduction in functionality.

Another potential mode of action of phosphates in increasing meat functionality was thought to be through their chelating activity on cations, such as calcium, magnesium and zinc (Hamm, 1960, 1971; Wierbicki et al., 1963). The speculation was that the cations were chelated and thus removed from the proteins, which opened up their structure and improved their water binding properties. The chelation process was suggested to break away one of the two proteins attached to the calcium or magnesium (Hamm, 1971). This chelation theory has been disputed by several researchers (Hellendorn, 1962; Inklaar, 1967; Kotter, 1960; Trout and Schmidt, 1986a). Polyphosphates did not reduce the amount of calcium and magnesium ions bound to protein (Inklaar, 1967). However, the method of exhaustive dialysis used by Inklaar (1967) has been questioned (deMan, 1971). In addition, meat treated with a chelating agent, such as ethylenediaminetetraacetic acid (EDTA), showed no increase in water retention (Hellendorn, 1962; Sherman, 1961), and addition of calcium and magnesium to meat did not reduce water binding (Bendall, 1954; Ellinger, 1972). Hamm (1971) suggested that the wide spreading EDTA molecule could not approach the protein bound bivalent cations because of steric reasons.

In the work of Siegel and Schmidt (1979b) on heat induced myosin binding, the effect of tripolyphosphate on pH was eliminated and the increase in binding ability was greater than expected due to the increased ionic strength offered by 0.5% tripolyphosphate when compared to NaCl. Therefore, the increased binding strength of myosin was attributed primarily to the specific interaction between polyphosphate and the protein. This interaction was not detailed in the investigation of Siegel and Schmidt (1979b), but this effect was suggested to cause increased myosin solubility by dissociating the actomyosin contaminating the crude myosin samples.

Many other workers have explained the action of phosphates on meat by the potential dissociation of actomyosin to actin and myosin by phosphates (for example, Hellendoorn, 1962; Shimp, 1981, 1983a, 1983b; Yasui *et al.*, 1964a, b). Actomyosin is dissociated only by pyrophosphate while other phosphates will dissociate actomyosin only when hydrolysed to pyrophosphate (Trout and Schmidt (1986a). Although direct evidence is lacking, only TPP may hydrolyse to pyrophosphate at a sufficient rate before product cooking to cause dissociation of actomyosin (Yasui *et al.*, 1964). Polyphosphates not believed to undergo rapid hydrolysis have also increased functionality in beef rolls (Trout and Schmidt, 1986a) and it was suggested that this protein phosphate interaction and actomyosin dissociation by phosphate may be involved in functionality of only certain products formulated with specific phosphates.

It was noted by Trout and Schmidt (1986a) that it was doubtful that pyrophosphate was completely hydrolysed in meat products as observed in the experiments of Morita *et al.* (1983a). Trout and Schmidt (1986a) suggested that the hydrolysis of pyrophosphate was an indication of actomyosin dissociation. Trout and Schmidt (1986a), on the basis of a reference to Graenicher and Portzehl (1964), stated that complete hydrolysis of pyrophosphate would indicate that actomyosin is not dissociated since pyrophosphate must be present for actomyosin to remain in the undissociated form. However, a close reading of Graenicher and Portzehl's text shows the statement of Trout and Schmidt to be unfounded. Graenicher and Portzehl (1964) did not mention the hydrolysis of phosphate or that the presence of pyrophosphate was required for actomyosin to remain in the undissociated form. This in turn casts doubt on the interpretation of the work of Morita *et al* (1983a), who Trout and Schmidt (1986a) stated to have observed complete hydrolysis of pyrophosphate during the manufacture and initial stages of thermal processing of meat products. Unfortunately, the journal in which Morita *et al.* (1983a) published could not be accessed.

In the investigation of dissociation of actomyosin by Graenicher and Portzehl (1964) it was found that only the chelate, magnesium pyrophosphate, was capable of dissociating actomyosin. Dimagnesium pyrophosphate, magnesium ion and free pyrophosphate were not capable of causing any dissociation of the actomyosin. In contrast to magnesim pyrophosphate, the chelate, calcium pyrophosphate did not cause any dissociation of actomyosin. It is therefore proposed that it is unlikely that pyrophosphate caused actomyosin

dissociation in the absence of added magnesium as suggested, for example, by Bendall (1954) and Trout and Schmidt (1986a).

Although unable to dissociate actomyosin in the absence of magnesium, pyrophosphate alone has been shown to bind to many proteins, including actomyosin (Nauss *et al.*, 1969; Vandegrift and Evans, 1981). Binding of phosphates to meat proteins (Hamm, 1971; Trout and Schmidt, 1986a) has been used to explain the action of phosphates on meat. Shorter chain length phosphates were thought to react with actomyosin; longer chain hexametaphosphates were thought to bind directly to the myosin (Yasui *et al.*, 1964a). However, Trout and Schmidt (1983) stated that while the polyanionic nature of phosphates allows them to attach to protein sites and attract more water molecules, there was no direct relationship between the extent to which phosphates bound to meat proteins and their effect on functionality.

The binding of strongly negative polyvalent anions, such as ATP, ADP and pyrophosphate, with myosin prevented precipitation of the myosin. This was taken as evidence (Morita and Shimizu, 1969) that ATP or pyrophosphate, on binding changed, to some extent, the secondary and tertiary structure of the myosin molecule. However, Gratzer and Lowey (1969) studied myosin and HMM using optical rotary dispersion (OR) and far UV absorption spectroscopy in the presence and absence of ATP and pyrophosphate and found no change by either method. It was concluded that any change in the α-helix content was less than 0.2%. Changes in the binding capacity for bromothymol blue, supported the view that a small conformational change occurred.

In contrast, Egelandsdal *et al.* (1986) stated that pyrophosphate was a denaturant, based on the work of Wright and Wilding (1984) and Tonomura *et al.* (1963), and as such it apparently had an effect in the temperature range 54-58°C. However, this statement requires clarification. Wright and Wilding (1984) used DSC to study the thermal denaturation of myosin. Samples of myosin in 0.04 M potassium chloride, 0.5 mM DTT, 100 mM maleate at pH 6.5 had 5 mg/ml OP, pyrophosphate and TPP added to the systems. The samples containing OP and TPP resulted in a single denaturation transition at approximately 57°C, while the pyrophosphate sample resulted in a double peaked transition at about 47 and 57°C. While these samples differed from each other, the conditions used were different to those studied

elsewhere in the paper. Most other myosin samples were tested in potassium phosphate buffers and at different ionic strengths or pH. Therefore, there was no data for denaturation of myosin alone to be used as a standard.

To explain the results presented, Wright and Wilding (1984) studied the time course of hydrolysis of TPP in a myosin system. However, the pH and salt concentration differed from the above solution (pH 6.0 and 0.4 M potassium chloride, respectively) and 1 mM calcium chloride was added. The TPP and pyrophosphate samples were suggested to both contain myosin bound pyrophosphate. The sample with OP was suggested to contain myosin bound ADP because of the extraction of myosin from prerigor muscle without the use of pyrophosphate in the preparation of the myosin. If the differences apparent in the thermograms of the three myosin samples reflected the effects of active site-bound phosphate, then it was suggested that TPP or its hydrolysis product, pyrophosphate, was bound in a similar fashion to ADP, whereas incipient pyrophosphate bound at alternative or additional sites on the myosin heads.

Wright and Wilding (1984) stated that myosin presented a complex picture in its denaturation behaviour because of the number of separate events occurring and the different extent to which they were effected by solution conditions. Based on this statement, it is suggested that unless samples were studied under equivalent conditions, the results obtained were not necessarily comparable. Therefore, the statement concluding that pyrophosphate is a denaturant (Egelandsdal *et al.*, 1986) is not an accurate description of the results presented in the study by Wright and Wilding (1984). Wright and Wilding (1984) did, however, suggest that pyrophosphate destabilized the myosin molecule by about 9°C compared to the effects of OP and TPP, even though the latter was probably hydrolysed to OP and pyrophosphate.

In contrast to the work of Wright and Wilding (1984), Tonomura *et al.* (1963) using optical rotary dispersion and exposure of 'abnormal' tyrosine, showed that the secondary and tertiary structure of the myosin molecule changed on its binding with pyrophosphate or ATP. These experiments were all conducted at 0.5 to 0.6 M potassium chloride or NaCl. Therefore, at high salt concentrations (0.5 to 0.6 M) pyrophosphate acted as a denaturant of myosin. In an earlier paper (Tonomura *et al.*, 1962), it was observed that in potassium chloride solution, the

helical content and reduced viscosity were almost unchanged. However, the effect of pyrophosphate alone was not studied for comparison.

More recently, results of investigations on beef rolls with different phosphates, ionic strengths, and pH values, led Trout and Schmidt (1986a) to advance a theory to explain the activity of phosphates as meat binders. Based on regression equations, it was found that 93% of the variation in cooking yields and 86% of the variation in tensile strength of beef rolls could be explained in terms of pH, molar sodium chloride and molar phosphate concentrations. It was concluded that phosphates increased protein functionality mainly by altering hydrophobic interactions that stabilized the protein structure. A linear decrease in the effectiveness of phosphates with increasing chain length was observed which was attributed, at least in part, to changes in hydrophobic interactions. If increased protein functionality in the presence of phosphates was due to changes in electrostatic interactions, all treatments of the same ionic strength and pH, (even without phosphate) would have increased functionality to the same extent. Regression analysis of the results showed that the decrease in functionality with increasing phosphate chain length at equal weight concentrations of all phosphates was linearly related to the decrease in molar phosphate concentration. Sofos (1989) noted that with increasing chain length of phosphate, molar concentrations decreased at equal weight concentration. Thus, the linear decrease in effectiveness may have been due to either or both of these changes, that is increased chain length and decreased molar concentration. Increased protein functionality by phosphates, through changes in hydrophobic protein interactions, was also supported by the lack of an effect by pH on the effectiveness of phosphates. This was suggested to eliminate the potential for phosphates to be active through alteration of electrostatic interactions. Thus, the hydrophobic effects of phosphates had the predominant effect on functionality at ionic strengths greater than 0.15 (Trout and Schmidt, 1986a).

The investigation of Trout and Schmidt (1986a) was based on the assumption that phosphates act as other salts would. The assumption that hydrophobic bonding was responsible for gelation was based on elimination of electrostatic effects. The basis for this argument is the conclusion that at high salt concentrations, salt primarily affects hydrophobic interactions, since salts have little effect on electrostatic interactions at ionic strengths greater than 0.1 (Melander and Horvath, 1977). There was no mention of hydrogen bonding involvement in

gelation. At pH 5.50 to 6.35 it is not expected that the phosphates would be completely dissociated (Trout and Schmidt, 1986c) and therefore hydrogen bonding is possible in gelation.

The assumption that phosphates act as other salts do, appears too simple. This is evident when considering the large effects on, for example WHC, as observed by other authors (including Fukazawa *et al.*, 1961c, Hamm, 1971; Siegel and Schmidt, 1979b) at low ionic strengths compared to other salts.

There has been a lot of work undertaken on the effects of phosphates on meat and myofibrillar protein, especially in terms of water holding capacity, cook yield and bind strength. The complex nature of meat and myofibrillar protein has made it difficult to interpret the results of these investigations and has resulted in many theories to explain the effects of phosphates on meat and myofibrillar proteins. However, it is generally accepted that the addition of phosphates to meat and myofibrillar protein results in effects not expected by such a small change in ionic strength when compared to, for example sodium chloride addition. It is these effects that have prompted the many investigations undertaken, of which many are contradictory. The complex nature of meat, protein systems and phosphates themselves has resulted in over-interpretation of results and conjectures based on weak evidence. Bearing in mind the highly speculative nature of the theories proposed, the only work which appears reliable is that of Tonomura *et al.* (1963) where pyrophosphate was suggested to have acted as a denaturant to myosin. This suggestion was a direct interpretation of the data obtained from optical rotary dispersion experiments.

6.3.2 The effect of addition of phosphates on GdL-induced gelation of myosin

The addition of TSPP to myofibrillar proteins in Chapter Four did not result in any significant effect on Young's Modulus until the complete dissociation of actomyosin had been achieved. In myosin, the addition of TSPP enhanced the G' values of the gels obtained in the presence and the absence of GdL. When compared to a ten-fold greater concentration of NaCl, the effect of phosphate on myosin gelation was greater than NaCl, even at this much lower ionic strength. It was assumed that the phosphate had an unusually large effect, and not that the

salt had a small effect, on myosin gelation for the relative ionic strengths. This assumption was based largely on the results of the research discussed in the above section (6.2.1).

Strain sweep data and s value were inconclusive (Table 6.1) and two types of network formation were suggested to explain this high G'; gel or entanglement. If a gel formed, it was suggested that the TSPP may have acted as a denaturant and subsequent to denaturation, gelation may have occurred. Tonomura *et al.* (1963) observed that pyrophosphate addition to myosin resulted in denaturation of the protein, providing support for this suggestion. The denaturation observed, suggested the exposure of hydrophobic sites. However, the work of Tonomura *et al.* (1963) was undertaken at ionic strengths of 0.5 to 0.6 M NaCl or potassium chloride. According to Trout and Schmidt (1986a) the formation of a gel at ionic strengths of greater than 0.15 would involve hydrophobic interactions, but at lower ionic strengths, as used in the present experiments, electrostatic interactions would be expected to cause effects and not hydrophobic interactions. This would suggest that work similar to that of Tonomura *et al.* (1963) needs to be undertaken in the absence of high concentrations of salt. This, however, results in difficulties because the myosin must be solubilised for optical rotary dispersion.

In contrast to gelation, if an entanglement network was formed, it was suggested that this network could have occurred with the addition of dissociated TSPP to the myosin. At the pH of the myosin system with the addition of TSPP in the presence and absence of GdL (pH 6.0 to 8.8) much of the TSPP would have been dissociated. The molecule, unlike NaCl, would present a concentration of negative charge. It was suggested that addition of this polyanion to myosin would have resulted in the movement of myosin molecules. The high molecular mass of myosin (approximately 500 kDa) and the heads protruding from the coiled tail would have allowed the myosin to form an entanglement in the essentially monomeric state. However, the rigid rods of filamentous myosin would not likely have permitted the flexibility required for entanglement formation. Therefore, if the myosin was to have formed an entanglement network with the addition of TSPP, myosin must have been in the essentially monomeric form. It was suggested that if an entanglement network had formed, the change in pH may have induced this essentially monomeric state of myosin, from the aggregated strands which were observed for myosin alone (Figures 5.7a to d).

The TSPP-induced network was maintained as the pH was lowered with the inclusion of GdL in the myosin plus TSPP system. Added hydrogen bonding provided extra rigidity to the GdL-induced gels formed and provided stability to the gel when the pH was lowered to values less than 4.3.

Similar to the work of Trout and Schmidt (1986a), it was suggested that the use of several phosphates might provide information about effects of phosphates on gelation. In the present experiments, the phosphates added to the myosin were all of the same molar concentration. In a study by Trout and Schmidt (1986c) the percentage of dissociation of phosphates used in food products was presented. An adaptation of this data measured at pH 6.0 is presented in Table 6.2. Similar data can be calculated for pH 4.5 using dissociation constants to determine the ionic strengths of the phosphates used. These results are presented in Table 6.3. The dissociation constants of HMP could not be found in the literature and this prevented calculation of the ionic strength for this phosphate.

The addition of OP, TSPP, TPP and HMP all resulted in varied initial G' values (Figure 6.1). The greater the initial pH of the myosin with added phosphate, the greater the initial G' obtained. If gelation occurred, these results could indicate that pH increase resulted in increased denaturation.

Some workers have disputed the theories that increased pH was responsible for the effects of phosphates on meat protein functionality. An example of evidence used to dispute this theory is that the addition of sodium hydroxide to meat protein did not increase water binding (Knipe *et al.*, 1985). However, even if the addition of sodium hydroxide resulted in the denaturation of myosin, gelation may not have occurred subsequent to denaturation. The polyanionic nature of the phosphates may have provided crossbridges required for the formation of a network resulting in gelation subsequent to denaturation. The lack of these polyanions may prevent the formation of a network.

Table 6.2 The degree of dissociation of phosphates at pH 6.0.

Phosphate	Chain length (n)	Concentration (%, w/v)	Molar concentration x10^3	Dissociation (%)
Disodium phosphate	1.0	0.15	10.5	91
		0.30	21.1	92
Tetrasodium pyrophosphate	2.0	0.15	5.6	89
		0.30	11.3	88
Sodium tripolyphosphate	3.0	0.15	4.0	83
		0.30	8.1	81
Sodium hexametaphosphate	12.8	0.15	1.1	45
		0.30	2.2	46
		0.45	3.3	47

Adapted from Trout and Schmidt (1986c)

Table 6.3 Characteristics of the phosphates used and calculated data relating to the phosphates at pH 4.5.

Phosphate	Formula	MW	Chain Length	Conc. (x10^{-3} M)	Conc. (% w/v)	% Diss.	Ionic Strength
OP	$NaH_2PO_4.2H_2O$	156	1	5.4	0.09	33.4[a]	0.0055
TSPP	$Na_4P_2O_7.10H_2O$	446	2	5.5	0.25	51.4[a]	0.0174
TPP	$Na_5P_3O_{10}$	368	3	5.5	0.21	61.3[b]	0.0527
HMP	$(NaPO_3)_6$[c]	612	6	5.5	0.34	-	-

[a]based on dissociation constants from Weast (1973)
[b]based on dissociation constants from Corbridge (1978)
[c]from Stecher *et al.* (1968)
OP = orthophosphate
TPP = tripolyphosphate
TSPP = tetrasodium pyrophosphate
HMP = hexametaphosphate
Conc. = concentration of phosphate in solution
% Diss. = degree of dissociation of the phosphate

The lack of effect of OP on myosin could be explained by the small increase in pH compared to the addition of TPP and TSPP and the small size of the OP molecule which could favour intramolecular, not intermolecular, interactions. This phosphate has been shown to raise the

pH of meat, without effecting the functional properties of the sample (Shimp,1981; Trout and Schmidt, 1986a). However, in the present experiments, the pH was raised little in comparison to the other added phosphates of the same molar concentration (Figure 6.1) and may not have resulted in as much, if any, denaturation of the myosin compared to the other phosphates investigated.

In contrast to gelation, if an entanglement network was formed, pH would have played a different role in network formation. Firstly, the increased pH with phosphate addition would have resulted in increased dissociation of phosphate hydroxyl groups. However, the presence of these groups could have only induced an entanglement if the myosin was in the essentially monomeric state. If an entanglement network had formed, then the results suggest that the pH may not have been raised sufficiently with HMP and OP to cause dissociation of myosin filaments (Figure 6.1) and hence the low G' that was observed.

The G' versus pH and time curves of the TPP plus GdL data was similar to that for the TSPP plus GdL data, but was slightly higher at given pH or time. The similarities in the shape of the TSPP plus GdL and GdL only curves suggested that acid-induced gelation occurred after the phosphate-induced network formation. The phosphate-induced network appeared to be maintained throughout the acid-induced gelation and provided the added G' observed when compared to the G' of the GdL curve at a given pH, down to pH 4.3. There also appeared to be some enhancement of the G' by hydrogen bonding as the pH was lowered. The similarities between the TSPP plus GdL and the TPP plus GdL curves of G' provided explanations for the effects of TPP on myosin in terms of both entanglement or gel network formation.

Similar to TSPP addition to myosin, if a gel was formed the presence of TPP might have resulted in denaturation and then gelation immediately upon addition of the phosphate to myosin. The increased initial pH compared to TSPP, could explain the greater G', if alkaline pH induced denaturation. If an entanglement network had formed, addition of the largely dissociated TPP molecule could have resulted in movement of the myosin molecules. The concentration of negative charge would be greater than for TSPP possibly inducing tighter network formation and the higher pH may have induced greater dissociation of the myosin.

The HMP curve differed somewhat from the TSPP and TPP curves in its shape. A lag period prior to gelation resulted in a slow increase to about pH 5.0. This data resembled the G' curve of GdL alone and the G' curve with OP added to myosin plus GdL. The HMP curve of G' then increased similar to TSPP or TPP addition to myosin plus GdL. It was suggested that the hydroxyl groups of the HMP molecule may not have been significantly undissociated until about pH 5.0. Upon reaching pH 5.0, some of the hydroxyl groups might have formed allowing hydrogen bonding to occur. With the large number of hydroxyl sites, a greater amount of hydrogen bonding might occur, compared to that observed in the presence of TSPP or TPP addition, explaining the final high pH observed without the initial network formation. The lack of increase in pH to values greater than 6.0 could explain the lack of a network formed initially as indicated by the immediate G' value observed. A lack of increase in G' would then indicate small, if any, denaturation preventing gelation, or possibly a lack of dissociated myosin filaments and negatively charged myosin preventing entanglement formation.

In terms of chain length and ionic strength, the G' curves of OP, TSPP and TPP increased with the increasing parameter, suggesting that these factors could account for the effects of phosphates. However, assuming the ionic strength of HMP was greater than the other phosphates, the HMP molecule did not coincide with either of the factors. The assumption that HMP has a greater ionic strength can be based on the work of Trout and Schmidt (1986c) at pH 6.0 (Table 6.2) and following the trend observed in the present calculations (Table 6.3).

6.3.3 Summary

The addition of TPP or TSPP to myosin with GdL resulted in a large G' immediately upon addition of the phosphate. It was suggested that the phosphate may denature some of the myosin and result in immediate gelation. Alternatively, addition of the concentrated anionic charge of the dissociated phosphates to myosin may have resulted in movement of myosin and entanglement formation. This entanglement could only have occurred if the alkaline pH resulted in dissociation of myosin from a filamentous to an essentially monomeric state. The rigid rods would not likely have permitted the required flexibility for entanglement formation.

Electrostatic and hydrophobic interactions may be responsible for the gels formed, whereas, electrostatic interaction would largely have been responsible for entanglement network formation if formed according to the suggested mechanisms. The more alkaline the pH induced by the addition of phosphate, the greater G' values that resulted. For gel formation this suggested that denaturation may be pH-induced. If entanglement network formation had occurred, more alkaline pH could have resulted in greater dissociation of the phosphate, greater negative charge on the myosin and greater dissociation of myosin filaments.

The network formed with the addition of TSPP and TPP appeared to remain intact throughout the acid-induced denaturation and gelation. This initial network provided stability to the acid-induced gels and with added hydrogen bonding prevented a decline in the G' at pH 4.3 as was observed with GdL only (Figure 5.1).

The addition of HMP did not result in as alkaline pH values as measured for TSPP and TPP. Lower immediate G' was also observed compared to the G' values of TSPP and TPP. A lag in the development of G' was observed down to pH 5.0 where G' then increased similarly to that for TSPP and TPP (Figure 6.2). This increase was suggested to be a result of formation of hydroxyl groups which could enhance hydrogen bonding.

The small size of the OP molecule which would favour intermolecular interactions and small effect on pH might explain the lack of initial network formation and the lack of enhancement of the acid-induced gelation.

The small amount of data presented in this chapter does not provide enough evidence to conclusively show the mechanism of action of phosphates on myosin, and therefore meat and myofibrillar protein. It is suggested that further and more detailed studies should be conducted to investigate the mode of action of phosphates on myosin. These experiments might include electron microscopy to observe the filamentous or monomeric state of myosin at alkaline pH, or obtaining more s values and strain sweep data for statistical significance to show the type of network formed, that is, gel or entanglement.

CHAPTER 7

THE REVERSIBILITY OF GdL-INDUCED MYOSIN GELS

7.1 INTRODUCTION

The most important feature of restructured meat products is the ability of the protein matrix formed to effectively bind the meat pieces together. Effective binding is essential for the product to retain its structural integrity during subsequent handling and slicing (Schmidt and Trout, 1982). Maintenance of structural integrity encompasses the ability of the gel to remain intact if the subsequent handling involves reversion of the conditions used to form the gel, back to the state prior to gelation.

Thermal reversibility is observed in the study of many proteins. Gelatin is a well known example of a thermally reversible protein gel (Glicksman, 1982). Gelatin gels are readily formed on cooling previously heated aqueous solutions of the protein. Reheating to about 40°C results in melting of the gels and these steps of cooling and heating can be repeated many times. By varying the thermal history of the gel, many different structures can develop through the continuous breaking and reforming of the links of the gel network. Since the gel melts so readily, it is believed that the links in the network involve only secondary forces and not covalent bonding.

In contrast to reversible gelation, some proteins form thermally irreversible gels which soften or shrink with subsequent heating (or cooling), but melting does not occur under practical conditions (Schmidt, 1981). Egg white coagulum is routinely characterised as a disulfide cross-linked thermoirreversible protein gel (Shimada and Matsushita, 1981; Egelandsdal, 1980).

It is theoretically plausible that a given protein system may possess the ability to form either type of gel depending upon the formation conditions. It is also possible that some parts of the gel process may be reversible and others irreversible. Beveridge *et al.* (1984) demonstrated that in both egg albumin and whey protein concentrate gels, a portion of the total elasticity formed on cooling was reversible when the gel was reheated.

Myosin and some of its fragments have shown reversible gel characteristics under certain conditions of pH, ionic strength and temperature. Wright and Wilding (1984), using DSC found that the thermal denaturation of the 'hinge region' of rabbit myosin at pH 6.0 and ionic strength of 1.0 was reversible. In a series of papers, Samejima *et al.* (1976, 1981) studied the thermal denaturation of myosin rod and reversibility of thermal denaturation was also reported. Rogers *et al.* (1987) reported that the thermal unfolding of myosin rod obtained from four different organisms was highly reversible in the temperature range 0 to 80°C. Arteaga and Nakai (1992) observed full reversibility of the thermal denaturation of turkey breast myosin when heated for up to 30 min at 40°C, for 5 min at 50°C and incubated for 24 h at 4°C.

Similar to gels induced thermally, gels formed through pH alteration or chemically-induced gels may also be reversible. However, reversibility of conditions of pH or chemical modification is generally more difficult to achieve than for thermally-induced gelation. The temperature of a protein system and the rate of increase or decrease of temperature can readily be regulated. Removal of ions or change of pH is more difficult to control, especially in terms of rate. The aim of this chapter was to investigate the reversibility or otherwise of GdL-induced myosin gels.

7.2 RESULTS

7.2.1 Myosin dialysis

Samples of myosin which had gelled with added 0.70% GdL were placed in quiescent water. After several hours the surfaces of the sample had dispersed into the water. The amount of myosin dispersed into the solution increased with increasing time and with stirring.

Myosin and additives were formed into cylinders in dialysis tubing, allowed to stand for 24 h to form gels, and were then suspended in water. After 24 h, rubbery, translucent layers, several millimetres thick, had formed at the surface of the myosin gels exposed to the water through the dialysis tubing. The inner volume of the tube of myosin did not change from that of the myosin sample prior to dialysis.

In order to overcome this lack of uniformity in the gels formed upon dialysis, the shape of the gel was changed. The cylindrical myosin shape was flattened to approximately 1 mm thickness and the myosin was gelled and then dialysed as these flat discs. Use of this shape overcame the problem of non-uniformity and was used hereafter. The small size of this sample and the inconsistencies in the uniformity of thickness of the dialysed samples made it difficult to determine any change in volume. Subjectively, dialysed samples appeared to occupy greater volume compared to that occupied prior to dialysis.

Samples of myosin which had been allowed to stand for 24 h in dialysis tubing with added combinations of GdL, 0.25% TSPP and 2.50% sodium chloride (NaCl) were dialysed in water. Table 7.1 presents the results of tensile tests undertaken on an Instron Universal Testing Machine. The samples were subjected to tensile force to the point of destruction at which the force and displacement were recorded.

Myosin alone formed a stronger gel after 24 h dialysis (Table 7.1) than if allowed to stand at 4°C for the same time. The sample which was not dialysed was a solution and therefore, could not be suspended from the clip used to subject the sample to the tensile force. The pH of myosin decreased from a pH of about 7.0 prior to dialysis, to 5.7 after dialysis. The gel was transparent and colourless, and was firm to the touch.

Solutions of myosin containing either NaCl or NaCl and TSPP became firm after 24 h dialysis (Table 7.1). The appearance of the gels was transparent and colourless, similar to the solution prior to dialysis.

The addition of 0.18% GdL to myosin resulted in a sample which appeared similar to that of myosin alone. After 24 h of dialysis, the sample had become clear and colourless, but was too

Table 7.1 Results of tensile tests of GdL-induced myosin gels.

Additives	Original Gel	Final Gel	Force (N, average)	Force (N, s.d.)	Displacement (mm, average)	Displacement (mm, s.d.)	Final pH
Not Dialysed							
0.70% GdL S	white solid		0.046	0.011	26.3	5.6	
0.70% GdL T + S	white solid		0.079	0.011	34.4	2.7	
Dialysed							
-	white suspension	clear solid	0.083	0.006	21.2	2.9	5.7
0.18% GdL	white suspension	clear solid	-[a]	-	-	-	
0.35% GdL	white suspension	clear solid	0.038	0.008	17.8	8.0	
0.70% GdL	white suspension	clear solid	0.097	0.021	39.0	7.0	5.7
S	clear solution	clear solid	0.138	0.043	15.5	5.7	
0.70% GdL S	white solid	clear solid	0.100	0.012	25.5	4.0	4.9
T	viscous white	clear solid	0.053	0.015	15.6	0.4	
S + T	clear solution	clear solid	0.089	0.020	14.0	1.2	
0.70% GdL S + T	white solid	clear solid	0.164	0.024	34.6	7.2	

[a]subjectively stronger than sample which had not been dialysed, but too weak to suspend for testing

Terms pertaining to Table 7.1:
0.18, 0.35, 0.70% GdL = concentration of GdL used
T, S = 0.25% TSPP, 2.5% NaCl used
s.d. = standard deviation
Force, Displacement = measured at the point of destruction

fragile to be suspended from a clip to enable tensile tests to be undertaken. Dialysis of myosin with added 0.35 and 0.70% GdL resulted in translucent, colourless gels from a myosin-like suspension. These gels were stronger than that at the lower GdL concentration (Table 7.1). The greater GdL concentration resulted in greater maximum force required to

disrupt the gel and the gel was able to be extended to greater lengths than the sample with 0.35% GdL added. Dialysed myosin, while extended to only half the length of the sample with 0.70% GdL added, required force comparable to that at 0.70% GdL to result in destruction.

The opaque gels formed with the addition of 2.50% NaCl plus 0.70% GdL, 0.25% TSPP and 0.25% TSPP plus 2.50% NaCl plus 0.70% GdL, also became clear, colourless gels after 24 h of dialysis. Both samples of myosin with added 2.50% NaCl plus 0.70% GdL and 2.50% NaCl plus 0.25% TSPP plus 0.70% GdL, which had and had not been dialysed, were tested on the Instron Universal Testing Machine. The dialysed samples showed that significantly greater tensile force was required to disrupt the samples.

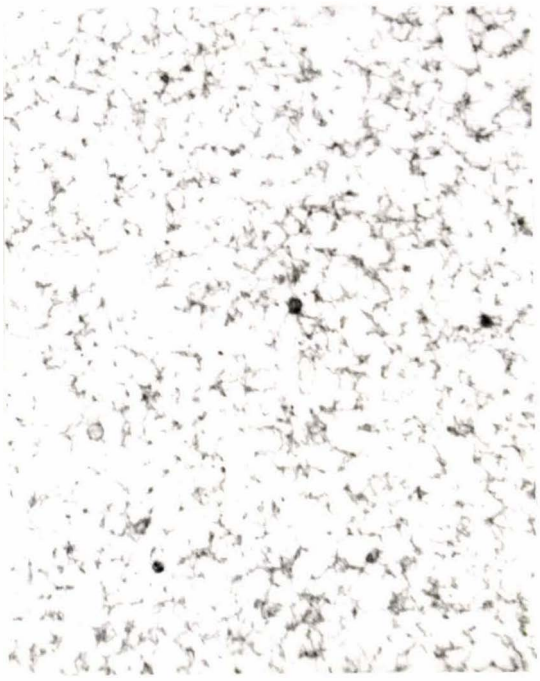
Samples of myosin were dialysed in sodium hydroxide, sodium hydrogen carbonate, and in sodium hydroxide solutions in which the addition of sodium hydroxide was stepwise resulting in pH from approximately 4.0 to about 10.0. The gels formed did not appear different to gels that had formed upon dialysis in water. However, the final pH values of the samples were generally greater than 6.0. A sample of myosin with added 0.011% PCMB was gelled with the addition 2.50% NaCl and 0.70% GdL. A white solid formed. Dialysis of this sample resulted in a sample similar to that without added PCMB.

Figures 7.1 to 7.3 are transmission electron micrographs of dialysed samples of myosin with 0.70% GdL in water and sodium hydroxide, myosin alone and with 0.70% GdL and 2.50% NaCl, respectively. The networks formed appeared uniform in appearance and were finer compared to networks prior to dialysis (Figures 5.8a-c, 5.7a-d and 5.18a and b).

7.2.2 Analysis of washed and dialysed myosin

A sample of myosin was washed with 50 times its volume of water. The sample was centrifuged as in the final step of the myosin preparation and the pellet was retained. It was noticed that there was a significant change in the appearance of the myosin. The original myosin sample was subjectively a white liquid-like suspension. The washed and centrifuged myosin had the consistency and appearance of hair gel, a translucent, colourless and very

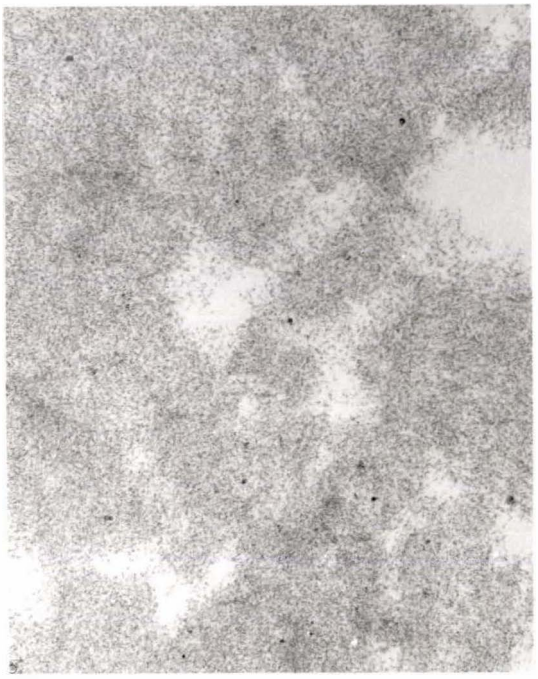
a



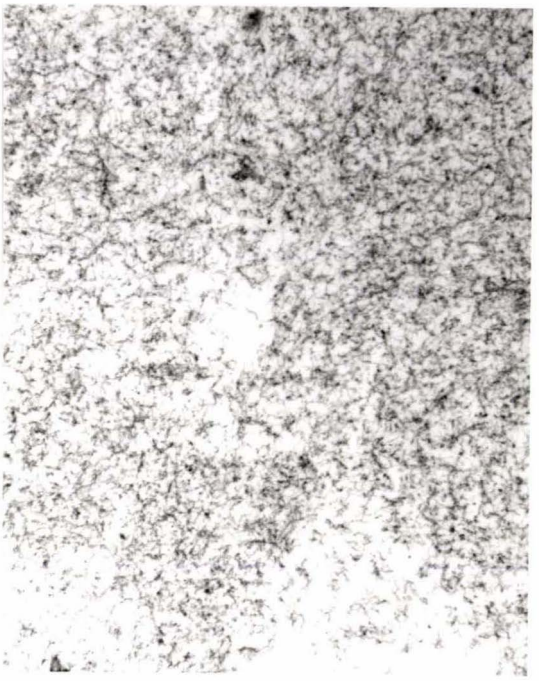
b

c

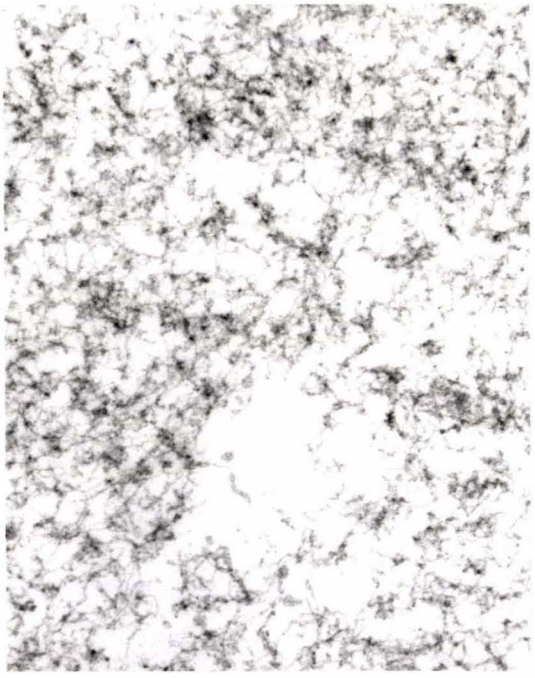
Figure 7.1 Transmission electron micrographs of myosin gels with 0.70% GdL, after dialysis (pH 5.7). The micrographs are at magnifications of a) 5200x, b)21200x, c)48600x.



a



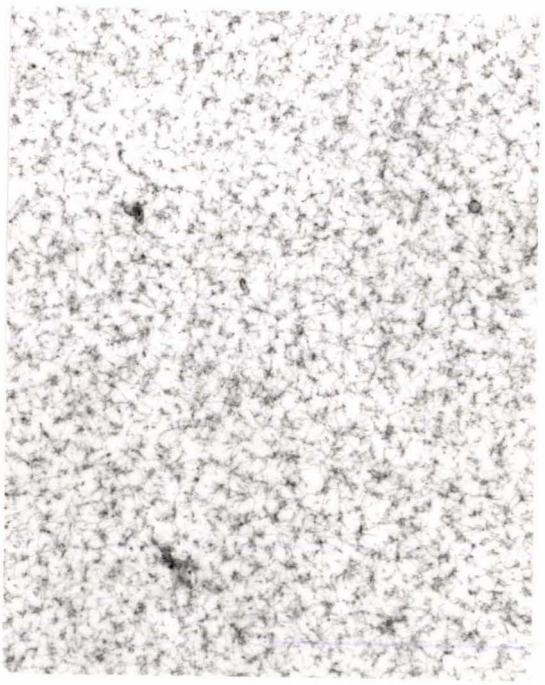
b



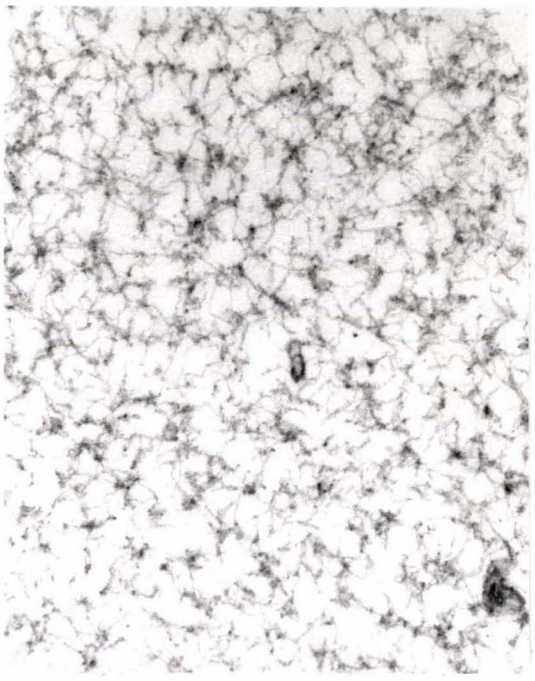
c

Figure 7.2 Transmission electron micrographs of myosin after dialysis (pH 5.7). The micrographs are at magnifications of a) 5200x, b) 21200x, c) 48600x.

a



b



c

Figure 7.3 Transmission electron micrographs of myosin with 0.70% GdL and 2.5% NaCl, after dialysis (pH 4.9). Micrographs are at magnifications of a) 5200x, b) 21200x, c) 48600x.

viscous solution or rubbery gel, but was not sticky like hair gel. The concentration of myosin prior to the washing step was 34.6 mg/ml. After washing and centrifugation, the concentration of the gel was 17.2 mg/ml and therefore, without loss of myosin occupied a significantly greater volume.

Analysis of the sodium and potassium ion contents of the solution obtained from the final centrifugation of the washed myosin gave:

5.10 μg/ml sodium

62.80 μg/ml potassium.

These values represent 0.23 mg sodium/ml myosin and 3.14 mg potassium/ml myosin removed from the myosin in the washing process.

In an attempt to understand the significant physical changes observed when myosin was washed in 50 times its volume of water, concentrations of sodium and potassium equivalent to that removed by washing were added to the washed sample as sodium and potassium chlorides. No apparent effect on the physical appearance of the myosin was observed. The sample remained translucent and colourless in appearance and was subjectively rubbery, the volume of the sample did not change and no water was observed to exude from the sample. Samples of the washed myosin alone and with the addition of 0.70% GdL were dialysed in water for 48 h. Similar samples of myosin which had not been washed, were dialysed. The results of the two elements, sodium and potassium for which there was a significant change measured by an Inductively Coupled Plasma (ICP) analysis are presented in Table 7.2.

Assuming that the ions added in the myosin preparation were not concentrated in the solids retained after centrifugation and no chemical interaction of the ions with myosin occurred, the concentrations of sodium and potassium present in the final myosin preparation were approximately:

3.910 mg potassium/ml myosin

0.391 mg sodium/ml myosin.

In the unwashed samples with and without GdL, high amounts of potassium and sodium were removed by dialysis (Table 7.2). The concentration of sodium removed from the unwashed

Table 7.2. ICP analysis of dialysates of washed and unwashed dialysed myosin and myosin with 0.70% GdL addition.

Sample	potassium (μg/ml)[a]	potassium (mg/ml myosin)[b]	sodium (μg/ml)[a]	sodium (mg/ml myosin)[b]
washed	<0.60		0.58	0.077
washed 0.70% GdL	<0.60		0.54	0.091
control[c]	<0.60		0.21	
unwashed	3.80	at least 1.64	0.88	0.344
unwashed 0.70% GdL	5.10	at least 2.09	3.10	1.437

[a]weight (μg) of element per volume (ml) of dialysate
[b]weight (mg) of element per volume (ml) of myosin, assuming that for myosin, 1 g = 1 ml.
[c]control = dialysis tubing which was clipped at both ends with bulldog clips and treated as though it contained a myosin sample in the tubing (stood at 24 h at 4°C, then dialysed for 48 h in water at 4°C).

sample with 0.70% GdL was greater than the calculated concentration of sodium based on the solutions used in the myosin preparation. Samples with added GdL resulted in a greater concentration of elements in the dialysate than samples without GdL. The washed samples resulted in sodium present in the dialysates, however the concentrations were less than in the unwashed samples. Similar to the unwashed samples, the presence of GdL resulted in greater concentrations of sodium in the dialysates.

7.2.3 Dialysis of myofibrillar proteins

Myofibrillar proteins were gelled and dialysed as cylinders in dialysis tubing or in PVC tubes with dialysis tubing at the ends of the tubes. In both types of sample, differences, when observed, were only observed at the surface of the gel in contact with the dialysis tubing. The inner volume of the gels remained similar in appearance to the sample prior to dialysis and the pH of the samples did not change at the centre.

A gel of myofibrillar protein with 0.3% TSPP, 2.3% NaCl and 2.0% GdL was dialysed in water for 44 h. The resultant gel was grey, crumbly at the surface, not sticky and had a pH of 4.6. The gel also had an 'off' odour. The addition of azide to the dialysis solution did not

prevent the formation of the odour. Dialysis in sodium hydroxide resulted in a similar gel to that in water and final pH of 4.5 after 66 h.

Myofibrillar protein gelled with 2.0% GdL and then dialysed in sodium hydrogen carbonate resulted in a gel with a white surface which was not sticky. The inside of the sample was sticky, pink and glassy, similar to the sample prior to dialysis. The pH of the core of the sample was approximately 4.0 while the surface was 5.7. The sample which had not been dialysed had an overall pH of 4.5. The white surface of the dialysed sample was strong and rubbery to the touch. After 168 h of dialysis, the sample had not changed.

Dialysis of myofibrillar protein alone did not result in an observable difference in physical appearance compared to that of myofibrillar protein which had not been dialysed. The addition of 2.3% NaCl resulted in a stronger gel after dialysis, than that which had not been dialysed in water. However, the gel was not strong enough to suspend by the clip for tensile testing.

The addition of 2.0% GdL and 2.0% GdL with 2.3% NaCl resulted in gels with strong white surfaces which were able to be tested using tensile force to the point of destruction. The samples which were not dialysed could be suspended, but were very fragile and disintegrated. Results of the two samples tested are given in Table 7.3.

Table 7.3 Tensile test of dialysed myofibrillar gels.

Additives	Force (N, average)	Force (N, s.d.)	Displacement (mm, average)	Displacement (m, s.d.)
2.0% GdL	0.153	0.018	17.6	4.0
2.0% GdL 2.5% NaCl	0.106	0.019	5.6	1.2

Terms pertaining to Table 7.3:
s.d. = standard deviation
Force, Displacement = measured at the point of destruction

7.2.4 Dialysis of minced meat

Gelled samples of minced meat (50.0 g) with added GdL (3.0 g) in PVC tubes were suspended in a flow of water. The surface of the gel in contact with the dialysis tubing became white and strong and the pH approached 5.0. The centre of the gel remained red and glassy and the pH did not change from 4.0.

7.3 DISCUSSION

Reversal of pH alteration or ion addition is difficult to obtain compared to thermal reversibility where the cooling or heating rate can be controlled. Addition of GdL to minced meat, myofibrillar protein or myosin resulted in the slow lowering of the pH of the samples. The exact rate of this lowering and the rate of addition of salts to the samples was uncertain and would have been affected by the buffering capacity of the sample and the effect of ions already present in the system. To raise the pH at a similar, but reverse rate, to the change induced by GdL is virtually impossible. Addition of salts was stepwise in the myosin preparation and the gel formation of the three systems. Removal of these ions at similar rates is also difficult, if at all possible. However, the rate of addition may not affect the reversibility of the gel, or may only partially affect the reversibility. Regardless of the reversible state achieved, removal of ions and the alteration of the pH to values greater than the IEP would provide an indication of the structural integrity of the gels formed using GdL.

7.3.1 Myosin gel reversibility

Addition of myosin gels directly to water, without being contained in dialysis tubing, resulted in the dissolution of the gels into the water. This could have indicated a disruption of the gel network formed with subsequent reversal of the state of the myosin to a monomeric or filamentous conformation. Containment of the myosin sample during dialysis appeared to result in the formation of a gel of different characteristics to the sample prior to dialysis. This containment was achieved using dialysis tubing.

The dialysis of cylinders of myosin resulted in a strong rubbery layer at the surface of the gel in contact with the dialysis tubing. The centre of the gel remained at the pH of the myosin sample which had not been dialysed and the sample centre did not change in appearance. Hence, it may be deduced that this strong surface was impermeable to movement of protons and solution into the sample and ions outwards. This impermeable layer did not allow the centre of the myosin sample to change as the surface was able to. This phenomenon appeared to be similar to that of case hardening in tanning. Changing the shape of the sample from cylindrical to flat discs overcame this problem of case hardening and the dialysed gels were uniform in appearance across the whole of the gels. This disc-like sample was used thereafter.

The pH of the dialysis solution appeared to have little effect on the physical appearance and the rate of formation of dialysis-induced myosin gels over a 24 h period. Stepwise addition of sodium hydroxide from pH 4.0 to 10.0 to the dialysis solution surrounding myosin with added 0.70% GdL gels, immersion of gels of myosin with 0.70% added GdL in solutions of sodium hydroxide or sodium hydrogen carbonate of pH 8 to 10, or immersion of these myosin gels in water resulted in no observable differences in the gels formed. All were translucent, colourless, strong gels compared to samples subjected to similar treatments, but which had not been dialysed, which were white suspensions.

Dialysis of ions out of the systems is slow relative to removal by washing, which is almost immediate. Repeated washing of the samples of myosin alone resulted in a translucent gel, similar in appearance to that formed by dialysis. The washed sample also occupied a greater volume than that prior to dialysis. Volume changes were difficult to observe in the flat discs of myosin that were dialysed, due to the lack of uniformity in sample shape and the thinness of the discs. Subjectively, the samples did appear to increase in volume with dialysis.

The washed sample was of myosin alone and not of the acid-induced gelled myosin in which myosin was denatured by lowered pH and then gelled. Placing a gelled myosin sample, such as that of myosin plus GdL plus NaCl, in quiescent water resulted in the dissolution of the myosin. This suggested that a disruption of the myosin network had occurred and possibly a reversal of the myosin network to essentially monomeric or filamentous myosin. These results indicated that the rate of ion diffusion was important in obtaining reversibility of the

GdL-induced gel. A slow rate of ion diffusion, imposed by the barrier of dialysis tubing, resulted in a stronger gel which was dense to the point of being effectively impermeable. However, at a sufficiently fast rate of ion diffusion, the myosin gel appeared to be reversible.

It should be noted that while the gel appeared to be reversible, the myosin might not have returned to its native state. It is possible that the denaturation of myosin was irreversible. This could have resulted in a different conformational state of the washed and acidified myosin, to that of native myosin upon dispersion of the gel. Experiments involving optical rotary dispersion (OR) and circular dichroism (CD) could provide information about the folding of the different myosin molecules.

The potassium removed by dialysis of the unwashed myosin probably was sourced from the solutions added during the myosin preparation and can be accounted for based on uniform distribution of the potassium throughout the myosin and its washings during the myosin preparation. Washing removed 3.14 mg potassium/ml myosin from myosin alone. This translates to approximately 80% of that added in the myosin preparation. Dialysis resulted in approximately 50% removal of the amount of potassium removed by washing, or 40% of the potassium added in the preparation of myosin.

Washing removed 0.23 mg sodium/ml myosin and dialysis after washing removed a further 0.077 mg sodium/ml myosin. This gives a total of 0.307 mg sodium/ml myosin removed. Similar to the removal of potassium by washing, the total sodium removed equates to approximately 80% of that added in the myosin preparation. Dialysis alone resulted in approximately 90% removal of sodium from the myosin. However, the amount of sodium removed from the unwashed myosin with 0.70% GdL added, suggested that sodium interacted with the myosin during the myosin preparation. The sodium removed in the dialysate (1.437 mg/ml of myosin) was greater than that added in the myosin preparation (0.391 mg/ml myosin) assuming that the sodium was uniformly dispersed throughout the supernatants and pellets in the centrifugation steps of the preparation of myosin and assuming there was no interaction of these ions with myosin. These results suggested that the sodium added was not uniformly removed as assumed, but was concentrated in the myosin pellets during the myosin preparation and the presence of GdL acted to remove some of the sodium. This then

suggested that myosin had a greater affinity for sodium than potassium, which to the best of the author's knowledge has not been previously reported. Note that the sodium and potassium in the myosin system prior to gelation was only calculated based on that added. There would also likely be some sodium and potassium inherent in the myosin system, which was not quantified.

The addition of GdL to the washed myosin samples resulted in a greater loss of sodium and potassium via dialysis than from the myosin only samples. Raising the pH of the myosin sample would alter the charges on the protein and result in some movement of groups involved in electrostatic interactions. It is also important to note that anions and cations cannot migrate through a membrane in arbitrary amounts because electrical neutrality has to be preserved on both sides of the membrane. If an anion migrates in one direction, a cation must accompany it to maintain the charge balance, and vice versa. This is the Donnan effect (Atkins, 1984) which would appear a useful mechanism to explain the effects of dialysis on myosin suspensions, gels and solutions.

In the present experiments, washing of myosin resulted in the removal of potassium and sodium. Assuming that the myosin gel formed as a result of washing was a result of the removal of these ions by the Donnan effect, then the addition of these ions to the washed myosin should have produced a myosin suspension like that prior to washing. Concentrations of sodium and potassium similar to that removed by washing, when added back to the myosin did not result in a reversion of the translucent, rubbery myosin gel to the original white myosin suspension. These results indicated that the removal of ions from myosin did not produce a reversible gel with respect to ion content and it is suggested that the Donnan effect does not adequately explain the mechanism of myosin gel formation via dialysis. The fact that the Donnan effect does not take into account chemical effects could explain the inadequacy of this mechanism.

Studies by Chong (1994) involving diafiltration (10 kDa molecular mass cut off) of sodium cholate with unbuffered water resulted in precipitates and has presented an explanation for the effects of dialysis on the myosin systems. Diafiltration is similar to prolonged dialysis. The water, as was also used in the present experiments, was unbuffered and therefore, was

saturated with carbonic acid. Stirring the water and surface exposure to air meant there was a constant supply of carbon dioxide in contact with the water and the carbonic acid was constantly replenished, if used. The precipitate resulting from the diafiltration of the sodium cholate was a result of the ion exchange of sodium for protons, converting the sodium cholate to insoluble cholic acid. Similar displacement of sodium and potassium ions by protons in the myosin system could explain the change in the physical appearance of the solutions, suspensions and gels upon dialysis. The GdL added may not be in significant concentration to displace all sodium present, but would probably displace some of the sodium. Hence, the greater amount of displacement of sodium and potassium in the presence of GdL (Table 7.2) and possibly the greater 'strength' of the gel achieved.

Interactions could result in refolding of the molecules or strengthening of the network already in place. If refolding occurs, it may be only partial refolding of the myosin or network structure adding greater stability and strength to the gel network increased by the added interactions, such as repulsion. The varied force values which resulted for the dialysed myosin samples with the combinations of additives (Table 7.1) could be explained by the changes induced by dialysis, being additive to, or at least affected by, the systems present prior to dialysis. If the dialysis resulted in complete refolding and new network formation, it would be expected that the resultant gels would all be the same. Although all the gels were similar in appearance, the force required to destruct the gels formed after dialysis varied. It is suggested that this variability is a result of refolding and network formation of myosin, affected by the state of the myosin prior to dialysis. Perhaps the gels formed prior to dialysis are not disrupted, but built upon. With only relatively weakly bound ions removed, bonds or interactions forming a network prior to dialysis, such as in the myosin sample with added 0.70% GdL, 2.50% NaCl and 0.25% TSPP, possibly remained after dialysis. Strengthening of the already formed gel by extra hydrogen bonding arising from, for example, the generated carboxylic acid groups, and increased interaction could explain the high tensile force at the point of destruction of these gels after dialysis compared to similar gels which had not been dialysed. In addition, lowered ionic strength would have increased the concentration of insoluble protein in the myosin sample allowing more myosin to participate in gel formation.

The appearance of the dialysed gels in the micrographs of myosin, myosin with 0.7% GdL and myosin with 2.50% NaCl and 0.70% GdL (Figures 7.1 to 7.3) showed a fine network without the appearance of aggregates or filaments in sharp contrast to the samples which had not been dialysed. These results support the suggestion that there is a change in the nature of the network of myosin after dialysis.

The addition of PCMB to myosin prior to gelation with 2.50% NaCl and 0.70% GdL, did not result in apparent differences in the gel formed upon dialysis compared to those without the addition of PCMB. These results suggested that sulfhydryl bonding was not involved in gelation which occurred with dialysis.

The gels formed with dialysis have not been shown to be true gels according to the definition advanced by Glicksman (1982). There were no experiments in the present work which were directed at defining the 'gels' formed as true gels or entanglement networks.

7.3.2 Myofibrillar protein and minced meat gel reversibility

The myofibrillar protein did not form a gel when dialysed and was similar to the sample which had not been dialysed. The preparation of myofibrillar protein, unlike that of myosin, did not involve the use of buffers and therefore no added sodium or potassium would have been present in the myofibrillar protein. Without the lowering of the pH, myosin was not extracted to any significant degree (observed in Chapter Four). Without a critical amount of protein extracted from the myofibrillar structure, a three-dimensional network would not have formed.

The addition of NaCl to the myofibrils also had little effect on the myofibrillar gel when dialysed. However, the resultant gel was subjectively slightly stronger than the sample which had not been dialysed, perhaps due to disaggregation of myosin filaments that might be present and possibly dissociation of some myosin filaments. The displacement of the added sodium could result in partial network formation of any extracted myosin and reinforcement of the myofibrillar protein.

The samples with added 2.0% GdL, 2.3% NaCl and 0.3% TSPP at pH 4.5 have partially extracted and dissociated actomyosin. In Chapter Four, it was suggested that the extracted protein formed an impregnated composite system of myosin about the myofibrillar structure. With this network present, removal of the ions, as with the myosin gels, could result in stronger network formation (Table 7.3) via increased intermolecular interactions with the displacement of ions perhaps resulting in unfolding and refolding, and greater hydrogen bonding.

The myofibrillar sample with only 2.0% GdL added, also resulted in increased gel 'strength' (Table 7.3). This could be explained by the presence of extracted myosin in the myofibrillar sample. This extracted myosin would likely have sodium bound in the system and protons are able to displace these sodium cations upon dialysis.

Removal of ions from the minced meat sample resulted in a strong white layer at the surface of the sample in contact with the dialysis tubing. The fat and gristle present in the minced meat samples would provide barriers to dialysis of these ions from the sample centres. It also appeared that the strong surface may have prevented dialysis of ions from the centre of the meat sample, similar to the impermeable layer formed in the cylinders of dialysed myosin, which was suggested to be a result of case hardening.

7.3.3 Summary

When the rate of displacement of sodium and potassium was sufficiently high, the myosin gels appeared to disintegrate. The rate of reversion of pH and ion removal or displacement was therefore, suggested to be important in achieving reversibility of GdL-induced myosin gels. A slow rate of ion diffusion, imposed by a barrier of dialysis tubing, resulted in a strong, translucent gel, which was dense to the point of being effectively impermeable to ion migration. This significant change in density was not easily observed in the TEM micrographs, in which the structure of the network appeared more uniform and possibly more dense.

The enhanced tensile 'strengths' of the gels formed by dialysis of discs of myosin was hypothesised to be a result of the displacement of sodium and potassium ions with protons, thereby enhancing hydrogen bonding. The protons are present in excess, due to the carbonic acid - water equilibrium. The exposure of water to air provided a constant reservoir of carbon dioxide, and therefore carbonic acid to maintain the equilibrium.

The addition of GdL to myosin without dialysis, could have resulted in some network formation by the displacement of ions similar to dialysis. When dialysed, even more of the ions would have been displaced, resulting in increased interactions, such as hydrogen bonding, and therefore increased tensile 'strength' of the gels formed. Displacement of ions by protons and any unfolding or further interaction would have been affected by the state of the myosin prior to dialysis, explaining the variation in tensile 'strengths' of the gels obtained after dialysis.

It appeared that myosin had a stronger affinity for sodium, compared to potassium and that sodium did not appear to be uniformly removed from the pellets and supernatants in the centrifugation steps of the preparative procedure of myosin.

The myofibrillar gels with added 2.3% NaCl or decreased pH (2.0% GdL added), with or without added 2.3% NaCl plus 0.3% TSPP, resulted in stronger gel formation upon dialysis. At the lowered pH, partial extraction and dissociation of actomyosin would have occurred resulting in an impregnated composite network of myosin reinforcing the myofibrillar structure. Displacement of sodium from the myosin in the myofibrillar system would act in the same way as for myosin alone.

CHAPTER 8

DISCUSSION

8.1 DISCUSSION OF RESULTS

Acid dialysis into a myosin system caused slow lowering of pH and resulted in protein gel formation (Fretheim *et al.*, 1985; Hermansson *et al.*, 1986). It was proposed that GdL addition to a myosin system causing slow lowering of the pH, similar to dialysis, might also result in myosin gel formation. Myofibrillar proteins, in particular myosin, have been shown to be the major contributors to thermally-induced meat gelation (for example, Fukazawa *et al.*, 1961a,b; Hashimoto *et al.*, 1959; Schnell *et al.*, 1970; Vadehra and Baker, 1970; Siegel *et al.*, 1978a,b). Assuming that myofibrillar proteins also play a significant role in acid-induced gelation, it was further proposed that addition of GdL to a meat system might result in meat gelation.

Addition of GdL to minced meat did form gels. The lactone, GdL, is commonly added to food products, including varieties of sausage, as a method of lowering the pH. However, it appears that this additive has not previously been used specifically and solely as a gelling agent in meat systems. It was difficult to obtain useful data to quantitatively describe the minced meat gels formed. This was due to the large variance in the data caused by the presence of gristle and fat in this multicomponent system. It was therefore required that the investigation be focused on myofibrillar proteins because of the greater uniformity achieved using this protein group.

Addition of GdL to myofibrillar protein resulted in gel formation. Two mechanisms of gel formation were proposed to explain gelation at different pH values. At a pH value between 4.8 and 4.5, and at values less than this pH, partial extraction of the A-band occurred and a gel network formed. Results of TEM work were similar to those of Rao *et al.* (1989b) who studied the acid marinading of beef. This TEM showed that the myofibrillar structure was

present at pH 4.5. Comparison with work of Rao *et al.* (1989b) and using soluble protein data and Young's Modulus data, suggested that the gel formed was an impregnated composite system of myosin formed about the already present myofibrillar structure. It was suggested that a minimum amount of extracted protein was required for gel formation to occur.

At a pH value between 4.5 and 3.9 and below this value, complete extraction of the A-band occurred as actomyosin was depolymerised and filaments were dispersed. Dispersion of the filaments resulted in the dissolution of the myofibrillar structure and therefore the resultant gel was no longer an impregnated composite system, but was suggested to be a myosin gel incorporating components, such as actin, into the gel structure.

According to Ferry's theory of thermally-induced protein gelation, denaturation must precede aggregation and the aggregation step must occur more slowly than the denaturation to allow the denatured protein molecules time to orient themselves and interact at specific points, thus forming a three-dimensional network (Ferry, 1948). Fretheim *et al.* (1985) showed that at pH 5.5 and less, extracted myosin was denatured with time. This would have allowed new interactions to occur as the protein unfolded and exposed reactive groups. Decreased pH would provide stronger denaturing conditions. In the present experiments, a greater amount and a faster rate, with respect to pH, of denaturation was observed as the pH was decreased. Lowering the pH, and therefore increasing the amount of denatured protein, resulted in a greater rate of gel formation. The sequence of events of myofibrillar gelation is presented in Figure 8.1.

Measurement of Young's Modulus using an Instron Universal Testing machine is a crude measure of the characteristics of the gel formed. This crude measure, the assumption that extraction of the A-band results in gel formation and therefore myosin is largely responsible for gel formation of myofibrillar proteins, and the many investigations concluding that myosin was responsible for heat-induced gelation (including, Schnell *et al.*, 1970; Siegel *et al.*, 1978a,b; Vadehra and Baker, 1970) made the transition from the investigation of myofibrillar protein to myosin a logical step in the determination of the mechanism of meat gelation at low pH.

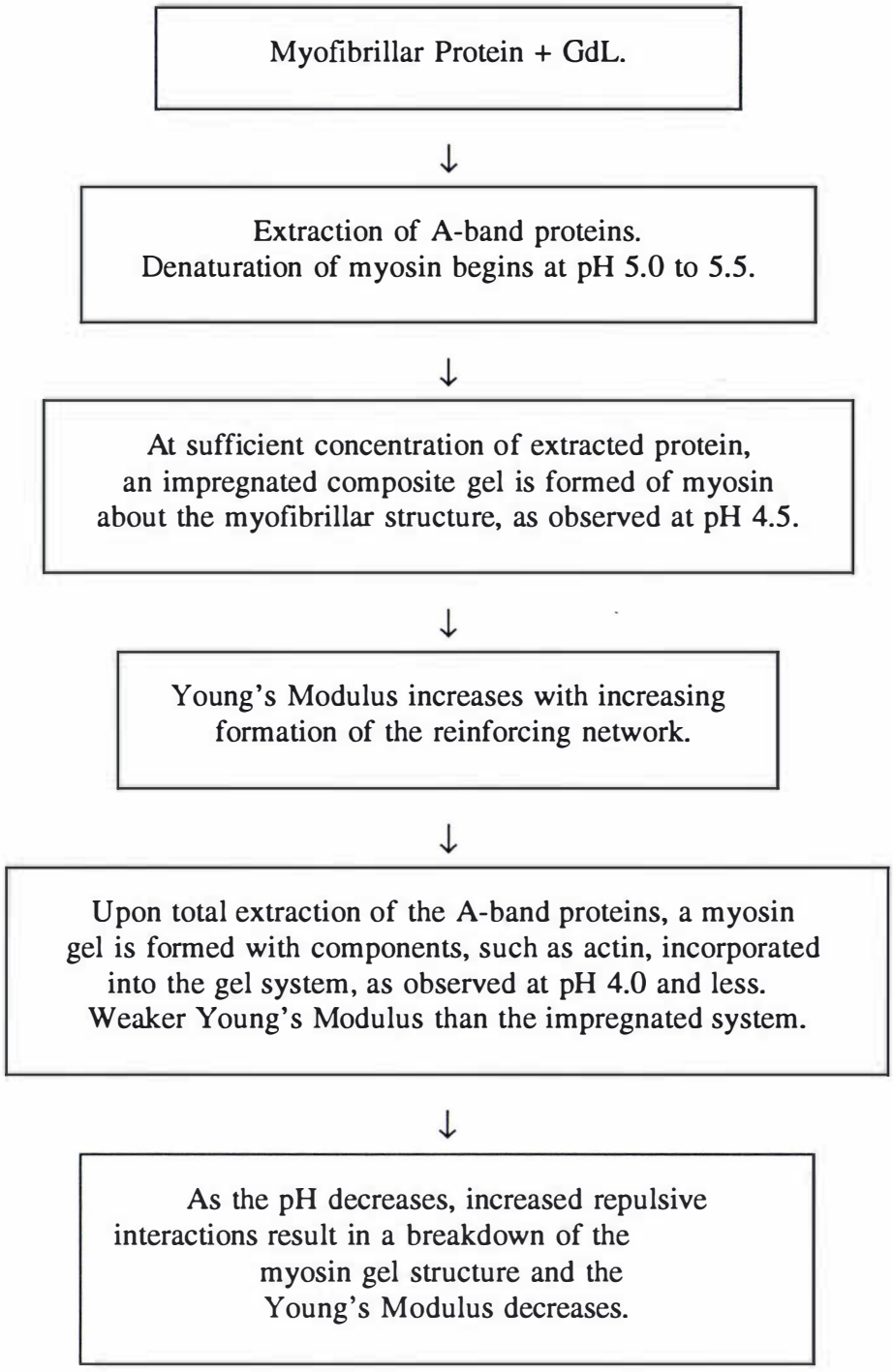


Figure 8.1. The sequence of events in GdL-induced myofibrillar gelation.

Use of myosin would allow investigation of the types of bonding and interactions involved in gelation. While bind strength, denaturation and sulfhydryl bond formation have often been investigated in the heat gelation of meat and meat proteins (for example, Siegel and Schmidt, 1979b; Fukazawa *et al.*, 1961c; Samejima *et al.*, 1983), other interactions, such as hydrogen bonding, have rarely been studied. It would appear that intermolecular interactions are the basis of the mechanism of gelation of these proteins, and therefore knowledge of the interactions involved in gelation would provide a better understanding of the gelation process.

It is difficult to compare the composite myofibrillar system with data from a crude myosin system, essentially a single myofibrillar protein, without knowledge of the interactions among the proteins or the dissociation state of the myofibrillar proteins. While direct comparison of data was not possible, trends could be compared.

The constraints of the myofibrillar structure are eliminated in a myosin system and myosin is present in an essentially monomeric or filamentous state. Myosin was shown to denature at pH 5.5 with time (Fretheim *et al.*, 1985) and at sufficient concentration of soluble protein formed a gel network. As the pH of solutions of myosin decreased with the addition of GdL in the present experiments, exposure of the hydrophobic sites increased, as measured by fluorescence spectroscopy, and indicated that denaturation had occurred. Parallel to denaturation was gel formation of myosin suspensions measured using a Bohlin rheometer. Denaturation stopped at pH 4.3 to 4.5. However, the pH continued to decrease and the gel formed at about pH 4.5 became liquid-like at about pH 4.0, suggested to be due to an excess of repulsive interactions.

In accordance with Ferry's theory (Ferry, 1948), it was assumed that denaturation had preceded aggregation if a gel formed. There were no measures taken to verify gelation at the gel-like stage of G' development. It was suggested that the molecules had unfolded exposing reactive sites on the protein, thereafter these sites reacted to form a three-dimensional network. Hydrogen bonding participated in the gel network as indicated by the use of sucrose, a competitor for these sites. Electrostatic interactions would likely have played a role in gel formation with the altered pH. Hydrophobic bonding would also have played a role with exposure of these sites observed by fluorescence spectroscopy. Sulfhydryl bonding did not

contribute to gelation. These results differ from the study of acid-induced gelation of myosin via dialysis undertaken by Fretheim *et al.* (1985) where sulfhydryl bonding was observed to be involved in gelation at 4°C. The sequence of events that is suggested for the formation of GdL-induced myosin gels is presented in Figure 8.2.

The myosin gel formed with GdL at pH 4.2 had a dense structure. Aggregates of myosin strands were not evident at pH 4.2, but were observed in myosin alone at pH 6.8. These aggregates may have been present in the densely packed structure of myosin with GdL, but the dense nature of the gel did not permit observation of these aggregates.

The formation of myofibrillar protein gels as a result of hydrochloric acid dialysis and the direct addition of hydrochloric acid suggested that the formation of gels upon hydrolysis of GdL is a general acid-induced mechanism and not a GdL- or gluconic acid-specific interaction with the protein. Further support for this mechanism is found in the experiments of Fretheim *et al.* (1985) and Hermansson *et al.* (1986) who used buffers consisting of combinations of phosphate and citric acid to lower the pH of myosin solution via dialysis, resulting in gel formation. Slow introduction of the acid allowed uniform gel formation to occur.

The addition of 2.3% sodium chloride (NaCl) to myofibrillar proteins with GdL resulted in the formation of an impregnated composite system at pH values greater than 4.5 due to the extraction of A-band protein, as in the absence of NaCl. The myofibrillar structure was retained down to lower pH values than observed in the absence of NaCl. This was attributed to the effect of salt on the IEP of myosin. At concentrations of 0.4 to 0.6 M potassium chloride, the IEP of myosin has been observed to be less than pH 4.5. While it is unlikely that the IEP of myofibrillar protein would be lowered as much as for myosin, due to the greater buffering capacity of myofibrillar protein, it is likely that the IEP would be lowered. The gel which resulted with the inclusion of NaCl in the myosin plus GdL system, had greater Young's Modulus at pH 4.0 compared to similar gels in the absence of NaCl. This increased Young's Modulus was possibly due to the impregnated composite system formed at pH less than 4.5 in the presence of NaCl. Unlike the system with GdL only, it was suggested that the impregnated composite system remained intact down to pH 4.0, due to the lowered IEP. In

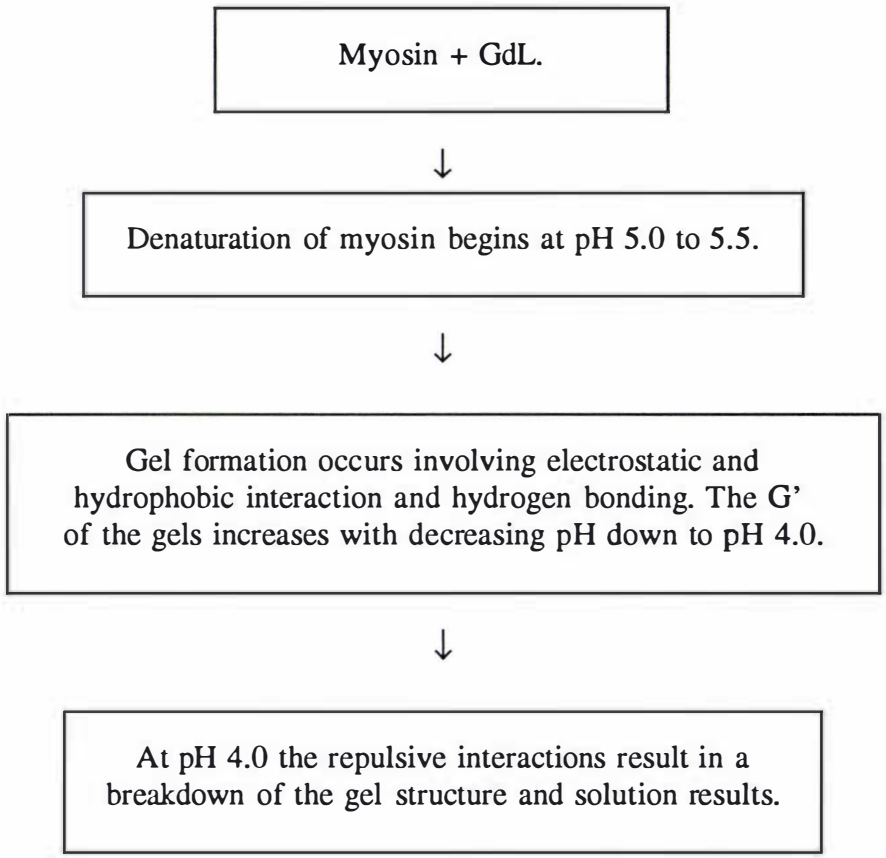


Figure 8.2. The sequence of events in GdL-induced myosin gelation.

the presence of NaCl, the greater solubilisation of myosin as the pH decreased suggested that more myosin would have participated in the gel network. With a greater amount of myosin participating in network formation, the gel formed would have a greater Young's Modulus than in the absence of NaCl. As the concentration of added NaCl was increased in the myofibrillar protein plus GdL system, the number of interactions would have increased at given pH, with dissociation of extracted myosin filaments, and the IEP of myofibrillar protein or myosin would have decreased. The gel network was suggested to have become more tightly bound and liquid was observed to exude from the samples. At a critical salt level, about 22.00% NaCl, salting out occurred. With the tighter network formed, liquid was forced from the protein matrix and the water holding capacity was decreased. The gel formed became less springy and more rigid with the increased bonding, and was crumbly from the salting out.

The addition of 2.50% NaCl to myosin alone resulted in a clear solution of solubilised protein. The inclusion of 2.50% NaCl in the myosin system with GdL resulted in less denaturation at given pH compared to in the absence of NaCl. This effect on denaturation was suggested to be a result of the lowered IEP of myosin. The amount of denaturation, as was observed in the absence of NaCl, increased with decreasing pH. The addition of NaCl lowers the IEP of myosin and stabilizes the protein molecules by shielding the charges of the protein as the pH is reduced. Therefore, in the presence of NaCl a lower pH is required to result in a given amount of denaturation compared to in its absence. With added GdL, the G' of the gels was essentially the same at pH above 4.5, with or without the addition of 2.50% NaCl. However, as the pH decreased below 4.5, there was no observable decline in G' until pH 3.1 when 2.50% NaCl was added. This was attributed to the lowering of the IEP of myosin. The shielding of the charges would lessen the repulsion between molecules and therefore the deleterious effect of excess repulsive interactions, which were suggested in the absence of NaCl, would not be achieved until lower pH.

Increased concentrations of NaCl resulted in increased concentrations of soluble protein and therefore would have initiated increased interactions upon denaturation. These increased interactions resulted in tighter matrix formation and therefore greater G'. Increased

interactions initiated by denaturation in the presence of salt explain the greater G' in the presence of NaCl and GdL compared to in the presence of GdL only.

The gels formed with the addition of GdL and NaCl were true gels and not entanglement networks. The networks formed involved electrostatic bonding, with lowered pH, and likely more so with the added salt, compared to in the absence of salt. Hydrophobic bonding was also involved with the exposure of hydrophobic sites upon denaturation. There was no evidence to support or oppose hydrogen bonding, while sulfhydryl bonding did not appear to be involved in gelation. Similar to the gel formed in the absence of NaCl, the gel formed in its presence consisted of aggregated strands.

The inclusion of 0.3% tetrasodium pyrophosphate (TSPP) in the myofibrillar protein system had no effect on the observed characteristics of the gels formed in the presence of GdL until the myofibrillar system had completely broken down (about pH 4.0). At this pH and less, the TSPP enhanced the Young's Modulus of the protein gel.

In the present experiments, the absence of the constraints of the myofibrillar structure with the addition of 0.25% TSPP to myosin resulted in a large initial G' in the absence and presence of GdL. Studies on the effects of phosphates have generally used meat or myofibrillar protein. The complex nature of these systems and phosphate behaviour has resulted in a large number of theories to explain the effects observed. Many of these theories are based on slim evidence and have been a matter of dispute.

Two mechanisms were suggested to explain the large G' observed with the addition of TSPP to myosin with and without GdL in the present experiments (Figure 8.3). The s values and the strain sweep data were not conclusive and suggested that the network formed could have been either a gel or an entanglement network. If an entanglement network was formed, it was proposed that it might be a result of the addition of TSPP, in a largely dissociated form, to myosin. At the pH of the myosin system with the addition of TSPP, pH 7.6 to 8.8, the TSPP would have been largely dissociated and would have been a concentration of negative charge when compared to, for example, NaCl. This polyanion could induce movement of the largely negatively charged myosin at this pH, and result in an entanglement. While the myosin

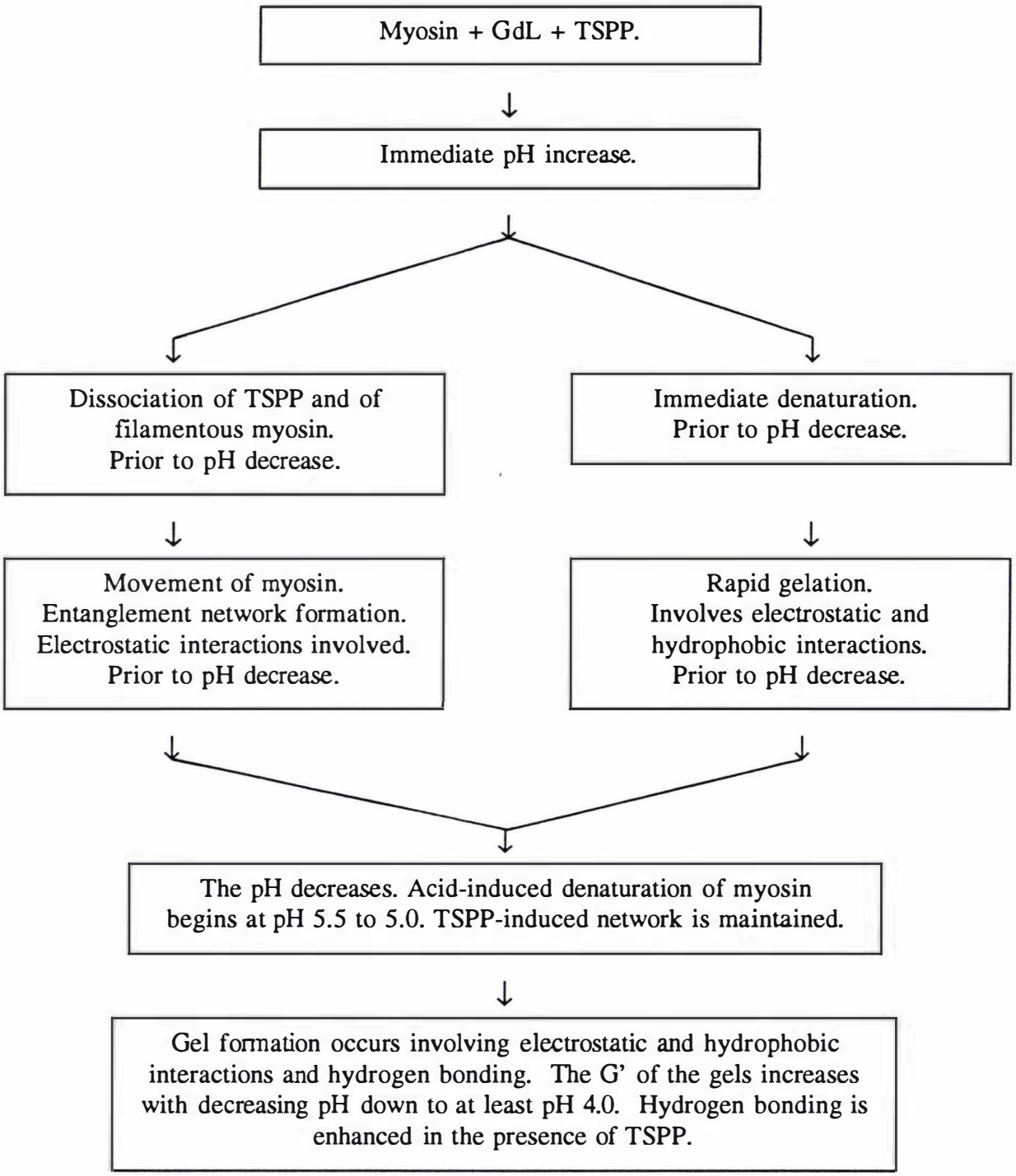


Figure 8.3. The sequence of events in GdL-induced myosin gelation with added TSPP.

molecule has a large molecular mass of approximately 500 kDa and has heads protruding from the coiled tail, this molecule would not likely form an entanglement network in the filamentous state. The rigid nature of the filaments would not permit the flexibility required to achieve entanglement formation. Therefore the conditions of pH must also induce the essentially monomeric state of myosin, from the filament-like state observed in the TEM micrographs of myosin.

In contrast to the explanation of the effects of TSPP on myosin via entanglement network formation, is the suggestion that the addition of TSPP might result in gel formation. According to Ferry's theory of gel formation, denaturation must precede aggregation for a gel network to form (Ferry, 1948). Denaturation was not observed using fluorescence. However, the immediate increase in G' observed suggests that gel formation was rapid. If immediate denaturation and subsequent rapid gelation occurred, this may have happened prior to commencement of measurement of fluorescence. Mixing of ANS and the additives, such as GdL, and transference of the sample to the fluorometer resulted in a delay of 2.5 min from the addition of GdL to the commencement of measurement of the fluorescence. If denaturation and gelation had occurred essentially immediately, this delay in measurement could explain why no denaturation was observed when measurement began.

The inclusion of GdL in the myosin plus TSPP system resulted in decreased pH and acid-induced denaturation occurred subsequent to the TSPP-induced network formation. Hydrogen bonding was enhanced with the inclusion of TSPP. The initial network formed with the addition of TSPP appeared to be maintained throughout the subsequent acid-induced denaturation and gelation. The stability provided by the TSPP-induced network and enhancement of the hydrogen bonding resulted in maintenance of the acid-induced gel to pH below 4.3.

It was suggested that the addition of further phosphates, such as tripolyphosphate, hexametaphosphate and orthophosphate, to myosin might provide data leading to an explanation for the effects of phosphates on myosin. The addition of tripolyphosphate (TPP) to myosin in the presence and absence of GdL resulted in increased initial G' and increased

pH. The effects of TPP on myosin appeared similar to the effects observed with TSPP addition to myosin and therefore, the same conclusions were drawn to explain the effects of TPP addition on myosin (as for Figure 8.3).

The addition of hexametaphosphate (HMP) to myosin in the presence of GdL showed a lag period in the G' curve prior to G' development compared to the addition of TSPP or TPP to myosin plus GdL. The addition of HMP did not induce a large increase in pH compared to TSPP or TPP addition, and this could explain the lack of initial high G' observed. The low pH with the added HMP, compared to the addition of TSPP and TPP, may not have resulted in the dissociation of myosin filaments required for entanglement network formation. In contrast to entanglement formation, low pH may not induce much, if any, denaturation of the myosin required for gel formation. The lag period observed may have been a result of the largely dissociated HMP molecule forming hydroxyl groups as the pH was lowered, which could then have enhanced hydrogen bonding. Without this enhancement of the hydrogen bonding, the G' curve might follow that obtained for GdL only.

The small size of the orthophosphate (OP) molecule favouring intramolecular interactions and the small increase in pH when added to myosin were used to explain the lack of G' development upon addition of OP and the lack of effect on the G' curve with acid-induced gelation.

Because of insufficient data to distinguish between the two proposed sequences of gel formation, it is suggested that further and more detailed studies should be conducted to investigate the mode of action of phosphates on myosin. These experiments might include electron microscopy to observe the filamentous or monomeric state of myosin at alkaline pH, or obtaining more s values and strain sweep data for statistical significance to show the nature of the type of network formed, that is, gel or entanglement.

The pH and NaCl effects dominated the myofibrillar system with GdL, NaCl and TSPP added. No significant effect as a result of the presence of TSPP was observed. However, this combination of additives in a myosin system resulted in intermediate G' values and decreased denaturation compared to GdL with TSPP or GdL with NaCl. The effects of addition of both

NaCl and TSPP with GdL appeared to be dominated by the effects of NaCl and therefore the effects of ionic strength. However, TSPP did influence the resultant G' and denaturation.

Reversibility of heat-induced myosin gels is not often investigated and involves cooling the myosin sample at the same rate that it was heated at. Reversibility of acid-induced gelation is more difficult to undertake in terms of removal of ions, in particular, at the rate at which the ions were added. Myosin gels appeared to revert to myosin filaments or monomers when allowed to stand in quiescent water, a condition of unlimited ion diffusion. The rate of reversion of pH and ion removal or displacement was therefore suggested to be important in achieving reversibility of GdL-induced myosin gels. A slow rate of ion diffusion, as imposed by a barrier of dialysis tubing, resulted in a strong, translucent gel, which was dense to the point of being effectively impermeable to ion migration. The structure of the network observed using TEM appeared more uniform than prior to dialysis.

The enhanced tensile 'strengths' and impermeability of the gels formed by dialysis of discs of myosin was hypothesised to be a result of the displacement of sodium and potassium ions with protons, thereby enhancing hydrogen bonding. The protons are present in excess, due to the carbonic acid - water equilibrium and the exposure of water to air providing a constant reservoir of carbon dioxide, and therefore carbonic acid to maintain the equilibrium. The added protons could result in increased intermolecular hydrogen bonding adding rigidity to the already present network, such as through the formation of more carboxylic acid groups. These effects and the effects of electrostatic interactions with the removal of sodium and potassium and the replacement with protons possibly resulting in some unfolding, would provide possibilities of added interactions, increasing gel rigidity The networks present prior to dialysis were not necessarily destroyed, but may have been built upon. At least, the state of myosin prior to dialysis would explain variability among dialysed sample tensile force values at the point of destruction.

Investigation of the dialysed myosin gel prompted experiments involving washing. As a result of these experiments, it appeared that myosin had a stronger affinity for sodium, compared to potassium and that sodium did not appear to be uniformly removed from the pellets and

supernatants in the centrifugation steps of the preparative procedure of myosin. To the best of the author's knowledge, these effects have not previously been reported.

Myofibrillar gels with added GdL, GdL plus NaCl or GdL, NaCl plus TSPP, resulted in stronger gel formation after dialysis than before. Displacement of cations from the system would act in the same way as for myosin alone, and the added interactions or network development of the myosin reinforcing the myofibrillar system could explain added tensile force at the point of destruction for these gels. In the myofibrillar preparation, no ions were added. In the absence of added sodium and potassium, the protons displace sodium and potassium inherent in the myofibrillar system. Without extraction of myosin, displacement of cations from myosin appeared to have little effect in the absence of a myosin network.

Application of GdL addition to meat at 4°C, to a process for the production of a chilled restructured meat product could result in a viable meat product. The amounts of GdL and other possible additives, such as NaCl, would be relatively low when compared to those currently used in such products, as the surface of the meat only would be required to bind. The lower the concentrations of NaCl, GdL and TSPP used, the greater the consumer appeal in terms of perceived health characteristics and cost. These additives are currently used in food products and at the estimated levels required would not breach health regulations. Phosphate did not appear to have much effect on the uncooked product, but may enhance some of the characteristics when cooked.

Problem areas in development of a product may include colour, flavour and texture characteristics. The colour at the joining surfaces may take on a grey appearance which as well as being an unattractive colour to the consumer would tend to highlight the joins. Cooking, crumbing and freezing could eliminate the problem, but are contradictory to the purpose of producing a chilled product. The hydrolysis products of GdL and GdL itself impart an astringent or metallic flavour to the meat. However, possibilities arise in terms of adding flavours to the mixture, such as mushroom and black pepper. The texture could be effected by the fibres not aligning which could also effect the appearance of the product.

No cooking was undertaken to determine if the pieces maintained their bind when heated or disintegrated. The process outlined in the present experiments is relatively simple, merely mixing GdL and any other additives into the meat pieces and standing at 4°C for at least 10 h. If development of the product could maintain this minimal amount of added labour, the possibility of adding value to high quality, but currently low value meat trimmings, is available for use. Unlimited opportunities exist, for example, in terms of adding flavourings, producing rolls of steaks for fast foods and institutional foods and the provision of a quality restructured steak at lower cost to the consumer than an actual steak.

8.2 FUTURE WORK

The postulated series of events describing GdL-induced gelation of meat, myofibrillar proteins and in particular myosin are based on the limited research undertaken in this study, the small number of papers in this area of acid-induced myosin gelation and acid marinading (such as, Fretheim *et al.*, 1985; Hermansson *et al.*, 1986; Rao *et al.*, 1989b) and the large amount of research undertaken on thermally-induced gelation. The postulated events are therefore in the 'hypothesis' stage and there is a great deal of future work which could provide a better understanding of the mechanism of acid-induced gel formation.

The assumption that myosin was the major protein involved in this gelation was based on heat-induced gelation. Myosin did form strong gels but it is suggested that actin and actomyosin be investigated. The effects of proteins, such as C-protein, tropomyosin and troponin, should also be investigated. Likewise with myofibrillar protein, the effect of slow lowering of pH on the other two protein groups of meat, sarcoplasmic and connective tissue proteins should be studied, and their contribution to gelation determined. The fractions of myosin, such as the head and rod portions, could show order of gelation, for example head binding first and then the tail. The use of fragments would also show the relative contributions of the different parts of myosin to gelation.

Muscles from different areas of the animal could be compared, for example fast twitch (such as cutaneus trunci) compared to slow twitch (such as masseter) and cardiac muscle. Not only the effects of use of different muscles could be examined, but also muscles from different

animals, such as bovine and ovine muscles, or mammalian and avian or piscine. These results would indicate the universality of the mechanism and show the effects of myosin isoforms on denaturation and gelation.

Using experimental procedures not used in this investigation could provide more information about the mechanism of gelation. For example, in the present experiments the concentration of myosin was too dilute for the sensitivity of the differential scanning calorimeter (DSC) available for use. This sort of data could provide information about the denaturation of the protein, such as shifts in denaturation temperatures. Scanning electron microscopy and the use of different preparative procedures for electron microscopy could show greater structural detail of the gels formed. The use of a more refined tool than the Instron Universal Testing machine could provide a better rheological description of the myofibrillar protein gels formed.

The use of different acids could enable the effects of rate of change of pH to be studied as well as the effects of different types of acids. The addition of sodium acid pyrophosphate, for example, could provide conditions of added phosphate and acid, reducing the number of additives required in the gelation process. The effects of salts could be further investigated using, for example, the Hofmeister series of salts. The effects of the addition of salts and sugars on myosin could also be studied. The effects causing increased G' might have applications in the manufacture of restructured meat products. The observed stronger affinity of myosin for sodium than potassium could also be investigated. These experiments would all provide information about the effects of different anions and cations on gelation.

The effects of pH changes on dilute systems of myosin and the gelation of myosin with the added washing step, further lowering the ionic strength, could be investigated. Gelation at various concentrations of myosin could also be studied to determine the effects of low pH on dilute myosin solutions and suspensions and to determine the critical myosin concentration required for gel formation. As well, the effects of increased myosin concentrations could be examined.

The effects of heating of the gels formed at low pH should be studied with restructured meat products in mind. If the protein has already denatured, is there any further denaturation upon

heating and is the network formed heat sensitive or able to retain its structural integrity when heated?

Investigation of the interaction of myosin with GdL or gluconic acid and its salts could determine the effective binding of these compounds with the protein and how this binding effects the gel structure.

These are some of the many aspects which could provide a greater understanding of the mechanism of gel formation. Development of a restructured meat product would entail a whole new area of investigation and a great deal more work of an applied nature. The application of the results presented to other proteins, such as dairy and fish proteins, could also add another dimension to the possible research stemming from this project.

CHAPTER 9

CONCLUSIONS

The mechanisms of acid-induced gelation of myofibrillar protein and myosin were investigated. Glucono-δ-lactone (GdL), was used to achieve acid-induced gelation at 4°C. While used in for example, salami as an acidulant, it appears that this food additive has not been previously used specifically and solely as a gelling agent in meat systems.

The addition of GdL to minced meat resulted in gelation of the meat. However, it was difficult to obtain useful data to quantitatively describe the gels formed. Addition of GdL to myofibrillar protein also resulted in the formation of gels. Two mechanisms of gel formation were proposed to explain gelation at different pH values. At pH 4.8 to 4.5, and at values less than this pH, extraction of the A-band occurred and a gel network formed of the extracted myosin. The gel was suggested to be an impregnated composite system of myosin reinforcing the myofibrillar structure. At a pH value between 4.5 and 3.9, and at pH values less than this critical pH, complete extraction of the A-band occurred as actomyosin was depolymerised and filaments were dissociated. Dissociation of filaments and extraction of the A-band proteins resulted in dissolution of the myofibrillar structure and the resultant gel was a network of myosin. This myosin gel had a lower Young's Modulus than that of the impregnated composite system. Denaturation of extracted myosin was suggested to have occurred at pH 5.0 to 5.5 and lower allowing new interactions to have occurred as the protein unfolded and exposed reactive groups.

Use of myosin allowed the investigation of the types of bonding and molecular interactions involved in gelation. While bond types involved in myosin gelation have rarely been studied, it would appear that intermolecular interactions were the basis of the mechanism of gelation of these proteins. The addition of GdL to myosin resulted in the exposure of hydrophobic sites as the pH decreased suggesting that denaturation had occurred. Gel formation and

development occurred parallel to denaturation. As the pH decreased below pH 4.5, denaturation was stopped and the gel deteriorated to become liquid-like. This deterioration of gel structure was attributed to excess repulsive interactions at pH approximately 4.0. Denaturation was assumed to have preceded aggregation to have formed a gel network. Hydrogen bonding and hydrophobic interactions were shown to have participated in gel formation. Electrostatic interactions were proposed to have played a role in gel formation, especially as a result of the altered pH. Sulfhydryl bonding did not appear to have taken part in gelation. The mechanism of denaturation and subsequent gelation was suggested to be acid-induced.

The addition of sodium chloride (NaCl) to myofibrillar protein plus GdL resulted in retention of the impregnated composite system to pH at least 4.0. The retention of this reinforced structure was attributed to the lowering of the IEP induced by the introduction of NaCl. Increasing concentrations of NaCl increased the Young's Modulus of the acid-induced gel which was suggested to be a result of the dissociation of extracted myosin filaments. In excess concentration, the addition of NaCl resulted in salting out and the gel structure deteriorated.

The addition of NaCl to myosin resulted in solubilisation of the myosin and a translucent solution was obtained. The addition of NaCl to myosin with GdL resulted in similar acid-induced gelation to that observed in the absence of NaCl, but the gel network was retained to lower pH values. This effect was attributed to the lowered IEP of myosin in the presence of NaCl. Increased concentrations of NaCl resulted in increased G' values as a result of increased dissociation of myosin filaments introducing more interactions upon denaturation.

The inclusion of TSPP in the myofibrillar protein plus GdL system had no observable effect, until the myofibrillar structure had completely dissolved. Thereafter, the inclusion of TSPP enhanced the Young's Modulus of the gel.

The addition of TSPP to myosin resulted in a large initial G' in the absence and presence of GdL. Two mechanisms of network formation were suggested to explain this large G'. The first was entanglement network formation. It was proposed that the largely dissociated TSPP

molecule might cause movement of the largely negatively charged myosin molecules and result in entanglement. The large molecular weight of myosin and the heads protruding from the coiled tail would be conducive to entanglement formation, but it is suggested that the alkaline pH induced by the addition of TSPP must also have dissociated the rigid myosin filaments to have allowed the flexibility required for entanglement formation. This network formation would have involved electrostatic interaction.

In contrast to the mechanism of entanglement formation, the second mechanism suggested was that of gel formation. It was proposed that TSPP induced denaturation of myosin, possibly as a result of the change in pH to alkaline values. Subsequent to this denaturation, rapid gelation could have occurred with the polyanionic TSPP forming intermolecular crossbridges. This gelation would possibly have involved electrostatic and hydrophobic interactions.

In the presence of GdL, acid-induced denaturation and gelation occurred subsequent to the TSPP-induced network formation. The TSPP-induced network appeared to be maintained throughout the acid-induced denaturation and gelation. Hydrogen bonding in acid-induced gelation was enhanced by the presence of TSPP.

The addition of tripolyphosphate to myosin resulted in increased initial G' and pH. The effects of TPP on myosin were similar to those of TSPP addition and the same conclusions were drawn to explain the effects of this phosphate on myosin.

The addition of orthophosphate and hexametaphosphate to myosin plus GdL resulted in low initial pH. This small effect on pH was suggested to explain the low initial G' of these systems. Orthophosphate did not enhance the G' of myosin plus GdL gels. Hexametaphosphate addition to myosin plus GdL resulted in a lag period to pH about 5.0, where the inclusion of hexametaphosphate resulted in G' data similar to that for added TSPP or tripolyphosphate. This may have been the pH at which the hexametaphosphate molecule formed hydroxyl groups and increased hydrogen bonding could explain the enhanced G' of the gel formed at lower pH values, compared to those formed in the absence of hexametaphosphate.

The characteristics of the pH and NaCl effects dominated the observed effects of addition of NaCl and TSPP to myofibrillar protein or myosin in the presence and absence of GdL. However, in the myosin system, TSPP did appear to influence denaturation and the resultant G'.

Reversibility of myosin gel formation was found to be dependent on the rate of ion diffusion. In the present experiments, it was observed that the acid-induced gel was reverted to myosin and myofibrillar protein when allowed to stand in quiescent water, a condition of unlimited ion migration. However, a slow rate of ion diffusion as imposed by a barrier of dialysis tubing, resulted in a strong, translucent gel, which was dense to the point of being effectively impermeable to ion migration. The enhanced tensile 'strengths' and the impermeability of the gels formed by dialysis of myosin was hypothesized to be a result of the displacement of sodium and potassium ions with protons, enhancing hydrogen bonding. The networks present prior to dialysis were not necessarily destroyed, but may have been enhanced by the effect of dialysis. As a result of these experiments, it was also observed that myosin had a stronger affinity for sodium than for potassium, which to the best of the author's knowledge has not previously been reported.

Experiments on many aspects of acid-induced gelation of meat, myofibrillar protein and myosin were carried out. Studies undertaken by other workers have generally focused on one type of bonding to explain gelation at given conditions. In contrast, the present work investigated hydrophobic and electrostatic interactions, and hydrogen and sulfhydryl bonding. All types of interaction except the covalent sulfhydryl bonding appeared to be involved in gelation of myosin and therefore myofibrillar proteins and meat. However, the data obtained was insufficient to allow a definite understanding of the sequence of events of gelation. Hence the foregoing discussions have necessarily been hypothetical. More definitive experimentation could determine the mechanism of acid-induced gelation at chilled temperatures.

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APPENDIX 1

Light microscopy was undertaken on myosin suspensions, solutions and gels in the presence of combinations of GdL, sodium chloride (NaCl) and tetrasodium pyrophosphate (TSPP). Nomarski differential interference contrast (NDIC) was used. The micrographs presented are all at magnifications of 100x.

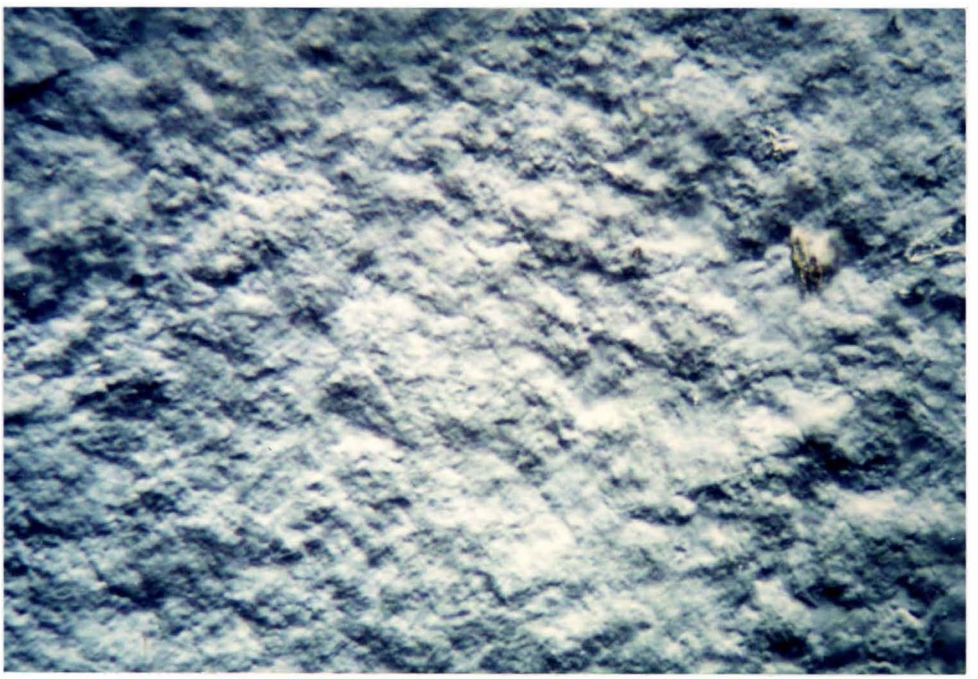

Figure A1.1 A light micrograph of myosin at 100x magnification and using NDIC.

Figure A1.2 A light micrograph of myosin with 0.70% GdL added at 100x magnification and using NDIC.

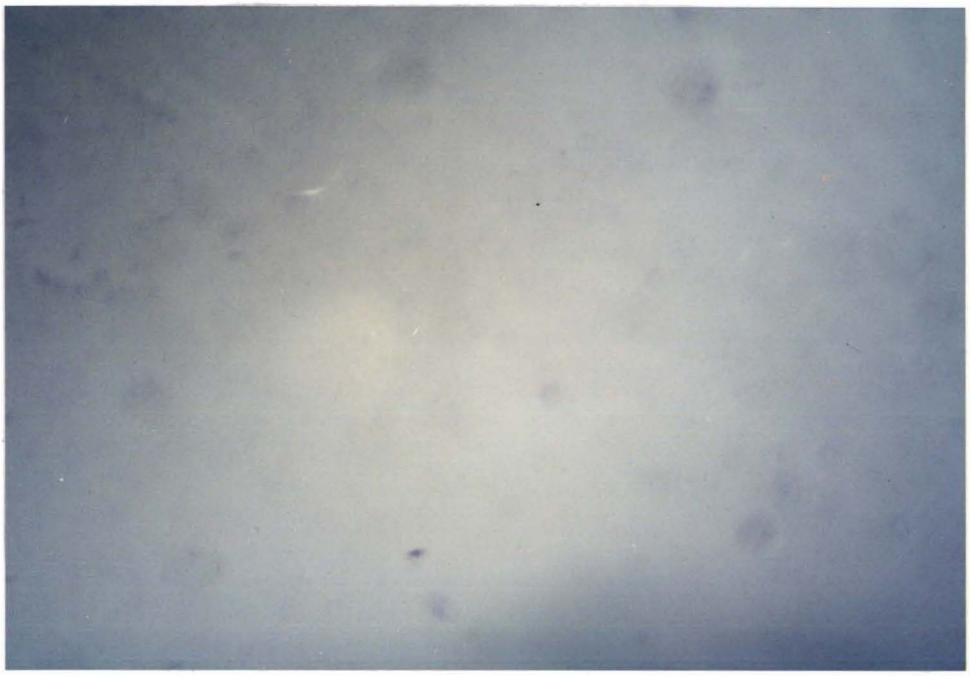

Figure A1.3 A light micrograph of myosin with added 2.50% NaCl at 100x magnification and using NDIC.

Figure A1.4 A light micrograph of myosin with added 0.70% GdL and 2.50% NaCl at 100x magnification and using NDIC.

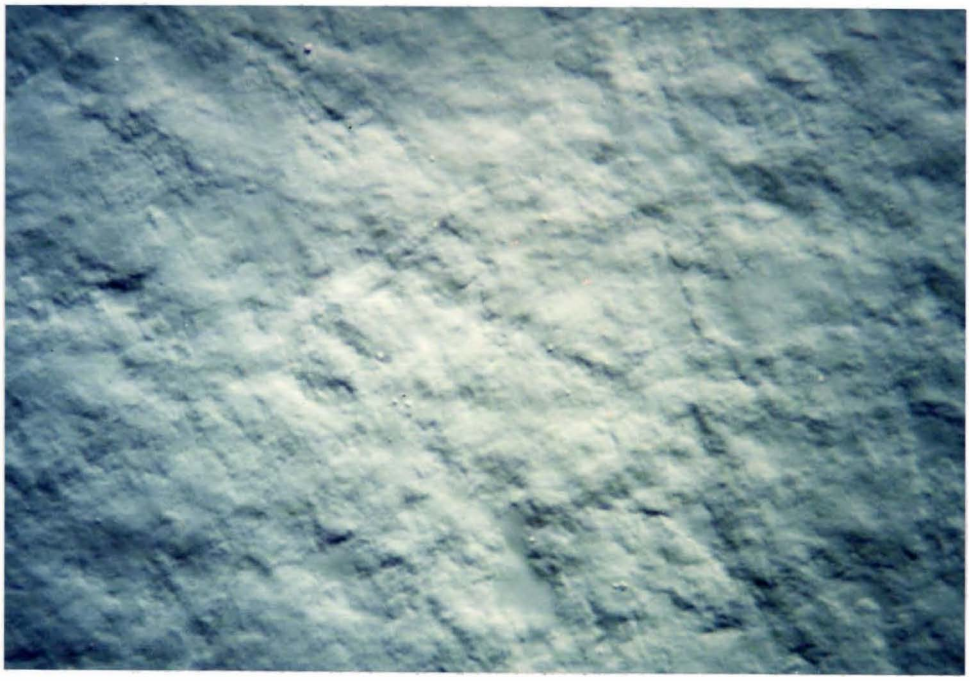

Figure A1.5 A light micrograph of myosin with 0.25% TSPP added at 100x magnification and using NDIC.

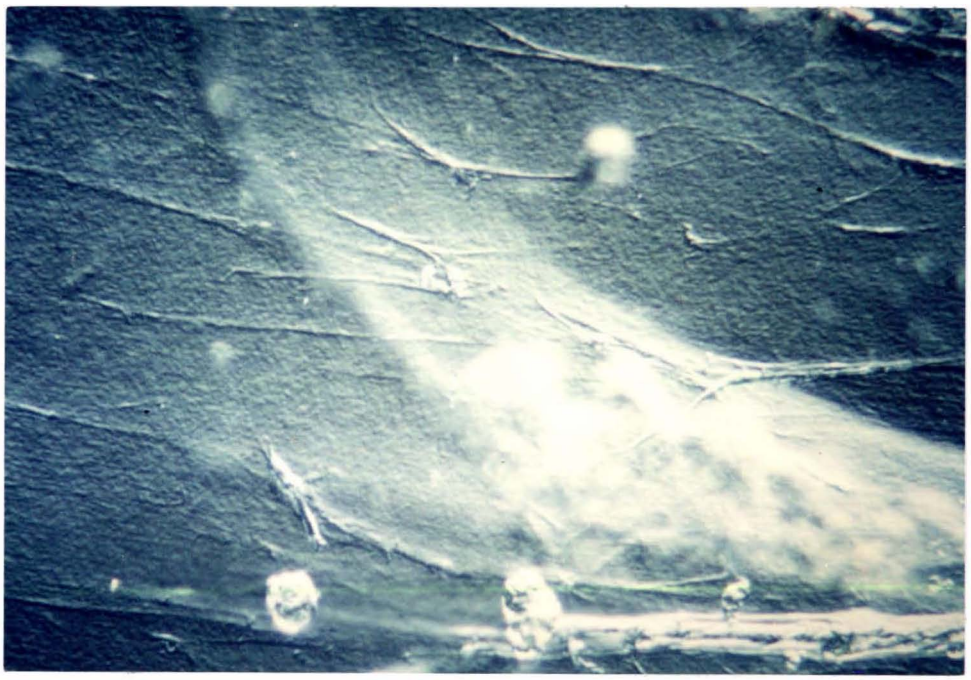

Figure A1.6 A light micrograph of myosin with added 0.70% GdL and 0.25% TSPP at 100x magnification and using NDIC.

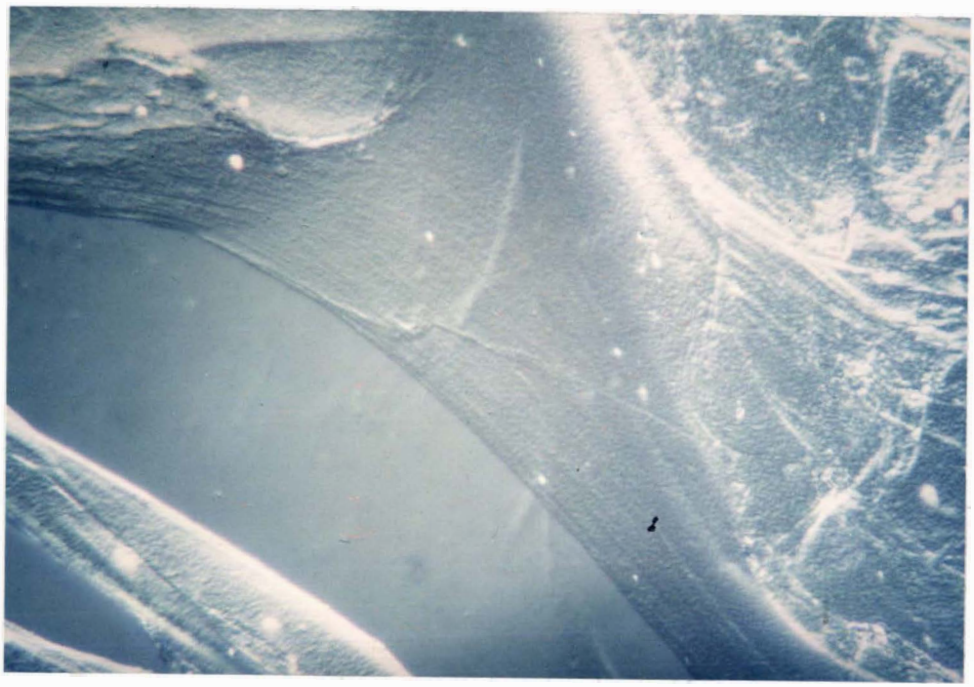

Figure A1.7 A light micrograph of myosin with added 0.70% GdL, 2.50% NaCl and 0.25% TSPP at 100x magnification and using NDIC.

APPENDIX 2

The terms "bind", "bind strength" and "gel strength" are commonly used in the literature on meat and protein gelation regardless of the appropriateness of these terms with reference to the rheological parameters measured. This is illustrated by the reference to gel strength in the studies of Samejima *et al.* (1981, 1988) using a band-type viscometer to measure gel rigidity. Gel and bind strength imply failure of the sample and using small strain testing failure of the sample does not usually occur. This inappropriate use by other authors has been preserved throughout this thesis, but when referring to the authors own results, care has been taken to use the appropriate terminology.

In the Chapter Four, Young's Modulus was measured to rheologically characterise the gels formed. Young's Modulus is the stress-to-strain ratio of a material in the linear region of the stress-strain graph for tension or, as in the present experiments, compression. Young's Modulus is therefore a small strain rigidity measurement enabling the determination of solid-like characteristics of the myofibrillar samples during gelation. However, this measure is limited in that it does not allow differentiation between rigid, but brittle gels (possibly arising from fast protein aggregation (Hermansson, 1979)) and rigid, but more cohesive gels (arising from slower aggregation). It is the property of cohesiveness, not rigidity, that determines gels with the most effective binding ability. Springiness, used in the present experiments, is a better measure of cohesiveness and therefore binding potential. Testing to failure either by torsion testing or uniaxial compression, would have provided a greater source of information with regard to binding potential of the gels than either of the two measures used, however, the present work was limited by the lack of instrumentation to undertake an appropriate torsion test, especially at the concentrations of myofibrillar protein and myosin used.

In Chapters Five and Six, the dynamic equivalent to Young's Modulus, the storage modulus (G') was used to characterise myosin samples. This small strain measure was also used to follow the development of solid character as the pH of the samples was lowered. However, the use of G' was complemented by the use of ANS fluorescence so that the development of solid-like character was compared to the amount of denaturation at a given pH or time enabling interpretation of molecular events and their sequence during the gelation process. In addition, failure tests were conducted at the final pH of the myosin samples and are presented as apparent strain (γ) values at the point of destruction.

APPENDIX 3

In Chapter Five, sucrose, glucose and sorbitol were added to myosin samples in an attempt to investigate the contribution of hydrogen bonding to GdL-induced myosin gelation. The G' was decreased at given pH in the presence of the carbohydrates for samples of myosin with added 0.70% GdL and 0.70% GdL plus 0.25% TSPP (Figures 5.37 and 5.38, respectively). It was concluded that hydrogen bonding was involved in GdL-induced gelation of these samples, but that it was not the only form of interaction responsible for gelation. In the presence of 2.50% NaCl and 0.70% GdL the observed increase in G' could not be explained (Figure 5.39).

This investigation proposed that the carbohydrates would act as competitive inhibitors for hydrogen bonding and therefore a reduction in the G' of the myosin sample would indicate less intermolecular hydrogen bonding. However, the mechanisms by which carbohydrates are hypothesised to act as protein cryoprotectants (reviewed by MacDonald and Lanier, 1991) suggests that the observed decrease in G' values may be a result of stabilisation of the protein against denaturation. It was concluded that generally the polyols and sugars which increase the surface tension of water may act to stabilize proteins dually - by favouring solute (polyol and sugar) exclusion from the protein surface and by enhancing the strength of intramolecular hydrophobic interactions. The latter effect arises from the unfavourable decrease in entropy that occurs when water molecules experience a decreased mobility (enhanced hydrogen bonding) in the vicinity of exposed hydrophobic side chains.

Timasheff and Arakawa (1989) also stated that if in the course of the denaturation reaction, the chemical nature of the interactions between protein and stabilizer does not change, the situation should become even more unfavourable thermodynamically in the unfolded state of the protein. The zone of exclusion of the stabilizing co-solvents (low molecular weight polyols and sugars) becomes greater as the protein-solvent interface increases during denaturation, due to an increase in the asymmetry of the protein.

This stabilization of the protein to denaturation could partly explain the decrease in the G' of the myosin gels in the presence of GdL and GdL plus TSPP. However, the increase in G' observed in the presence of NaCl and GdL still cannot be explained.