

THE MECHANISM OF ACTION OF POLYPHOSPHATES

THE MECHANISM OF ACTION OF POLYPHOSPHATES

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MEMORANDUM

Apart from the acknowledged assistance and advice received, this dissertation is the result of my own work. It is submitted in accordance with the regulations prescribed by the University of Bristol for the Degree of Master of Science.

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ABSTRACT

Polyphosphates in small concentrations allow the use of lower concentrations of salt in the manufacture of meat products reducing their salty taste and the health risk from over-consumption of sodium. In their presence a better bind is achieved and the water present in raw meat or taken up during processing is retained more completely after cooking. The aim of this thesis was to study the molecular mechanism by which polyphosphates function.

Rabbit myofibrils and mince were used to investigate the effect of pyrophosphate on swelling and protein extraction in a range of NaCl concentrations from 0.1-1.0 M at pH 6.0. 1 mM pyrophosphate had little effect on the swelling of myofibrils in 0.1 to 0.4 M NaCl but, at higher NaCl concentrations it caused a marked inhibition of swelling. In the presence of 10 mM pyrophosphate swelling was higher than in NaCl alone in 0.3 and 0.4 M NaCl, but again at higher NaCl concentrations pyrophosphate caused a marked inhibition of swelling. Protein extraction was higher at NaCl concentrations above 0.2 M in the presence of pyrophosphate than in NaCl alone; this was more marked in the presence of 10 mM pyrophosphate. Phase-contrast light micrographs showed pyrophosphate to extract the A-band as the NaCl concentration rose to 0.3 M NaCl and above, and in NaCl alone at concentrations of 0.4 M and above only images of a disordered myofibrillar structure were observed.

The results of Bendall (1954) that pyrophosphate increases the water uptake of mince were confirmed. In mince washed to remove endogenous ions and sarcoplasmic proteins, 10 mM pyrophosphate produced more swelling and extraction of protein than NaCl alone were produced in the 0.1-0.3 M NaCl concentration range but, at higher concentrations it produced inhibition of swelling. However, swelling in washed mince was less than in myofibrils under the same ionic conditions.

The results are interpreted by supposing that pyrophosphate promotes the dissociation of actomyosin, changing the myofibrils from a rigor to a relaxed state allowing limited expansion of the filament lattice. In NaCl concentrations above 0.3 M pyrophosphate is also thought to co-operate with salt to depolymerise the thick filament allowing myosin to be extracted and causing a depression of swelling.

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1. INTRODUCTION

1.1 Manufacture of meat products

The manufacture of meat products has many objectives. Firstly, for meat from joints which are already of high quality it aims to introduce better characteristics, *e.g.* the manufacture of cooked hams and the creation of a new texture, a new colour and a new flavour. Secondly it may promote a better utilization of less valuable cuts by incorporating them in products, *e.g.* reformed or restructured meat, with improved or new organoleptic characteristics (Jolley & Purslow, 1988). This is achieved partly by reduction, through comminution, of the negative effect produced by connective tissue structures, *i.e.* reducing toughness, and partly by the use of certain unit operations, *e.g.* massaging and tumbling, and addition of certain additives, *e.g.* salt and polyphosphates, which further improve, not only texture but also flavour, colour and general appearance (Jolley & Purslow, 1988).

1.2. Water-holding in meat and its importance

Water has a marked influence on the quality of meat products. It represents about 75% of the composition of lean meat at slaughter (Marsh & Leet, 1966), and its content and distribution profoundly affect the properties of meat, especially toughness, juiciness, firmness and appearance (Davey & Gilbert, 1974). The role played by water is due not only to its natural presence in meat, but also because during the manufacture of meat products it is commonly taken up (Siegel *et al.*, 1978). This addition further changes the characteristics of the meat, mainly texture, by diluting the effect of the structural components of muscle and connective tissue (Dransfield, pers. comm.).

1.3. Adhesion between meat pieces

In many meat products the division of muscular masses is carried out either by hand or mechanically through the use of a mincer, flaker, bowl chopper, colloid mill or other means. Pieces or particles varying in size have in the end product to be bound together and reformed or restructured into a mass with adequate texture and suitable for cutting or eating (Jolley & Savage, 1985; Jolley & Purslow, 1988).

A sticky exudate is normally present on the surface of meat which has been submitted to the action of salt and polyphosphates and the mechanical effects of massaging and tumbling (Maas, 1963; Theno *et al.*, 1978). Myofibrils and solubilised myofibrillar proteins may be found in its composition (Siegel *et al.*, 1978; Theno *et al.*, 1978). Myosin is particularly important, not only because it represents about 40% of the proteins of muscle [calculated from data from Scopes (1970) and Yates & Greaser (1983)], but also because it promotes a better adhesion between meat pieces than actin or sarcoplasmic proteins by forming a gel on heating together with other salt-soluble proteins (Fukazawa *et al.*, 1961; Nakayama & Sato, 1971; MacFarlane *et al.*, 1977).

1.4. The use of NaCl in meat product manufacture

Sodium chloride is used in the manufacture of many products in a concentration between 0.5 and 5.0% (0.1 and 0.9 M) (Hughes, 1988). It can be introduced into the meat in a dry form by rubbing its surface, or in the form of a solution which is either injected or allowed to soak into the meat. The penetration of salt, when a brine is used, is facilitated by mechanical agitation, *i.e.* massaging (Siegel *et al.*, 1978; Theno *et al.*, 1977) and tumbling. When present in meat, it acts (in certain concentrations) as a preservative, enhances its flavour (Hannan, 1975; Schwartz & Mandigo, 1976), cooked colour, and eating texture (Schwartz & Mandigo,

1976). It also promotes a better bind by dissolving myosin and making it available in the sticky exudate (Jolley & Savage, 1985). But sodium chloride is most important because it promotes an increase in water uptake from 0 to 40% or more (Offer & Knight, 1988) and reduces the losses of water on cooking (Wierbecki *et al.*, 1976).

1.5. The use of polyphosphates in meat product manufacture

Polyphosphates are widely used in the industry in association with salt (Hamm, 1960; Jolley & Purslow, 1988). Pyrophosphate is the active form by which the effects attributed to polyphosphates are produced (Yasui *et al.*, 1964 a,b), but in practice tripolyphosphate, due to its high solubility, is preferably used (Hannan, 1975). Blending of polyphosphates can give better results than the use of tripolyphosphate alone, the most effective blends containing this compound and glassy metaphosphates (Mahon *et al.*, 1971).

Longer chain polyphosphates are hydrolysed by enzymes present in meat to pyrophosphate and inorganic phosphate. Yasui *et al.* (1964 a,b) demonstrated that the longer chain polyphosphates can exert an activity similar to pyrophosphate if they are hydrolysed. Sutton (1973) also showed that the myosin molecule acts as an enzyme to hydrolyse triphosphate into pyrophosphate, which is then converted to orthophosphate by a muscle pyrophosphatase.

Polyphosphates are used for several reasons. In the presence of salt and polyphosphate the water present in the raw meat or taken up during processing is retained more completely after cooking (Bendall, 1954; Trout & Schmidt, 1984). Polyphosphates are added to meat in concentrations up to 0.5% (Mahon *et al.*, 1971). At this low level of addition they allow the use of lower concentrations of sodium chloride, which reduces the salty taste of the products and the health risk from over-consumption of sodium (Jolley & Purslow, 1988). They are also important in that they increase the extraction of protein (Bendall, 1954; Grabowska &

Hamm, 1979; Turner *et al.*, 1979), in particular myosin (Jolley & Savage, 1985), from meat pieces, improving bind in reformed or restructured meat products. Extracted protein is additionally important because, by tightly holding water when on heating it forms a gel, it may be the reason why cooking losses are reduced (Kotter & Fischer, 1975; Offer & Knight, 1988).

1.6. General mechanism of water-holding by myofibrils

As was previously mentioned, water is the major component of meat. Many changes taking place in meat itself - *e.g.* pH changes in PSE and DFD and meats - or in the treatments applied to the meat - *e.g.* increased pressure, chilling, freezing, thawing or cooking - are known to lead to a loss in water. The implications of losses in weight, or a reduction in sensory characteristics make it important for the exact place where water is stored and the mechanism by which it is held in meat both to be identified.

A relatively small amount of water (4-5%) in a muscle fibre is bound to muscle proteins (Elford, 1970; Cook & Wein, 1971), the rest existing as bulk water. In a live mammalian muscle (in the guinea pig) the myofibrils occupy 82% of the volume of fast white fibres and 87% of the volume of slow red fibres (Eisenberg & Kuda, 1975). Since water is the predominant component of muscle, it may be concluded that most of the bulk water in living muscle is located in the myofibrils, and that any changes in the distance between the thick and thin filaments will produce changes in the water held by muscle (Wismer-Pedersen, 1971; Offer & Trinick, 1983). Therefore the arrangement of the thick and thin filaments in a hexagonal unit cell as shown in Fig. 1.1 may be regarded as the structural unit of water-holding.

Hanson & Huxley (1953), showed that myofibrils can swell in salt solutions, at a pH value of

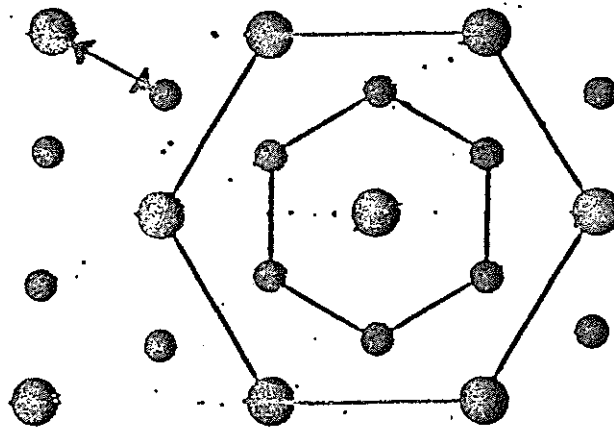


Fig. 1.1. Cross-section of vertebrate skeletal muscle showing the hexagonal arrangement of thick (O) and thin (o) filaments, and the distance between them (\longleftrightarrow) (not to scale) that may be reduced or increased and, therefore, produce changes in the water-holding capacity in muscle (adapted from Bagshaw, 1982).

7, and that this is accompanied by extraction of myosin from the A-band. Offer & Trinick (1983), by irrigating individual myofibrils in different NaCl solutions, at a pH value of 5.5, *i.e.* pH nearer that of meat, observed that little change in myofibrillar diameter occurred up to, and including 0.5 M NaCl. At a concentration of 0.6 M NaCl a substantial increase in the diameter of the myofibrils is seen both in the A- and I-bands, and maximum swelling is observed in about 1 M NaCl. From their observations these authors concluded that, in meat processing, in the presence of NaCl, water uptake occurs by the entry of water into the myofibrils as they swell laterally by an expansion of the filament lattice.

Offer & Trinick (1983) also observed concomitant changes in band pattern such as partial extraction of the A-band from its centre in 0.6 M NaCl, its nearly complete extraction in 1.0 M NaCl and invisibility of the Z-line in 0.6 M. This was not true in all myofibrils. In certain cases, in 0.8 M NaCl, where the Z-line kept its integrity, the A-band appeared less swollen and bowed with the I-band being flared on either side of the Z-line suggesting that the integrity of the Z-line influences the expansion of the A- and I-bands and consequently swelling.

1.7. Mechanism of salt-induced swelling of myofibrils

The myofilaments carry ionisable groups. At a pH value of 5, near their isoelectric point, their net charge is zero. If the pH is raised above 5 they become negatively charged, while at pH values below 5 they become positively charged (Hamm, 1975). Therefore in untreated muscle electrostatic forces are present tending to expand the lattice (Rome, 1967; Rome, 1968). The further from the isoelectric point of the myofilaments the pH is taken, the greater the repulsive force, and the greater the expansion of the filament lattice, which is thought to be resisted by transverse constraints such as the cross-bridges (in rigor muscle), the M-line and the Z-disc (Offer & Trinick, 1983). Hamm (1975) also showed that water-holding is lowest at a pH value of 5 and increases the further the pH changes from this value and that addition of NaCl

to the system alters the capacity of muscle to hold water, decreasing the water-holding capacity at pH values below 5, but increasing it at pH values above 5. From these observations he suggested that NaCl and other salts would increase water-holding by increasing the number of charges on the surface of the filaments which by being repelled would promote an increase in the interfilament spacing. The resulting increase in volume of the myofibril would, nevertheless, be expected to be small and doesn't account for the relatively large increases in water uptake observed when salt is added (Offer & Knight, 1988). The high concentration of ions would also tend to produce a screening effect outweighing any increases in the electrostatic repulsive force (Ledward, 1983; Offer, 1984).

The assumption, previously made, on the weak effect electrostatic forces would have on lattice expansion, and the observations by Hanson & Huxley (1953, 1955) and Josephs & Harrington (1966) that high salt concentrations depolymerise thick filaments, together with the above observations (Hanson & Huxley, 1953; Offer & Trinick, 1983) concerning changes in the sarcomeric band pattern, lead to an explanation on how salt may increase water-holding in meat.

Offer & Knight, (1988) proposed that when sodium chloride is added to meat in high concentrations, in the salting-in region, the depolymerisation of the thick filament to myosin molecules takes place (see Fig 1.2). The corresponding extraction of the centre of the A-band (see previous section: Offer & Trinick, 1983) is explained since in the M-line the myosin molecules are not attached to the thin filament and may diffuse away, while in the region of the thick filament overlapped by the thin filament, the attachment of the myosin heads to actin will not allow their escape. Their tails have, nevertheless, initially, a limited freedom of rotational movement but this is hindered by the thin filaments. As the thick filaments no longer exist it is possible for the actin filaments to become further apart and so a constraint to the movement of the myosin tail gradually ceases to exist. The tails themselves, entropically exploring more space, hit the actin filaments and by doing so become the driving force which expands the

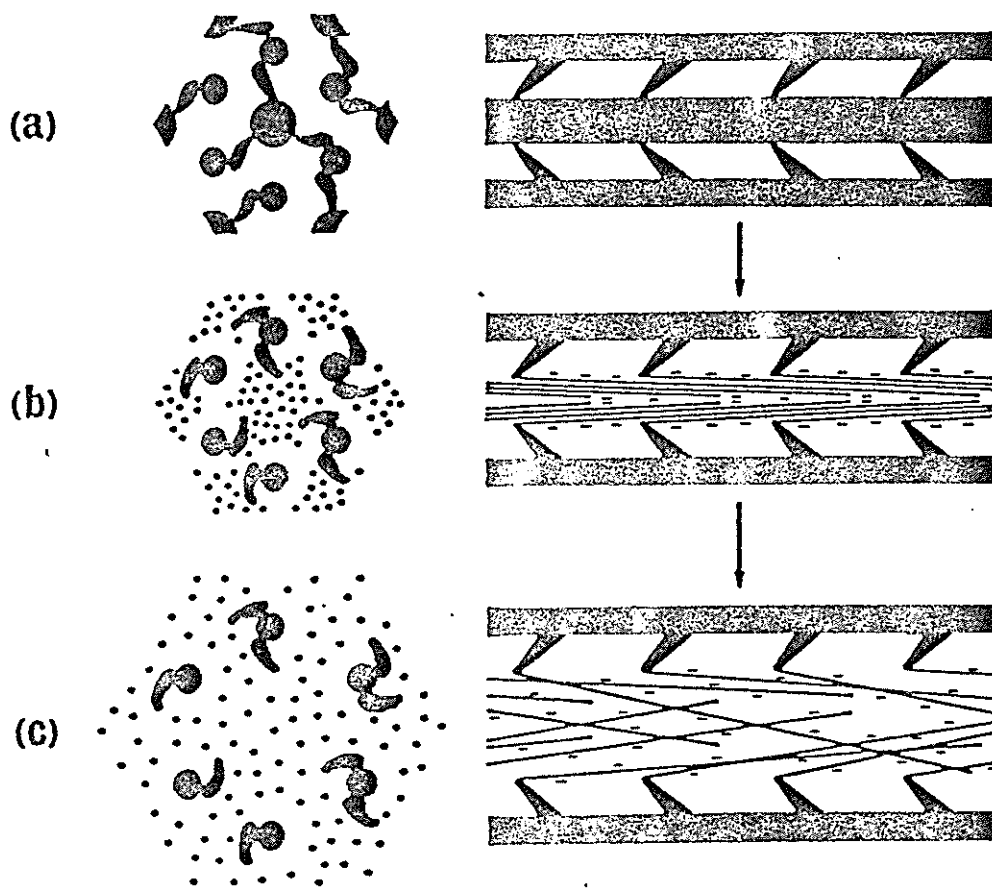


Fig. 1.2. Mechanism of salt-induced swelling of myofibrils. (a) Rigor muscle before addition of salt. On the left a transverse section through the filament lattice is shown: • represents the thin filaments, ● represents the thick filaments and ● represents the cross-bridges. On the right a longitudinal section through one thick filament and two thin filaments with connecting cross-bridges. (b) Before swelling. Depolymerisation but the motion of the tails of the myosin molecule is still little and restricted by the presence of neighbouring thin filaments. (c) After swelling. The tails of the myosin molecules are free and able to have a larger rotational movement. [adapted from Offer & Knight (1988)]

filament lattice. An entropic pressure is thus created, producing swelling, which is limited by the transverse structural constraints (M-line and Z-disc) already mentioned. Sodium chloride may have an additional effect, which is to extract material from the Z-disc, weakening it, and by doing so facilitating filament lattice expansion with the consequent swelling of the I-segments.

1.8. Action of polyphosphates on swelling and protein solubilisation in model systems

Polyphosphates are known to increase both water-holding and bind in processed meat products (Trout & Schmidt, 1983) as was previously mentioned. The extent to which this occurs depends on the type and concentration of the polyphosphates used and the concentration of other added salts (Shultz *et al.*, 1972) and ingredients used (Brebner, 1981). Studies on the effects polyphosphates have on meat products have been made using different polyphosphates under different conditions of concentration and pH; in association with other salts in different concentrations; in different practical or experimental systems, submitted to different incubation periods at different temperatures, and also submitted to different mechanical actions, *e.g.* centrifugation, massaging and tumbling. All these factors plus the fact that it has been known for a long time that phosphates are very effective in increasing the functionality of processed meat products, makes it difficult to describe the action of polyphosphates in raw meat and compare its effects with those of other additives. This is so, not only because of the variety of products in which polyphosphates have been tested, but also because the interaction of various factors also makes it difficult to understand which actions must be attributed to each additive, or whether the results obtained are really the effect of the additives used. However, the existence of work on the functional properties of polyphosphates can be combined with the existing experimental results of the action of polyphosphates on the structure of muscle and the molecular mechanisms which correspond to

that action. Reviews on both aspects of the role of polyphosphates have been made by Hamm (1960), Mahon (1971), Ellinger (1972), Trout & Schmidt (1983), Offer & Knight (1988), amongst others. Based on the existing information three different aspects referred to in the literature can be discussed, namely: (a) the effect of polyphosphates on water-holding capacity; (b) the effect of polyphosphates on protein solubilisation; (c) the relation between protein solubilisation and water-holding when polyphosphates are used; (d) the effect of polyphosphates on the sarcomeric structure of muscle.

Polyphosphates bring about marked swelling when extra water is added to meat [Ellerkamp & Hannerland (1952), cited by Bendall (1954)]. Exploring this information Bendall (1954) mixed rabbit rigor mince with an equal volume of brine and after incubation submitted this mixture to a centrifugation of 1200 x g, so that by measuring the volume of the supernatant, the amount of water taken up by the mince pellet could be calculated. He observed that 0.61% orthophosphate, 0.5% pyrophosphate, 0.5% Calgon® (containing 12-14 phosphate residues per molecule) and 0.5% glass (containing 20-30 residues) used individually promoted water uptake in raw systems at a pH value of about 5.7. Testing the effect of these polyphosphates when added to mince in association with 1% NaCl, he observed that pyrophosphate had a greater effect than the other polyphosphates, increasing the minced meat volume by 55% while the rest only produced a 30% increase. Using different mixtures of pyrophosphate and NaCl, or Na Cl alone with the same ionic strength, he also observed that pyrophosphate in conjunction with 1% sodium chloride solution produced a swelling effect twice as great as that of a concentration of sodium chloride of the same ionic strength. With 1% NaCl plus 0.5% pyrophosphate, this corresponded to a 64% average increase in the volume of the mince.

Experimental work has also been done comparing the effect of NaCl alone in the 3.6-8% concentration range and that of 1.7% NaCl plus 1% of tetra or disodium pyrophosphate (Swift & Ellis, 1956). At a pH between 5.5 and 9 on minced thawed raw beef Swift and Ellis (1956) confirmed that NaCl plus pyrophosphate promoted an higher increase in the initial volume of the mince than NaCl alone. This difference increased as the incubation period was

increased. These authors also found that conditioning increased the effect of both 3% NaCl and 1.7% NaCl plus $\text{Na}_2\text{P}_2\text{O}_7$ or $\text{Na}_2\text{H}_2\text{P}_2\text{O}_7$. An increase in the temperature of conditioning in the 0-20°C interval markedly decreased the volume of the samples treated with NaCl plus pyrophosphate and orthophosphate, but did not have this effect when NaCl was used alone. In their experiments they also showed that 1.7% NaCl plus 1% pyrophosphate solution at pH 7.5 and $\mu=0.66$, had a greater effect in promoting swelling than a 1.7% NaCl and 1.68% orthophosphate solution at pH 7.0 and $\mu=0.66$, in muscle that had been stored for two hours. But orthophosphate was shown to have a greater effect if the storage period was of 20 h. No interpretation of such results was given.

Another comparison was made between the effect of pyrophosphate plus NaCl and the effect of NaCl at the same ionic strength over a range of pH values (Hellendoorn, 1962). Minced beef at a pH value of about 5.5 was mixed with an equal volume of 2% NaCl plus pyrophosphate up to 0.5%. At final pH values below 5.5 in the minced systems, when compared with NaCl alone, pyrophosphate exerted a depressing effect on the water-holding capacity of the uncooked mince. At pH 5.5 after centrifugation 2% NaCl plus 0.5% pyrophosphate ($\mu=0.48$) was seen to have made the mince pellet retain as much of the supernatant as 3% NaCl alone ($\mu=0.51$). At pH values just above 5.5, however, 2% NaCl plus 0.5% pyrophosphate were seen to have started having a greater effect in water-holding than 3% NaCl alone. Unfortunately, at higher pH values all the supernatant was retained and the positive effect of pyrophosphate seen in the experiments by Bendall (1954) and Swift & Ellis (1956) was not permitted to exhibit itself.

Using mince but of pig origin, Sherman (1961) studied the effect of NaCl, a commercial mixture of polyphosphates (sodium pyro and polymetaphosphate) and tetrasodium pyrophosphate, under uncontrolled pH and ionic strength conditions. The additives were used separately and in different concentrations, calculated from his work to be between 0.1 and 3.33%, over a range of 1:2 to 4:1 brine-to-meat volume ratios. The commercial

polyphosphate mixture, which at its highest concentration promoted a final pH of 7.5 in the minced systems, was more effective than NaCl in promoting water uptake, which at the highest concentration only promoted a final pH value of the minced systems of about 6.0. Pyrophosphate, was more effective than the commercial polyphosphate mixture, but the final pH of the minced systems at the highest concentration of pyrophosphate reached values of about 7.9. In treatments with the commercial polyphosphate mixture and pyrophosphate in the lowest brine-to-meat ratios it was seen that 100% of the brine had been taken up by the mince, which means that if the volume of brine had been larger more swelling of the mince might have been observed and the difference between the additives might have been of a greater magnitude.

Muscle homogenates have also been used to compare the difference in effect between NaCl alone and NaCl plus 0.3% pyrophosphate in the pH value range between 5.1 and 6.7 (Hamm, 1979). In NaCl alone, the water-holding capacity of the system reached a maximum at a 5-6% NaCl concentration and fell again at higher NaCl concentrations. NaCl plus 0.3% pyrophosphate did not increase water-holding when compared with similar concentrations of NaCl alone. Even though the brine-to-meat volume ratio in these experiments was not stated, these results do not coincide with those previously shown in mince and in another homogenised muscle system where only one concentration of NaCl and pyrophosphate were used (Brebner, 1981). In the work by Brebner (1981) it was observed that 1.6% NaCl plus 0.3% tripolyphosphate significantly reduced the amount of supernatant on both normal and cold-shortened centrifuged beef homogenates at a pH value of 5.81 in normal meat and pH 5.87 in cold-shortened meat, when compared with 1.6% NaCl alone.

Besides these two physical states, minced and homogenised, muscle has also been used in the form of meat blocks principally to study the effect of polyphosphates on the structure of meat (Voyle *et al.*, 1984). These authors soaked blocks of meat in 1:3 brine-to-meat volume ratio in brines with or without pyrophosphate. A brine composed of 0.6 M (3.5%) NaCl, 1 mM

MgCl, 10 mM sodium acetate at pH 5.5 produced less weight gain than another composed of 0.6 M NaCl, 10 mM (0.22%) pyrophosphate, 1mM MgCl, 10 mM sodium acetate at pH 5.5 or 8. In the presence of 10 mM pyrophosphate at the higher pH of 8, gain in weight was greater than in salt alone salt plus pyrophosphate at 5.5 in fresh meat used 24 h after slaughter. But very little difference was found between gain in weight in the presence of 0.6 M NaCl plus 10 mM pyrophosphate and 0.6 M NaCl alone at pH 5.5 and pH 8.0 in muscle used 72 h after slaughter. The value of pH was, therefore, seen to influence the action of pyrophosphate in fresh meat but lost its influence as conditioning progressed. This observation may mean that, as conditioning progresses changes in the chemistry and structure of muscle have taken place which alter the way in which pyrophosphate can act and produce its effects at different pH values. This may also be the reason why Swift & Ellis (1956) observed orthophosphate to have a greater effect than pyrophosphate in swelling when storage of the meat was prolonged.

Jolley & Purslow (unpublished results), also using meat pieces observed 1.5% tripolyphosphate plus 8% NaCl to produce weight gains up to 90% of the initial weight while 8% NaCl alone produced only a 60% weight gain. They also observed that the rate of water uptake was faster at all NaCl concentrations in the presence of the phosphate than when NaCl was used alone. But agitation of the meat pieces in 8% NaCl produced a weight gain very similar to that produced by 8% NaCl plus 1.5% tripolyphosphate.

Many information exists on the effect polyphosphates have on protein solubilisation due to, as previously mentioned, the importance protein has on both cooked yield and bind in meat products. Besides his observations on swelling Bendall (1954) also studied the effect of salt and pyrophosphate in solubilising protein in rabbit rigor mince. When the brine consisted of 1% (0.17 M) NaCl and 0.5% (22.5 mM) pyrophosphate, he noticed that after a 24 h incubation period followed by centrifugation at 1200 x g for 30 min, the supernatants that were removed were very viscous. This was observed together with the fact that a tenfold

dilution of the supernatants in water produced a flocculation that is characteristic of myosin or actomyosin. The measurement by refractometry of protein present in the supernatant showed it to be found there in a concentration of 5 mg.100 ml⁻¹. None of these signs of the presence of myosin were referred with NaCl alone. He, therefore, suggested that the behaviour of pyrophosphate in producing swelling was attributable to its ability to dissociate actomyosin, but did not give an explanation on how this might happen.

Pyrophosphate, a commercial mixture of polyphosphates (sodium pyro and polymetaphosphate) and NaCl were also used separately, in concentrations between 0.1 and 3.3%, to treat pork mince by Sherman (1961). At the same concentrations and brine-to-meat volume ratio only polyphosphates were found to solubilise actomyosin to any extent. These results are not reliable, however, because the pH was not controlled. It is not possible to attribute the solubilisation solely to pyrophosphate since in the presence of this additive the pH was higher than in the presence of NaCl or commercial polyphosphate.

The effect of the concentration of NaCl and pyrophosphate addition on the solubilisation of myofibrillar protein was studied by Hamm (1979) on a different system, a frankfurter type homogenate of post rigor beef. Where the equilibration period between the brine and the meat is short and diffusion of protein is facilitated. A rise in NaCl concentration solubilised myofibrillar protein, reaching a maximum at 5-6% (0.86-1.03 M) NaCl, irrespective of the brine-to-meat volume ratio. Above that concentration it was seen to fall again. Under the experimental conditions used 19% of the total protein and 36% of the myofibrillar protein were solubilised in 6% NaCl. In 2% (0.34 M) NaCl those values were 11 and 19% respectively. In 2% NaCl it was also found that in the pH range between 5.1 and 6.7 the solubility of myofibrillar protein of the systems increased as the pH value rose. The presence of 0.3% pyrophosphate plus NaCl at different concentrations in the homogenates increased the solubility of muscle protein when compared with the presence of NaCl alone.

Brebner (1981) studying the effect of tripolyphosphate in products made from cold-shortened meat, found that in centrifuged beef homogenates 1.6% (0.72 M) NaCl alone extracted between 11.9 and 18.7% of the total protein in two cold-shortened muscle samples. 1.6% NaCl plus 0.3% tripolyphosphate, at a pH value between 5.81 and 5.87, produced an extraction of between 22.8 and 29.2% of the total protein of the meat.

Meat pieces have also been used to compare the effect of polyphosphates and NaCl on protein solubilisation (Voyle *et al.*, 1984; Jolley & Purslow, unpublished results). Voyle *et al.* (1984) compared the effect of 0.6 M (3.5%) NaCl, 1 mM MgCl₂, 10 mM sodium acetate at pH 5.5 with that of 0.6 M NaCl, 10 mM (0.22%) pyrophosphate, 1mM MgCl₂, 10 mM sodium acetate at pH 5.5 or 8.0 on protein solubilisation. The presence of pyrophosphate at pH 5.5 had no effect on the amount of protein extracted, but at pH 8.0 there was increased extraction of protein which achieved a value of 38% of the total protein of the meat, while in the presence of NaCl alone at pH 5.5 only 23% of the protein was extracted. This indicates that the value of pH is important in the promotion of solubilisation of protein by pyrophosphate. It also indicates that at a pH near that of the isoelectric point of the muscle proteins in these concentrations of NaCl and pyrophosphate, pyrophosphate is not more effective in producing protein solubilisation. But it does not prove that pyrophosphate promotes a higher level of protein solubilisation, since the two additives were not studied at the same pH value. It has also been observed, by Hamm (1979) cited earlier and van den Oord & Wesdorp (1978) cited ahead, that an increase in pH above 5 - the pH at the isoelectric point of the proteins of the meat - promotes an higher extraction of protein. NaCl plus sodium tripolyphosphate has also been found to produce a higher solubilisation of protein than NaCl alone in a time course of up to five days in meat pieces (Jolley & Purslow, unpublished results).

Of all investigations on the effect of pyrophosphate in protein extraction at different pH values and combined with NaCl in different concentrations the work of van den Oord & Wesdorp (1978) provides the widest range of information. Pyrophosphate was shown to be more

efficient in promoting the solubilisation of protein in both beef and pork homogenates. The beef and pork homogenates were not washed and so the effect of the additives used could not be totally attributed to their independent action. Nevertheless, their effect was compared at the same pH. In a 5:1 brine-to-meat volume ratio, over a 5.5 to 7.0 pH range. 2 or 4% (0.34 or 0.68 M) NaCl plus 0.2 (9), 0.5 (22.5) or 1% (45 mM) pyrophosphate always produced a higher total protein extraction than 2 or 4% NaCl alone or NaCl in combination with different concentrations of orthophosphate. The effect of pyrophosphate was only slight at the 1% (0.17 M) NaCl level and was maximal at the 4% (0.68 M) NaCl level, the maximum being closely approached with the 3% (0.51 M). Also relevant is the fact that maximum solubility in the presence of pyrophosphate was achieved at lower pH values than in salt alone and that in 2% NaCl concentration solubility tended to be maximum around pH 6.0, which contrasts with the results of Voyle *et al.* (1984) using meat pieces, where in the presence of 0.6 M (3.5%) NaCl, 10 mM (0.22%) pyrophosphate more protein was extracted at pH 8 .

Myofibrillar suspensions - where the penetration of salts and the diffusion of protein out from the myofibrillar structure are not hindered - have been submitted to the action of salt and pyrophosphate so that parts of the structure of the sarcomere may be identified and related with microscopic images of the action of NaCl and pyrophosphate. Offer & Trinick (1983) used SDS gel electrophoresis and showed that myofibrils treated in 0.6 M (3.5%) NaCl, 10 mM (0.22%) pyrophosphate, 1 mM MgCl₂, 10 mM sodium acetate, pH 5.5, had a substantial fraction of their myosin extracted, plus almost all the C-protein, tropomyosin, troponin and some α -actinin and actin. In the absence of pyrophosphate, nearly all the C-protein and some of the tropomyosin, troponin and α -actinin were extracted but little myosin was extracted. Only in 1 M (5.8%) NaCl was some myosin, actin and α -actinin removed and nearly all the C-protein, tropomyosin and troponin. The extraction of protein was , therefore, like in other systems, higher in the presence of pyrophosphate and occurred at a lower NaCl concentration.

As mentioned in the beginning of this section, the action of polyphosphates on the sarcomeric structure of muscle have also been studied. Hanson & Huxley, (1953) observed that if 0.47 M KCl, 10 mM pyrophosphate, 1 mM MgCl_2 and 0.1 M phosphate buffer, at pH 6.4, were used to irrigate rabbit myofibrils, the A-band largely disappeared in 1-2 s, leaving a myofibrillar structure with Z-lines and a pair of lines on either side of the H-band. They also observed that myofibrils swelled. If the same solution was used without Mg^{2+} , the A-band was not extracted but the swelling still occurred. It can, therefore, be concluded that swelling of myofibrils can occur without extraction of the A-band (thus myosin) and that pyrophosphate depends on the presence of MgCl_2 to produce extraction of the A-band.

Isolated myofibrils have also been irrigated for 3 min in the presence of different NaCl concentrations and 10 mM pyrophosphate, 1 mM MgCl_2 , 10 mM sodium acetate at pH 5.5 (Offer & Trinick, 1983). At concentrations up to and including 0.3 M little change occurred in the band pattern or in the volume. However, after irrigation in 0.4 M NaCl, the A-band was extracted from its ends and more completely than in salt alone, and the myofibril swelled laterally. The residue of these myofibrils consisted of strings of I-segments (Huxley, 1963) separated by gaps and with a detectable Z-line in the centre of each segment. Above 0.4 M NaCl little increase in diameter was observed but the Z-line gradually disappeared and the gaps between segments became more distinct. The extraction of the A-band occurred at a lower NaCl concentration (0.4 M) in the presence of pyrophosphate than in NaCl alone (0.6 M) where it occurred from the centre of the A-band and was only nearly completed in 1.0 M. Even though swelling took place at a lower salt concentration in the presence of pyrophosphate the swelling was less than the maximum in salt alone (at 1.0 M NaCl). Individual myofibrils, however, show a variable response to the action of NaCl and pyrophosphate (Offer & Trinick, 1983; Parsons & Knight, unpublished results). Some quantitative information of these phenomena was also recorded by Parsons & Knight (unpublished results). Rigor rabbit *M. psoas* myofibrils irrigated with NaCl alone showed an average increase in diameter of 42%

(SD 28%) when irrigated progressively in the 0.1-0.5 M NaCl concentration range, from the lowest to the highest concentration. In the 0.5-1.0 M NaCl concentration range there was an average diameter increase of 55% (SD 38%). The overall diameter increase by progressively irrigating myofibrils in higher NaCl concentrations from 0.1 to 1.0 M NaCl was 120% (SD 70%). Myofibrils of the same origin irrigated with NaCl plus 10 mM pyrophosphate showed an average diameter increase of 53% (SD 43%) when progressively irrigated in NaCl concentrations between 0.1 and 0.5 M. But they shrank by 6% (SD 19%) when progressively irrigated in NaCl concentrations between 0.5 and 1.0 M and the overall increase in diameter was only of 40% (SD 38%) in the 0.1-1.0 M NaCl concentration range.

Observations of the structural changes produced by NaCl and NaCl plus pyrophosphate at various depth levels in soaked blocks of pig meat have been made (Voyle *et al.*, 1984). A 0.6 M NaCl, 10 mM pyrophosphate, 1 mM $MgCl_2$, 10 mM sodium acetate buffer at pH 8.0 was shown by electron microscopy to produce complete or nearly complete extraction of the A-band from its ends, to a depth of at least one fibre from the surface. The same buffer used at pH 5.5 produced a less complete extraction of the A-band which appeared to occur only near the surface. The pH at which pyrophosphate acts has, therefore, influence in the extraction of protein by the additive as was seen previously in this section. In 0.6 M NaCl, 1 mM $MgCl_2$, 10 mM sodium acetate, no extraction of the A-band occurred. In the depth of the meat pieces treated with polyphosphate plus NaCl no extraction of the A-band could be detected. The Z-lines at the surface were also seen to break up along its length and this was the most common phenomenon related with the action of salt with or without pyrophosphate at high or low pH. The difference between the results obtained at the surface and in the depth of the meat were interpreted as the result of the lack of penetration of NaCl and pyrophosphate into the meat and so concentrations below those present in the surface of the meat - 0.6 M NaCl and 10 mM pyrophosphate - will have been achieved at different depths. The lack of structural changes at the myofibrillar level when compared with those observed by Hanson & Huxley (1953) and Offer & Trinick (1983) was attributed to the same reason, but were also suggested to be due to

the lower brine-to-meat volume ratio (1:3) used in this experiment, when compared with that of the irrigation of the isolated myofibrils. It was further suggested that in the intact tissue the possibility of myosin diffusing out of the meat blocks was diminished. The concentration of the myosin molecule would for that reason have been maintained constant over a substantial part of the meat block and only in the region near the surface did the concentration of the myosin molecules fall below a level at which the thick filament in that region would have become depolymerised by the salt and pyrophosphate.

Lewis *et al.* (1986) analysing the structure of cooked meat pieces of different origin, *i.e.* beef, chicken and pork, by means of electron microscopy, found that in most of the samples they treated with 0.5% of various polyphosphates in association with 2% NaCl the A/I-band overlap region was the most resistant to salt and pyrophosphate except in beef heart muscle and sometimes in other beef muscles. They also observed that in cooked muscle samples their images seemed to indicate that sarcoplasmic proteins precipitated onto the myofibrillar proteins a fact that was eliminated when polyphosphates were used. This led them to think that polyphosphates may reverse this precipitation by raising the pH of the meat and through this action increase water-holding. It was not, however, explained how the solubilisation of the sarcoplasmic proteins would influence water-holding by meat. The apparent contradictions between the results of these two approaches (Voyle *et al.*, 1984; Lewis *et al.*, 1986) lies in the fact that one was made in raw meat and the other made in cooked meat, which are two different systems. In cooked meat images of denatured myofibrillar structures will be expected to be found and can not be compared with those observed in raw meat. It may also be pointed out that the action of polyphosphates is already measureable in the raw state where it is therefore expected to find more reliable evidence of the changes that have occurred in the structure of muscle. In both these experimental approaches it is significant that artefacts due to preparation for electron microscopy make it difficult to interpret the images obtained.

The observations on the changes salt and polyphosphates produce on the structure of the sarcomere relate swelling with the extraction of the A-band. It can, for that reason, be deduced that swelling and protein extraction are related. This relationship is not, however, always the same in all systems that have been studied. Work done on mince (Bendall, 1954; Sherman, 1961) and homogenised meat (Brebner, 1981), and on meat pieces (Callow, 1932; Voyle *et al.*, 1984; Jolley & Purslow, unpublished results) shows that NaCl alone or associated with polyphosphates make swelling and protein solubilisation rise together in salt concentrations up to 8% (1.37 M). This is in contradiction with the observations by Offer & Trinick (1983) and Parsons & Knight (unpublished results), where swelling in the presence of NaCl plus 10 mM pyrophosphate was seen to be depressed in NaCl concentrations above 0.4 M NaCl at which a more complete extraction of the A-band (where myosin is located) was produced. A positive relationship between protein solubilisation and swelling was also not registered by Hamm (1979) in homogenised muscle treated with salt plus 0.3% pyrophosphate. While in 2% NaCl alone a highly significant correlation between water-holding capacity and protein extraction was found. In 2% NaCl plus 0.3% pyrophosphate, where more myofibrillar protein extraction was solubilised, water-holding capacity was not increased as the level of protein solubilised was increased. The reason for these results (Hamm, 1979; Offer & Trinick, 1983) lie in the capacity of pyrophosphate to promote a higher protein solubilisation than NaCl. Removing from the myofibrils the myosin molecules needed to promote swelling, as suggested by the hypothesis for salt-induced swelling of meat proposed by Offer & Knight (1988), would reduce swelling.

It is sensed by the listing of these various studies that certain uncontrolled conditions, in some cases unavoidable, have not always made it possible to compare the results observed and find them reliable. The pH of different meat samples is assumed to be uniform when it is predictable that variation must have existed. Common to almost all studies was the fact that polyphosphates were not compared with other additives under controlled pH conditions, *e.g.* Swift & Ellis (1956), Sherman (1961). Generally the pH conditions are irreproducible since

the temperature of measurement of pH values was not indicated, *e.g.* Bendall (1954), Swift & Ellis (1956), Sherman (1961) and Hellendoorn (1962). It is foreseeable that a wide range of concentrations to study the action of salt and polyphosphates - not used by many of the authors mentioned - will be more effective in detecting the effects polyphosphates produce, since the action of polyphosphates may only be noticed in a short range of concentrations (*e.g.* Bendall, 1954), contrary to what happened in most of the experiments that were revised. Amongst the unavoidable factors was the physical state of the muscle used, which influenced, *e.g.* in mince and meat pieces, the flow of molecules in and out of the meat. In the experiments with irrigated myofibrils, *e.g.* Offer & Trinick (1983), it was shown that different myofibrils have different swelling and protein extraction behaviours. This indicates that it is possible to study adequately swelling and protein solubilisation in myofibrils by using a volume of a myofibrillar suspension where the myofibrils showing different types of behaviour will be present, making it possible to obtain an average behaviour of myofibrils. Endogenous ions exist in meat in the concentrations (in moles.l⁻¹ of muscle) of: 100 mM K⁺; 27 mM Na⁺; 11 mM Mg²⁺; 20 mM carnosine; 100 mM lactate; 40 mM phosphate; 42 mM creatine and 32 mM other amino acids (Offer & Knight, 1988) and do most certainly influence the action of any additive used in meat.

1.9. Mechanism of action of polyphosphates

Polyphosphates have been thought by some authors to act in meat by raising the pH of the meat (Swift & Ellis, 1956; Lewis *et al.*, 1986) or its ionic strength (Swift & Ellis, 1956; Trout & Schmidt, 1984) but these explanations do not account for the results of Bendall (1954) and Hellendoorn (1962) in the first case, and those of Bendall (1954), van den Oord & Wesdorp (1978) and Lewis *et al.* (1986) in the second case. Bendall (1954) compared the effect of NaCl alone and NaCl plus pyrophosphate at the same ionic strength and found that in the presence of pyrophosphate more water was taken up by minced meat pellets. van den Oord &

Wesdorp (1978) studying the effect of pyrophosphate in protein extraction in pork and beef, calculated the ionic strength for each treatment at different salt and pyrophosphate concentrations, and pH values. They concluded that the effect of pyrophosphate on protein solubilisation was specific and that this effect could only to a small extent be attributed to the ionic strength promoted by the phosphate.

Lewis *et al.* (1986) studying the action of polyphosphates in meat products concluded that in the meat samples in which polyphosphates were effective in increasing yield, their capacity to maintain a high pH in the system was their most important mechanism of action, since other alkaline agents tested (sodium hydroxide, trisodium orthophosphate and Tris buffer) were also effective in increasing yield. The contribution to ionic strength of polyphosphates in the presence of an overall level of 2% salt was also concluded to have little effect in their study. Their conclusion that polyphosphates may act by their action in maintaining a high pH in the meat is doubtful in that they compared polyphosphates with the other reagents at high pH values (*e.g.* 12.1, 11.5 and 9.4) which are not found in meat processing. Though not explained on how such a mechanism of action would increase water-holding, it is also in that study suggested that polyphosphates by increasing the pH value would dissociate sarcoplasmic proteins which precipitate onto the surface of the myofibrils.

Chelation of Mg^{2+} and Ca^{2+} ions by polyphosphates and the breaking of links between metal ions and proteins, allowing an increase in space in the structure of proteins for water to be held, has been another mechanism proposed for their action (Hamm, 1960). This has been contradicted by the evidence that the chelating agent EDTA was less effective than pyrophosphate in promoting water uptake at the same pH value (Sherman, 1961). Hellendoorn (1962) also investigated the effect of the disodium salt of EDTA as a more efficient binder for Ca^{2+} , Zn^{2+} and Mg^{2+} and instead of the positive effect expected (Hamm, 1960) a marked depressing effect on water binding was observed. Substituting sodium chloride partially by ammonium oxalate with an equal ionic strength to investigate the influence

of removal of Ca^{2+} , led to the conclusion that the elimination of the calcium from muscle has nothing to do with water retention by meat in cooked systems, and that the specific activity of pyrophosphate cannot be explained in terms of its ability to complex Ca^{2+} ions as indicated by Hamm (1960). Addition of the three ions mentioned above in the same quantities as already present in the meat, was also tried, with minor depressing effects on water holding being produced in the presence of NaCl or NaCl and 0.5 % sodium pyrophosphate in cooked systems.

As previously also mentioned (section 1.5) pyrophosphate is considered to be the active form by which the effects attributed to polyphosphates are produced. Pyrophosphate has two major effects on actin and myosin systems: it promotes the dissociation of actomyosin (Weber & Portzehl, 1952) and helps sodium chloride depolymerise the shaft of the thick filament (Harrington & Himmelfarb, 1972).

Mg^{2+} promotes the dissociation of actomyosin by pyrophosphate (Weber & Portzehl, 1952). The degree of dissociation of actomyosin by pyrophosphate increases with the Mg^{2+} concentration in the 0.01 to 1 mM range (Gränicher & Portzehl, 1964). But it also slightly inhibits the depolymerisation effect (Harrington & Himmelfarb, 1972).

At the concentrations currently used in meat processing, polyphosphates may assist NaCl in causing the depolymerisation of the thick filament. This would explain their synergistic effect in water-holding when used in association with NaCl in certain concentrations, at certain temperature and pH values, and in the presence of certain concentrations of Mg^{2+} , Ca^{2+} and Cl^- ; while their action in freeing myosin heads from actin molecules, if great enough, would produce a depression in water-holding (Offer & Knight, 1988). The synergistic effect would correspond to more swelling being observed at lower NaCl concentrations since more myosin tails needed to promote swelling would be available because of an earlier depolymerisation of

the thick filament [see section 1.7, Offer & Knight (1988)]. The dissociation of myosin from actin on the contrary would lower the number of myosin tails available to promote swelling by allowing myosin to be extracted from the myofibrils (see Offer & Knight, 1988).

The observations (in previous section) by Offer & Trinick (1983) and Parsons & Knight (unpublished results) that pyrophosphate, even though producing swelling of irrigated myofibrils at a lower NaCl concentration than NaCl alone, decreased maximum swelling led Offer & Knight (1988), based on their hypothetical entropic mechanism of salt-induced swelling in meat, to suppose that the difference between the depression of maximum swelling in isolated myofibrils highly irrigated in a microscopic preparation and the increase in swelling noticed when polyphosphates are used in practice, on meat pieces, is due to the difference in solvent-to-meat volume ratio existing between the two systems.

In irrigated myofibrils the higher solvent-to-myofibril ratio would facilitate the solubilisation and extraction of greater quantities of myosin needed to promote maximum swelling. In meat pieces, however, it would be expected that pyrophosphate would be present in concentrations low enough to produce only a limited dissociation of actomyosin. At the same time the slower diffusion of myofibrillar protein, mainly myosin, in the meat and consequently its extraction to the exterior will be comparatively slower. Not only because of the physical barriers existing in intact muscle but also because of the thickness of the meat pieces.

When salt and polyphosphate diffuse into the meat the thick filaments will tend to depolymerise and actomyosin will tend to become dissociated, as expected; the myosin molecules produced will diffuse out into the salt solution and a gradient of concentration will be established in the meat piece; but as myosin molecules diffuse away from the surface of the meat they will be replenished by the dissociation of actomyosin and depolymerisation of more thick filaments in the depth of the meat volume; this reduces the depth over which the gradient of myosin molecules occurs. The effect produced is that the myosin molecule concentration is

maintained constant at a critical concentration over a substantial part of the meat portion and a balance will be created where myosin molecules will tend to be found as thick filaments rather than as single molecules (see Voyle *et al.*, 1984). Since, depolymerisation of the thick filament and freedom of the myosin tails are the phenomena required for swelling to happen, an increase in water-holding may be accompanied by only a small extraction of myosin as observed by Hamm (1971).

1.10. Outstanding problems concerning the interpretation of the mechanism of action of polyphosphates

Although the effect of polyphosphate has been investigated by several authors, major uncertainties exist. As explained above, controversy exists in the literature on whether or not pyrophosphate has a greater effect than salt in promoting water uptake by raw meat at equivalent ionic strength. Information on the pH and NaCl concentration at which polyphosphates exert their greatest effect on protein solubilisation and water uptake is deficient. In most cases swelling and protein extraction (especially myosin) have not been simultaneously investigated, even though they are suggested to be associated (Bendall, 1954; Offer & Trinick, 1988). Some of the model systems were not studied under a constant pH, endogenous ions naturally occurring in meat were not considered and different brine-to-muscle sample volume ratios were used and differences between the effects of the additives under practical conditions and those observed under experimental conditions have not been totally explained up to the present date. All these items and others concerning the action of phosphates in meat processing need to be critically tested.

The aims of the present work were firstly to re-examine whether pyrophosphate has a greater effect than NaCl, in both the swelling and myofibrillar protein extraction of uncooked meat. Secondly to explore the conditions of NaCl concentration at pH 6.0 required to get the

production of increased water uptake by pyrophosphate. A third aim was to investigate the discrepancy between the results for swelling observed in mince (Bendall, 1954) and myofibrils (Offer & Trinick, 1983; Parsons & Knight, unpublished results) . A fourth and final aim was to explore the relationship between protein solubilisation and swelling. To do this rabbit rigor muscle was used in two different physical states:

a) Isolated myofibrils prepared from *psoas* muscles, because of the characteristics of these muscles: (1) their low collagen content; (2) their high uniformity in fibre type and (3) the availability of a great amount of information existing on their physico-chemical and biological characteristics.

The reasons for employing washed myofibrils were that: (1) no endogenous ions, sarcoplasmic proteins, nucleotides, mitochondria, nuclei and sarcoplasmic reticulum will be present in sufficient amounts to influence the results; (2) a rapid equilibrium will be established between the salts and the fibrils so that the formation of gradients of salts penetrating the myofibrils or solubilised proteins leaving their structure will not be additional factors to consider; (3) swelling will not be restricted by connective tissue elements and (4) the interfilament space will be the only compartment where water will be held, unlike mince and meat pieces where water will be contained in other compartments - in the case of mince in between mince particles, in the case of meat pieces intracellularly between the myofibrils and the cytoplasmic membrane and endomysium, or in the extracellular space.

b) Back and hind-leg muscles of the same origin were used minced, and minced and washed. The reason of this choice was to repeat work previously done under uncontrolled conditions (Bendall, 1954) following the methodology then used and to repeat the same experiment but under controlled conditions, as was done with the myofibrils.

2. MATERIALS AND METHODS

- Origin of chemicals and measurement of pH

In all experiments chemicals, except where otherwise stated, were BDH AnalaR reagents and all measurements of pH were made using a Russell glass electrode (Russell pH, Ltd.).

2.1. Experiments with myofibrils

2.1.1. Preparation of buffer solutions

(i) Experiments 1, 2 and 3

Four different buffer solutions, shown in Table 1., were made.

Table 1. List of stock pH 6.0 buffer solutions used.

- (1) 0.1 M NaCl, 1 mM MgCl₂, 20 mM imidazole;
- (2) 2.0 M NaCl, 1 mM MgCl₂, 20 mM imidazole;
- (3) 0.1 M NaCl, 2 mM tetrasodium pyrophosphate, 1 mM MgCl₂, 20 mM imidazole;
- (4) 2.0 M NaCl, 2 mM tetrasodium pyrophosphate, 1 mM MgCl₂, 20 mM imidazole.

The pH of all four was adjusted to a value of 6.0, measured at 5.0 °C, with HCl. MgCl₂ in the pyrophosphate solutions was added just before their use so that precipitation of the pyrophosphate was avoided. This was followed by an immediate measurement of pH to detect if a rise in its value took place. The 0.1 and 2.0 M NaCl solutions with and without 2 mM pyrophosphate were mixed, in 20 separate plastic centrifugation tubes, in the ratios that would give NaCl concentrations with 0.1 M increment in the 0.1-1 M range (see Table 2). To each tube the same volume of a 0.1 M NaCl, 1 mM MgCl₂, 20 mM imidazole chloride

Table 2. Mixture of buffers (1) and (2) and (3) and (4) in various ratios, in order to obtain 50 ml of composed buffers which on addition to a volume of 50 ml of myofibrils suspended in 0.1 M NaCl, 1 mM MgCl₂, 20 mM imidazole chloride, pH 6.0 would permit the obtention of final NaCl concentrations with 0.1 M increment in the 0.1-0.1 M range.

required NaCl (M)	ml of buffer	
	(1) or (3)	(2) or (4)
0.1	50	0
0.2	44.74	5.26
0.3	39.47	10.53
0.4	34.21	15.79
0.5	28.95	21.05
0.6	23.68	26.32
0.7	18.42	31.58
0.8	13.16	36.84
0.9	7.89	42.11
1.0	2.63	47.37

solution, was added mimicking the addition of a volume of a myofibrillar suspension in 0.1 M NaCl, 1 mM MgCl₂, 20 mM imidazole chloride, pH 6.00. The pH of these mixtures was measured to detect if any change in pH had occurred at any NaCl concentration and to assess whether adjustments to the pH of solutions (1), (2), (3) and (4) were needed.

(ii) Experiment 4

The same procedures were followed except that 20 mM pyrophosphate were used instead of 2 mM in the solutions containing pyrophosphate, and the pH of the mixtures made, with the same objective as in the previous experiments, was measured after the addition of MgCl₂.

2.1.2. Phase-contrast light micrography of myofibrillar structure

Photomicrographs of the myofibrils in each incubation were taken in a cold room at a temperature between about 5 and 7°C, using a Wild Photoautomat MPS 45 photomicrographic unit coupled with a Wild MPS 51 S SPOT camera mounted on a Leitz Laborlux 12 phase-contrast microscope illuminated by an illumination unit fitted with a 12 V, 100 W Philips bulb.

10 µl of each myofibrillar system were taken after the incubation period, and put on a glass micro-slide with a 0.6 - 0.8 mm thickness. A glass cover slip, thickness nr. 1 1/2, was then compressed against the sample in the slide, and the preparation observed using a Phaco NPL phase-contrast 100 x oil immersion lens (NA 1.30).

The films used were Kodak Technical Pan film, ESTAR - AH Base, at a speed of 200 ASA. They were developed in Kodak HC-10 developer (dilution B) for 6 1/2 min and fixed for minutes in Kodak Unifix fixer. The images shown were magnified 1814 times.

2.1.3. Measurement of myofibrillar protein in the supernatants

No measurement of protein in the supernatants was done in Experiment 1, because of the longer incubation period and the mechanical action that were employed due to the need for re-suspension of the pellets in NaCl alone. In Experiment 2 an aliquot of supernatant from each tube was conveniently diluted in a NaCl solution of the same concentration as that of the supernatants. In Experiments 3 and 4 an aliquot of supernatant from each tube was conveniently diluted with and dissolved in 1% warm SDS (Sigma). The absorbancy of the solutions was measured in a Unicam (Unicam Instruments, Ltd.) SP 500, Series 2 spectrophotometer at 260, 280 and 320 nm in a 1 cm silica cell. Scattering was corrected for by subtraction of the value of the absorbance at 320 nm from the absorbance at 280 nm. The absorbance at 280 nm of a solution containing 1 mg/ml of myofibrillar protein was taken to be 0.7 (Knight & Trinick, 1982).

2.1.4. Preparation of myofibrils

(i) Experiment 1

New Zealand White rabbits were used in all experiments. After concussion stunning, slaughter and bleeding of the animals the carcasses were skinned partially, eviscerated, put on a tray, wrapped in a polythene bag and left for a period of 20 h in a cold room at a temperature between 5 and 7°C, to allow them to go into rigor. After this period, in the same room the *psoas* muscles were dissected, trimmed of fat, weighed, cut into about 0.5 cm size pieces, put into a 600 ml metal pot immersed in ice to avoid a rise in temperature and mixed with 10 volumes of 0.1 M NaCl, 1 mM MgCl₂, 20 mM imidazole chloride solution. The muscle was then homogenised in a Sorvall Omni-Mixer (Du Pont Instruments) with five 10 s bursts being carried out at maximum speed, and with a 10 s rest period between every two bursts to avoid a temperature rise. No significant rise in temperature was registered.

Phase-contrast microscopy was used at the end of the homogenisation period, to check if the muscles had been reduced to myofibrils or whether further homogenising was needed. 10 µl

of the suspension were diluted ten times in 0.1 M NaCl, 1 mM MgCl₂, 20 mM imidazole chloride, a drop of this diluted sample was placed on a 0.6 - 0.8 mm thickness glass slide, and then covered and compressed with a glass cover slip, thickness nr. 1 1/2. Phase-contrast light micrographs of the homogenised myofibrils were taken (see Results, Fig. 3.1 (a)).

The myofibrils were then pelleted by centrifuging the homogenate at 600 x g for 10 minutes in a refrigerated centrifuge at about 5°C, after which the supernatant was decanted. The pellet was re-suspended in the same solution in a 1:2 volume ratio and submitted to the same centrifuging treatment. This procedure of re-suspension followed by centrifugation and decantation of the supernatant was repeated two times more resulting in four centrifugations being carried out. The last myofibrillar pellet obtained was then resuspended in a volume of the 0.1 M NaCl, 1 mM MgCl₂, 20 mM imidazole chloride solution, corresponding to 25 % of the volume of the original homogenate. Lumps in the final suspension of myofibrils were eliminated by filtering it through a cotton mesh, with the help of constant manual agitation with a plastic spoon. Phase-contrast light microscopy was used to record the aspect of the suspended myofibrils, as a control (see Fig 3.1 (b)) and an estimation of the protein concentration of the myofibrillar suspension was made by reading the absorbance at 280 nm. The myofibrils in all experiments were stored in a 400 ml conical flask in a cold room at a temperature of about 4°C, and used within 24 hours of their preparation.

(ii) Experiment 2

The preparation in Experiment 2 was identical to that in Experiment 1 except for the following changes. Because of a limitation in size of the metal pot of the blender used, the cut muscles were, before homogenisation, diluted in a volume of 0.1 M NaCl, 1 mM MgCl₂, 20 mM imidazole chloride corresponding to only 8.5 times its weight. The washing procedure used was the same as that used in Experiment 1 with the difference that it was done in four 250 ml plastic centrifugation bottles and before the second, third and centrifugations the pellets were

dilluted and resuspended in of 0.1 M NaCl, 1 mM MgCl₂, 20 mM imidazole chloride in a 1:10 volume ratio. The aspect of the myofibrils obtained, after homogenisation and in the final suspension was identical to that of the myofibrils prepared in Experiment 1, which is shown in Figs. 3.1 (a) & (b).

(iii) Experiment 3

The same procedures as in Experiment 2 were used, except that the final volume of the buffer solution added to the washed homogenate corresponded to about 50% of the volume of solution added initially to the muscles before homogenising to obtain a sufficient final myofibrillar suspension volume to carry out the experiment. Filtration of the suspension was also not carried out since in Experiments 1 and 2 no lumps were present in the myofibrillar suspensions. The aspect of the myofibrils obtained after homogenisation and in the final suspension was identical to that of myofibrils in Experiment 1 (Fig. 3.1 (a) & (b)).

(iv) Experiment 4

The procedures employed were as those in Experiment 3, except that the final volume of the 0.1 M NaCl, 1 mM MgCl₂, 20 mM imidazole chloride solution was about 75% of the volume of the solution initially added to the muscles before homogenising. Myofibrils after homogenisation and in the final suspension had an identical appearance to that of myofibrils in Experiment 1 (Fig. 3.1 (a) & (b)).

2.1.5. Incubation of myofibrils in salt solutions

(i) Experiment 1

Sodium chloride solutions of 0.1 and 2.0 M NaCl, 1 mM MgCl₂, 20 mM imidazole chloride, with or without 2 mM tetrasodium pyrophosphate (pH 6.00 at 5°C), kept in a cold room at a temperature of about 4°C, were mixed in various ratios in pre-weighed 12 ml plastic centrifuge

tubes, in order to obtain a range of NaCl solutions in 0.1 M increments from 0.1 to 1.0 M NaCl and a total volume of 5 ml. After agitation of the myofibrillar suspension, 5 ml were then added with stirring to each tube, inside a cold room at about 4°C.

The myofibrillar suspensions in salt alone and in salt plus pyrophosphate were incubated for 200 and 245 min respectively, in a water bath at a temperature of about 4°C. The difference in time between tubes with and without pyrophosphate being caused by difficulties in focussing the preparations of myofibrils in salt plus pyrophosphate when collecting the microscopic images before centrifugation, and by the need to re-suspend the pellets in NaCl alone. The tubes were centrifuged at a temperature of about 5 °C.

In this experiment the tubes holding myofibrils in salt alone were first centrifuged for 5 min using a centrifugal speed of 1500 x g. After this period the formation of a defined pellet was not achieved. Re-suspension of the pellets followed by re-centrifugation was tried under different conditions of time and centrifugal speed. The final centrifugation consisted on the use of 3000 x g for 60 min. Because it was found that myofibrils bathed in salt alone would not adequately sediment using a centrifugal speed of 1500 or 3000 x g, the tubes in which the action of salt and pyrophosphate was to be studied were directly spun at a speed of 3000 x g for 60 min.

After centrifugation the supernatants were removed by careful aspiration with a Pasteur pipette and their volume roughly measured in plastic (neutral) sample tubes, in a cold room at about 4°C. The tubes containing the pellets were re-weighed in a cold room at a temperature between 5-7°C.

Swelling was calculated using the following swelling index (S): $S = [(weight\ of\ tube\ (g) + pellet\ (g)) - weight\ of\ empty\ tube\ (g)] / myofibrillar\ protein\ mass\ (g)\ in\ the\ final\ system.$

(ii) Experiment 2

The incubation period was of 180 min in all treatments and the plastic centrifuge tubes used had a 100 ml capacity. A centrifugal speed of 9000 x g for 60 min was used to achieve sedimentation of the myofibrils.

(ii) Experiments 3 and 4

In Experiments 3 and 4 the volume of the myofibrillar suspension and of the solution that was mixed with it were each increased to 10 ml. Otherwise, like in Experiment 2.

2.2. Experiments with mince

In Experiment 5 New Zealand White rabbit mince from back and hind-leg muscles was used in a repetition of the Bendall (1954) experiment in a smaller scale, not only of the volume of the total system, but also in the number of samples used in each treatment. In Experiment 6 the same muscles were used, but materials and methods were changed in order to meet the controlled conditions and the range of NaCl concentrations (0.1-1.0 M) that were used in the experiments with myofibrils.

2.2.1. Preparation of buffer solutions

(i) Experiment 5 (mince)

Three buffers, which had their pH adjusted to 6.50 at 5°C with HCl, were prepared:

- (1) 1% NaCl;
- (2) 1% NaCl, 0.25% pyrophosphate ($\text{Na}_2\text{H}_2\text{P}_2\text{O}_7$);
- (3) 1% NaCl, 0.5% pyrophosphate ($\text{Na}_2\text{H}_2\text{P}_2\text{O}_7$) (Bendall, 1954).

(ii) Experiment 6 (washed mince) four buffers were prepared as in Experiment 4.

2.2.2. Preparation of mince

Following the instructions indicated by Bendall (1954) rabbit back and hind-leg muscles were dissected from a carcass, put on a tray, wrapped in two layers of polythene plastic, covered with ice and left in a cold room with a temperature of about 4°C for 24 h to allow them to go into rigor. The temperature of the muscles was carefully monitored so that it was kept at 0°C.

After this period the muscles were transported into a cold room at a temperature between about 5-7°C, trimmed of fat and minced at maximum speed in a domestic mincer [Kenwood Liquidiser A 288 G, Major model (Kenwood Manufacturing Company, Ltd.)] using a 3 mm plate.

2.2.3. Incubation of mince in salt solutions

Following the instructions indicated by Bendall (1954), inside a cold room at a temperature of about 4°C, a quantity of 20 g of mince was put in each of 20 empty, preweighed 100 ml plastic centrifuge tubes. The tubes were divided into four groups. 20 ml of distilled water were added to the first group and 20 ml of the three prepared buffer solutions, all at about 4°C, were added to the each of the remaining three groups of tubes, with stirring.

The next step involved an incubation for 30 min at room temperature followed by another in ice at a monitored temperature of 0°C in the same cold room for 20 h. The suspensions were then centrifuged at 1200 x g for 30 min at a temperature of about 5°C. Still at about 4°C in the cold room, the supernatants were carefully removed with Pasteur pipettes and this volume measured in 25 ml glass measuring cylinders. The pH of a portion of the supernatants was measured at both 5 and 20°C. The tubes with the pellets were re-weighed. Finally the measurement of the protein concentration in the supernatants was carried out as in Experiment 3.

Swelling was calculated according to the method employed by Bendall (1954): volume of 100 ml of the mince after treatment = initial vol. of the system (ml) – vol. of the supernatant (ml) x 100/initial weight of mince (g) and the index used to calculate swelling in the experiments with myofibrils. To use this second method it was assumed that there are about 100 mg of myofibrillar protein per g of muscle [from data by Bailey (1955), Maruyama (1970)], and since 20 g of mince were mixed with 20 ml of buffer the concentration of myofibrillar protein in the mince systems would have been of about 50 mg.ml⁻¹.

2.2.4. Preparation of washed mince

The procedure followed was that of Experiment 5 with the difference that, after mincing, the meat was mixed, in four 1 l plastic centrifuge bottles, with ten volumes of 0.1 M NaCl, 1 mM MgCl₂, 20 mM imidazole chloride at pH 6.0, measured at 5°C. This was followed by stirring for 1 h in a cold room at about 7°C using a plastic paddle connected to a Heidolph (type RZR 1, Lab-plant Ltd.) stirrer at its lowest speed. A first wash by centrifugation at 1200 x g for 30 min in a cold room at a temperature between 5-7°C was then carried out, and after its completion the supernatant was removed and the absorbance at 280 was measured. The pellet re-suspended and the whole procedure repeated once again.

2.2.5. Incubation of washed mince in salt solutions

Solutions of a 0.1 and 2.0 M NaCl, 1 mM MgCl₂, 20 mM imidazole chloride, pH 6.00, measured at 5°C, with or without 20 mM tetrasodium pyrophosphate, were mixed (as in the experiments with myofibrils), in various ratios in pre-weighed 100 ml plastic centrifuge tubes, in order to obtain a range of NaCl concentrations in 0.1 M increments, from 0.1 to 1.0 M (see Table 2). The tubes were then refrigerated at a temperature of 4°C. 15 g of refrigerated washed mince (*ca.* 4°C) were added to each tube (with a different concentration), with stirring, inside a cold room at a temperature of about 4°C. An incubation period of 20 h in ice at a

monitored temperature of 0°C was then spent inside a cold room and followed by centrifugation at a temperature of 5°C for 30 min at 1200 x g. In the same room the supernatants were carefully removed using a Pasteur pipette and their volumes measured in 25 ml glass measuring cylinders. The tubes containing the sediment were then re-weighed and swelling calculated using the same methods as used in the experiments with myofibrils and as in Experiment 5, so that a comparison could be made. Lastly the pH of all the refrigerated supernatants was measured at 5.0°C, and the protein concentration in the supernatants measured following the instructions used in Experiment 3.

3. RESULTS

3.1. Experiments with myofibrils

3.1.1. pH of buffer solutions

As mentioned in the Materials and Methods section, in Experiments 1, 2 and 3 two solutions: (1) 0.1 M NaCl, 1 mM MgCl_2 , 20mM imidazole chloride and (2) 2.0 M NaCl, 1 mM MgCl_2 , 20 mM imidazole chloride, both at pH 6.0 at 5°C, were mixed in order to obtain a range of final NaCl concentrations needed to study the action of NaCl alone. For the study of the action of pyrophosphate two other buffers (3) and (4) like (1) and (2) but including 2 mM pyrophosphate were prepared. In Experiment 4 solutions (3) and (4) were made including 20 mM pyrophosphate.

To avoid the possible precipitation of the phosphate in the presence of magnesium chloride, MgCl_2 was only added just before these solutions were used. Since the study of the effect of the NaCl and pyrophosphate was to be made at a constant pH value of 6.0, a preliminary study was made to see whether the final addition of MgCl_2 would produce any pH changes. No significant changes in pH were found.

A preliminary investigation was also made to find out if when the low and high NaCl concentration solutions were mixed and then added to an equivalent volume of a 0.1 M NaCl, 1mM MgCl_2 , 20 mM imidazole chloride, pH 6.0 (mimicking the addition of an equivalent volume of myofibrils), to give NaCl concentrations with a 0.1 M increment in the 0.1-1.0 M concentration range, there were any significant changes in the pH value. It was found (see Table 3.1) that with NaCl alone the pH rose to a maximum of 6.15 at 0.8 M. The pH in the 2.0 M NaCl solution (2) was accordingly changed to a value of 5.85 in order to reduce any

Table 3.1. Experiment 1. Final pH after mixtures of buffers (1) and (2) without pyrophosphate, and mixtures of buffers (3) and (4) with pyrophosphate were added to an equivalent volume of buffer (1).

NaCl (M)	pH of mixture	
	no pyrophosphate (1) + (2)	pyrophosphate (3) + (4)
0.1	6.01	6.00
0.2	6.05	6.04
0.3	6.05	6.06
0.4	6.07	6.09
0.5	6.07	6.11
0.6	6.14	6.11
0.7	6.14	6.15
0.8	6.15	6.18
0.9	6.12	6.20
1.0	6.11	6.25

effect the pH increase produced by the mixture of the buffers might have in the expression of the effects of NaCl. Mixing the two buffers (0.1 and 2.0M NaCl) with 2 mM pyrophosphate the pH rose to a maximum of 6.25 in the 1.0 M NaCl concentration. A readjustment of the pH to 5.75 was made in the 2.0 M NaCl, 2 mM pyrophosphate solution for the same reasons as above. The same mixture of buffers but with 20 mM pyrophosphate was made in Experiment 4. The value of pH was measured and seen to have increased in the 0.5 M NaCl concentration to 6.14 and reached a maximum of 6.37 in the 1.0 M concentration (see Table 3.2). The pH of the 2.0 M, 20 mM pyrophosphate, 1 mM MgCl_2 , 20 mM imidazole chloride buffer was therefore adjusted to a value of 5.85.

3.1.2. Swelling, protein solubilisation and changes in structure of myofibrils treated with NaCl or NaCl plus 1 mM pyrophosphate

A volume of washed myofibrils was mixed with an equal volume of NaCl solutions, to give a final concentration of NaCl in the range 0.1 to 1.0 M, with or without 1 mM pyrophosphate. After this addition, the systems were then left to incubate, for a certain period of time. Micrographs of the incubated systems were taken in order to find if swelling and protein extraction were accompanied by any changes in the structure of the myofibrils. The incubation was followed by centrifugation to pellet the myofibrils and then removal of the supernatant. From the weight of the myofibrillar pellet the swelling of the myofibrils was determined. An estimation of the myofibrillar protein present in the supernatants was then made.

(i) Experiment 1

The appearance of the myofibrils in suspension before treatment is shown in Fig. 3.1 (a) & (b). The images of the changes in the structure of myofibrils obtained in the presence of NaCl alone (Fig. 3.2.) and NaCl plus 1 mM pyrophosphate (Fig. 3.3.) were unsatisfactory.

Table 3.2. Experiment 4. Final pH after mixtures of buffers (3) and (4) were added to an equivalent volume of buffer (1).

NaCl (M)	pH after mixture
0.1	6.06
0.2	6.01
0.3	6.01
0.4	6.02
0.5	6.14
0.6	6.15
0.7	6.15
0.8	6.18
0.9	6.27
1.0	6.37

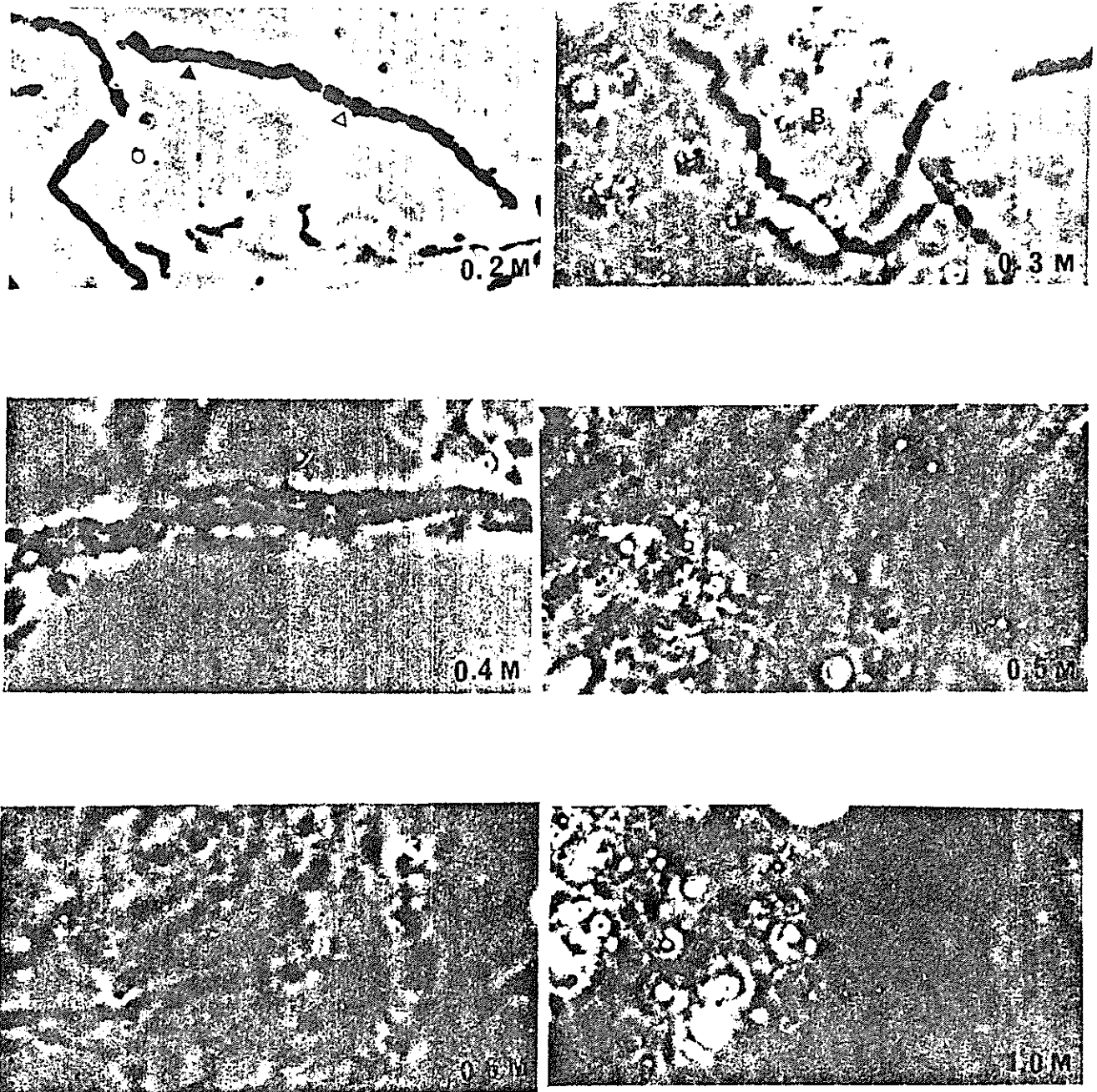


Fig. 3.3. Experiment 1. Phase-contrast light micrographs showing the effect of 0.2, 0.3, 0.4, 0.5 or 0.6 M NaCl, 1 mM pyrophosphate, 1mM MgCl₂, 20 mM imidazole chloride at pH 6.0 on myofibrils in a suspension, with a myofibrillar protein concentration of 12.5 mg.ml⁻¹, after an incubation period of 245 min at 4°C. In 0.2 M: Δ intact Z-line and \blacktriangle intact A-band. In 0.3 M: B represents area showing disorganised mass of myofibrils. Magnification 1814 x.



Fig. 3.1. Rabbit *M. psoas* myofibrils. (a) After homogenising muscle. (b) In suspension after being washed. Magnification 1814 x.

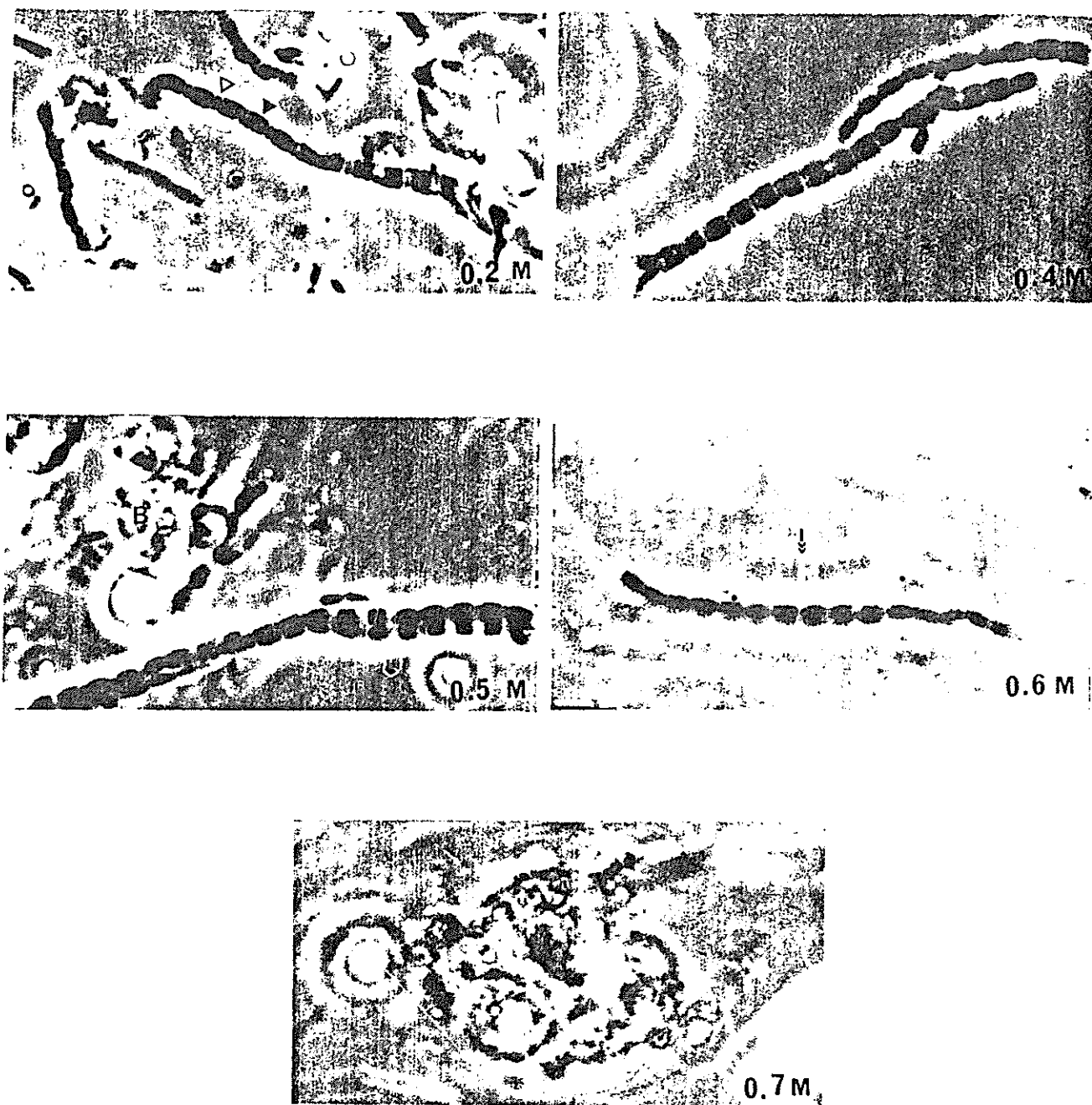


Fig. 3.2. Experiment 1. Phase-contrast light micrographs showing the effect of 0.2, 0.4, 0.5, 0.6, 0.7 or 1.0 M NaCl, 1 mM MgCl₂, 20 mM imidazole chloride at pH 6.0 on the structure of myofibrils in a suspension, with a myofibrillar protein concentration of 12.5 mg.ml⁻¹, after an incubation period of 200 min at 4°C. In 0.2 M: Δ intact Z-line and ▲ intact A-band. In 0.5 M: B represents area showing disorganised mass of myofibrils. In 0.6 M: ->> traces of myofibril suggesting extraction of the A-band. Magnification 1814 x.

Firstly, because under the low temperatures of operation (5-7°C) focussing was found to be difficult and secondly, because at a concentration of 0.4 M NaCl and above the field showed a disordered myofibrillar mass where individual myofibrils were difficult to isolate, but when found did not show signs of extraction of the A-band. This was accompanied by an higher viscosity of the supernatants. At 0.6 M NaCl myofibrils with an intact structure were found along with images suggesting extraction of the A-band. After 0.6 M NaCl no myofibrils could be found. In NaCl plus 1 mM pyrophosphate images of a disordered myofibrillar structure were registered already in 0.3 M NaCl, but some myofibrils with an intact structure were still found. The supernatants at that NaCl concentration were viscous and flocculent. The images obtained were thought to be due to an increase in viscosity of the supernatants at NaCl concentrations of 0.3 M and above in the presence of 1 mM pyrophosphate, and of 0.4 M and above in NaCl alone. This made the myofibrils stick together and resulted in that when myofibrils were compressed between a cover slip and a glass slide they could not be easily isolated, or isolated at all above 0.4 M NaCl plus pyrophosphate and 0.6 M NaCl alone. This was thought to be possible to avoid by diluting the myofibrillar samples. However, the dilution of the myofibrils was physically impossible to carry out in these experiments due to the amount of time and labour that would have to be spent and the consequent alteration of the schedule proposed.

The tubes containing 5 ml of a myofibrillar suspension plus 5 ml of a salt solution, with a final myofibrillar protein concentration of 12.5 mg/ml, were initially centrifuged at 1500 x g for 5 min. After this period of time it was found that no satisfactory separation of a pellet had been achieved at NaCl concentrations above 0.3 M, and so the partially sedimented mass was re-suspended and re-spun for 10 min at 1500 x g. This second period produced the same result, and a second resuspension was carried out and followed by a third centrifugation at the same g force but for 60 min. No better effect on sedimentation being accomplished, another re-suspension of the contents of the tubes was made, this time followed by a centrifugation at 3000 x g for 60 min.

The formation of a well packed pellet after centrifuging at 3000 x g for 60 min was still not achieved at NaCl concentration values above 0.3 M. It was possible to obtain pellet weight values at 0.4 and 0.5 M NaCl but this was difficult due to lack of cohesion in the pellet mass. Due to this difficulty, those values cannot be confidently used and are not shown in Fig. 3.4 where pellet weight is seen to have slightly increased from 0.1 to 0.3 M NaCl. Centrifugation of the tubes in which the 1 mM pyrophosphate was present was carried out for 30 min at 3000 x g, a period after which the tubes were checked. At the end of this period well pelleted pellets were already obtained, but the centrifugation was carried out for another 30 min, fulfilling a centrifugation period of 60 min. In this experiment the tubes containing myofibrils in 0.4 and 0.7 M NaCl, 1 mM pyrophosphate broke during centrifugation, which made them unavailable for subsequent measurements. The swelling index (Fig. 3.4) was seen to increase slightly from 0.1 M to 0.5 M NaCl. A decrease in pellet weight is observed between 0.5 and 0.8 M, after which values rise to a maximum at 1.0 M.

Due to the problems encountered in separating the supernatants from the pellets in this trial and the repeated manipulations exercised upon the tube contents, no measurement of protein concentration in the supernatant was made.

It was concluded that: (a) changes in the structure of myofibrils as observed in other experimental work (Offer & Trinick, 1983; Parsons & Knight, 1990) could not be found with the technique used for the reasons above mentioned; (b) images of a disordered mass of myofibrils were coincident with a rise in swelling of myofibrils in 0.3 and 0.4 M NaCl plus pyrophosphate, and difficulties in pelleting myofibrils in NaCl alone at a concentration of 0.4 M NaCl; (c) there was a need for a higher centrifugation speed since swollen myofibrils under the conditions used and in the presence of NaCl alone did not sediment at the speeds previously used; (d) swelling in the presence of 0.1 to 0.3 M NaCl plus 1 mM pyrophosphate was greater (Fig. 3.4) than that produced in 0.1 to 0.3 M NaCl alone; (e) swelling of myofibrils in NaCl plus 1 mM pyrophosphate at NaCl concentrations above 0.3 M is

EXPERIMENT 1

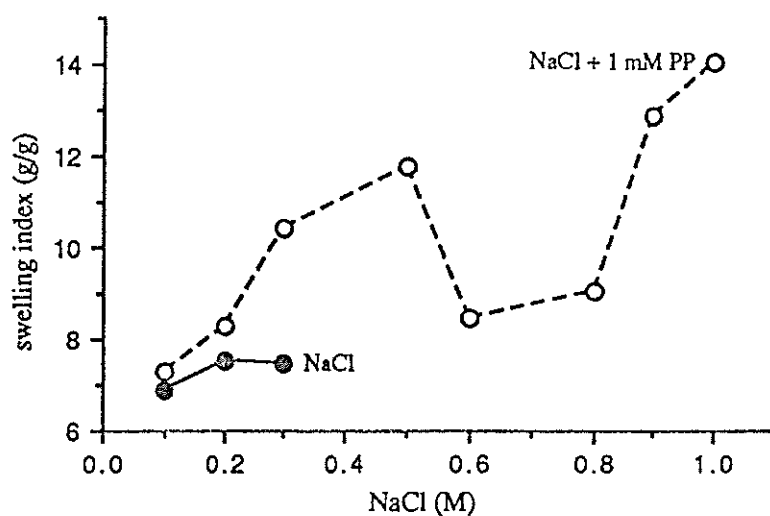


Fig. 3.4. Effect of NaCl and NaCl plus 1 mM pyrophosphate (PP) on swelling of myofibrils at pH 6.0 and a myofibrillar protein concentration of 12.5 mg.ml⁻¹. The incubation period was 200 min in NaCl alone, and 250 min in NaCl plus PP, followed by centrifugation at 3000 x g for 60 min.

suggested to be greatly depressed by the presence of pyrophosphate, otherwise it would have been equally impossible to pellet the myofibrils in the presence of pyrophosphate.

(ii) Experiment 2

Myofibrils in NaCl alone have been found to sediment at 9000 x g (Mitchell & Egelensall, 1989), and the confirmation that myofibrils would not swell at 3000 x g in NaCl alone in Experiment 1, led to the use of 9000 x g in Experiment 2. Besides this change in centrifugal speed and the reduction of the incubation period to 180 min, the same experimental conditions as in Experiment 1 were used, since the results obtained did not show the need for any other alteration. However, the concentration of myofibrillar protein in the myofibrillar suspension was of only 11.7 mg.ml⁻¹.

Microscopic images of systems after incubation (Fig. 3.5) showed myofibrils to have an intact structure up to a concentration of 0.3 M NaCl in NaCl alone. After that concentration images suggesting a disordered myofibrillar mass were observed. These images were more defined than in Experiment 1 and two different areas, A and B, could be seen in 0.4 M NaCl (see Fig. 3.5 - 0.4 M). In A, which only occurred in certain parts of the field, a striped pattern was seen indicating that B, which occupied most of the field, corresponded to a packed and structurally disorganised mass of myofibrils. In 0.4 M NaCl it was difficult but possible to find signs of myofibrils, which were not found after that salt concentration. In NaCl plus 1 mM pyrophosphate (Fig. 3.6) at a 0.3 M NaCl concentration images of extraction of the A-band were found along with images of myofibrils with intact sarcomeres. After that NaCl concentration, images with an appearance similar to area B in 0.4 M NaCl alone were observed.

Centrifuging the myofibrillar suspension at 9000 x g for 60 min in NaCl alone resulted in well sedimented pellets being observed at NaCl concentrations 0.1- 0.4 M and 0.9 - 1.0 M.

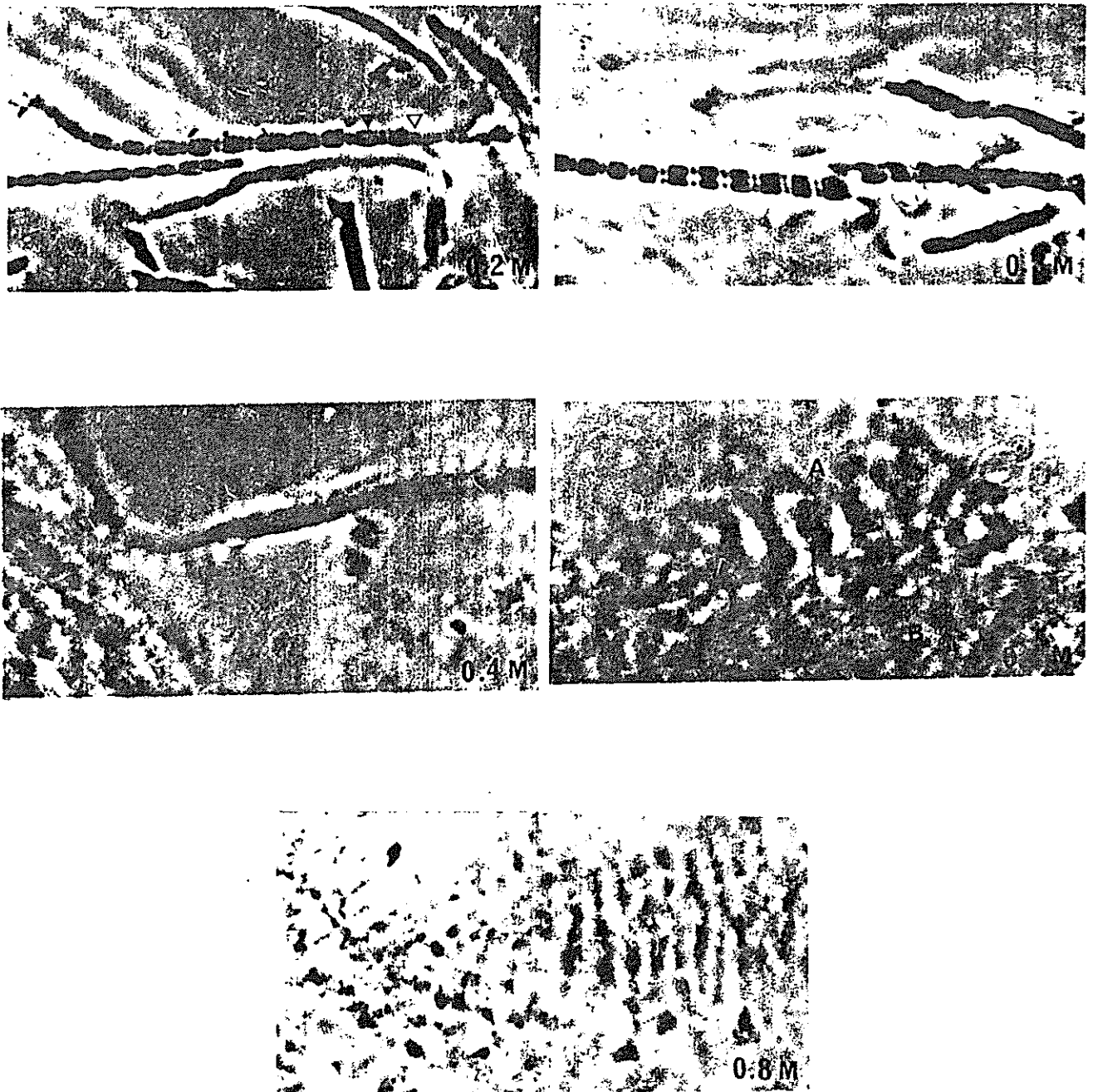


Fig. 3.5 Experiment 2. Phase-contrast light micrographs showing the effect of 0.2, 0.3, 0.4 and 0.8 M NaCl, 1 mM MgCl₂, 20 mM imidazole chloride at pH 6.0 on myofibrils in a myofibrillar suspension, with a myofibrillar protein concentration of 11.7 mg.ml⁻¹, after an incubation period of 180 min at 4°C. In 0.2 M: Δ intact Z-line and \blacktriangle intact A-band. In 0.4 M: A disorganised myofibrillar mass showing traces of a band pattern and B represents area showing disorganised mass of myofibrils. Magnification 1814 x.

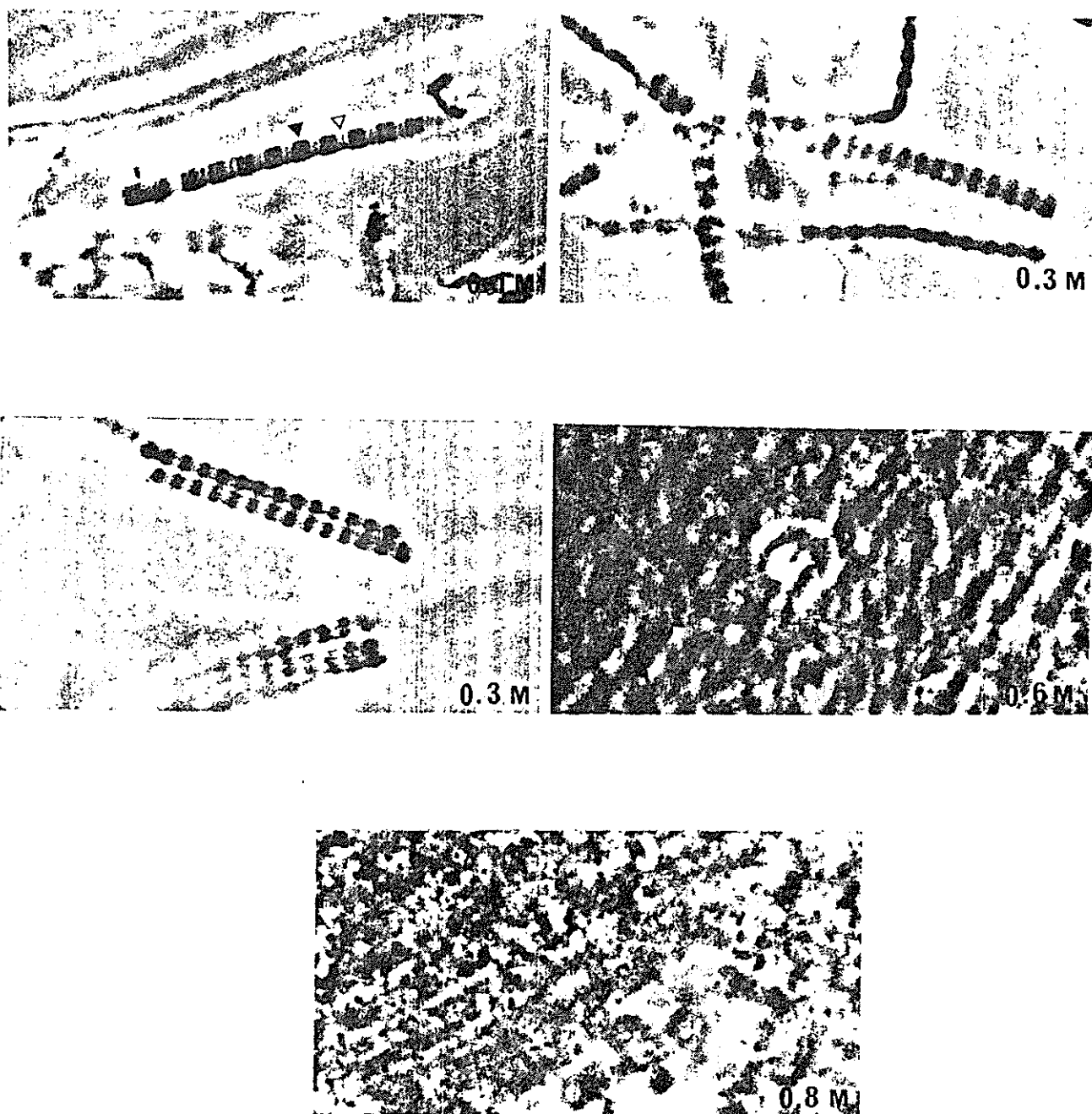


Fig. 3.6 Experiment 2. Phase-contrast light micrographs showing the effect of 0.1, 0.3, 0.6 and 0.8 M NaCl plus 1 mM pyrophosphate, 1 mM MgCl_2 , 20 mM imidazole chloride at pH 6.0 on myofibrils in a suspension, with a myofibrillar protein concentration of 11.7 mg.ml^{-1} , after an incubation period of 180 min at 4°C . In 0.1 M: Δ intact Z-line and \blacktriangle intact A-band. Magnification 1814 x.

Difficulties were still found in the packing of the pellet in the 0.5-0.8 M NaCl tubes since the pellets were still soft, very viscous, and lacking a firm cohesion which caused the suction used to remove the supernatants to drag parts of their mass. The results (Fig. 3.7 (a)) show that swelling did not increase from 0.1-0.3 M NaCl concentrations. At 0.4 M NaCl there was a sharp increase to a plateau up to 0.8 M NaCl. The fluctuation in the range 0.4 to 0.8 M is probably due to the difficulties experienced in recovering the supernatant. From 0.8 to 1.0 M there is a sharp decrease in pellet weight down to about half of the highest value registered in 0.4 M NaCl.

In the presence of 1 mM pyrophosphate little swelling was observed at any NaCl concentration and maximum swelling was registered in 0.4 M NaCl (Fig. 3.7 (a)). Above this concentration there is a sharp fall in pellet weight to a minimum at 0.6 M, which is maintained, with some fluctuation, in the 0.6 - 1.0 M interval, and corresponds to a shrinkage of the myofibrils, since pellet mass is lower than that initially observed at 0.1 - 0.3 M. The main conclusion from a comparison of these results (Fig. 3.7 (a)) with those obtained when NaCl is used alone show 1 mM pyrophosphate to depress swelling strongly at NaCl concentrations above 0.3 M. It may also be concluded that swelling was accompanied by a disorganisation of the myofibrillar structure.

Protein extraction (Fig. 3.7 (b)) increased in the range 0.1 - 0.8 M NaCl, in NaCl alone. There was a steep rise between 0.3 and 0.4 M NaCl, which like in swelling coincided with changes in the microscopic images of the myofibrillar systems. In the 0.9 and 1.0 M NaCl concentrations measurements of protein were not available due to light scattering. The values for protein extraction using NaCl plus 1 mM pyrophosphate were also seen to increase steeply in the 0.3 - 0.4 M NaCl concentration interval, which once more coincided with a change in the aspect of the myofibrils in suspension. From this concentration on the extraction values progressively increase to a maximum at 0.8 M NaCl, 1 mM pyrophosphate and falling at 0.9 M NaCl, 1 mM pyrophosphate. Scattering, once more, made the measurement of protein

EXPERIMENT 2

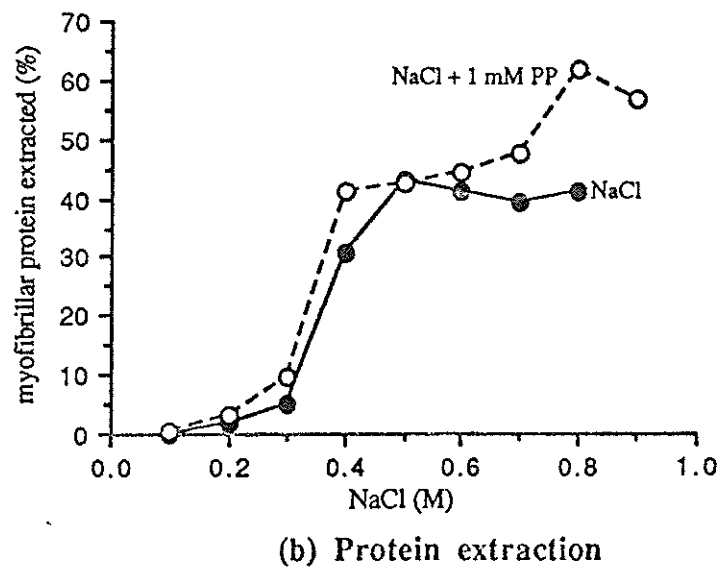
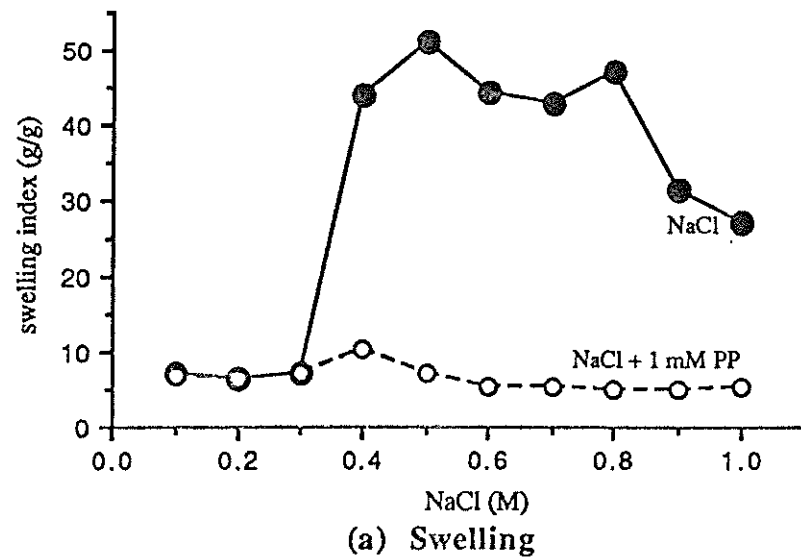


Fig. 3.7 Effect of NaCl and NaCl plus 1 mM pyrophosphate (PP) on (a) swelling of myofibrils and (b) extraction of protein at pH 6.0 and a myofibrillar protein concentration of 11.7 mg.ml^{-1} . The incubation period was 180 min and centrifugation was for 60 min at $9000 \times g$.

extraction in the 1.0 M NaCl concentration unavailable.

Plotting protein extraction against swelling (Fig. 3.8. (b)) shows the swelling index to have increased up to protein extraction values up to about 40% and to fall after that point in NaCl plus pyrophosphate. In NaCl alone (Fig. 3.8 (a)) the swelling index increased greatly when about 30% of the myofibrillar protein had been extracted and then slightly rose after about 40% had been extracted to a maximum, in 0.8 M NaCl. It is also seen that swelling in salt plus pyrophosphate at a level of protein extraction of also about 40% was lower than swelling at the same level of protein extraction in the presence of salt alone.

Two other conclusions may also be drawn: (1) by raising the centrifugal speed it is seen that in the 0.1 to 0.3 M NaCl concentration range, swelling in the presence of 1 mM pyrophosphate was no longer greater than in NaCl alone in the 0.1 to 0.3 M NaCl concentration range - this observation may have been caused by a decrease in interfibrillar spacing which is observed when pressure is exerted upon myofibrils (Millman, 1981) or by better packing - and the hint of an effect as that observed by Bendall (1954), seen before in Experiment 1, may have been depressed by the higher centrifugal speed; (2) the rise in pellet weight in 0.8-0.9 M NaCl observed in the presence of 1 mM pyrophosphate in Experiment 1 is no longer observed here, probably for the same reasons stated above; (3) like in Experiment 1 an increase in swelling and protein extraction were found to accompany images of a disorganisation of the myofibrillar structure in both NaCl alone and NaCl plus 1 mM pyrophosphate.

(iii) Experiment 3

This experiment was carried out to confirm the differences that existed between Experiments 1 and 2 and to confirm the results of Experiment 2. It was confirmed that 1 mM pyrophosphate in association with NaCl did not at any NaCl concentration promote greater swelling than

EXPERIMENT 2

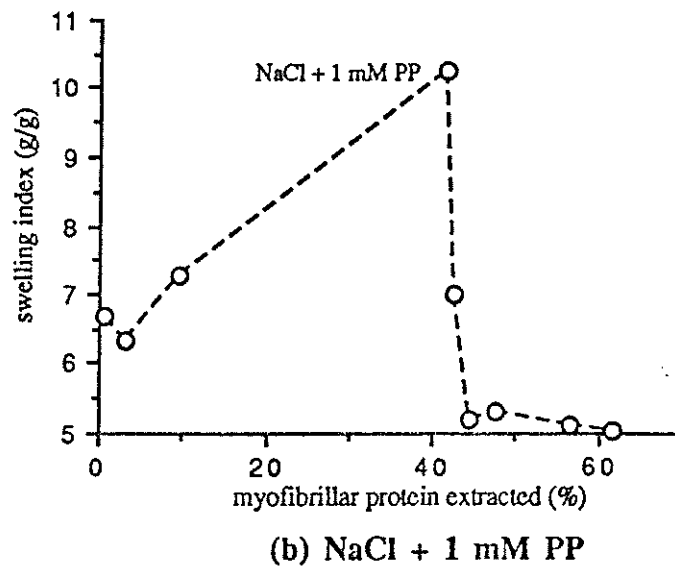
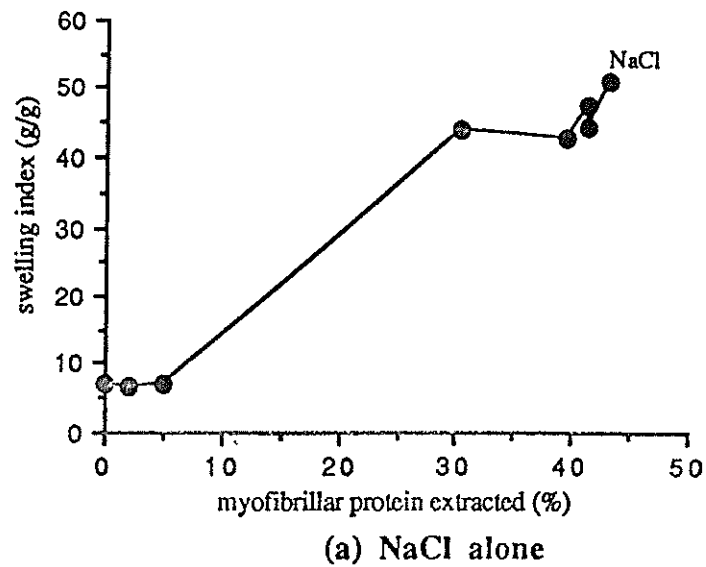


Fig. 3.8. Effect of protein extraction on swelling of myofibrils treated (a) with NaCl alone and (b) with NaCl plus 1mM pyrophosphate (PP). The incubation period was 180 min at $\sim 4^{\circ}\text{C}$ and centrifugation was for 60 min at $9000 \times g$. The initial myofibrillar protein concentration was 11.7 mg.ml^{-1} .

NaCl alone. The experimental conditions used in this trial were a repetition of those used in the previous experiment, the difference between the two lying in the lower myofibrillar protein concentration (8.5 mg.ml^{-1}) and the higher total volume (20 ml) of the systems used in the present experiment. These changes had the advantage that pellets packed better. The scattering noticed in Experiment 1 was avoided in this experiment by the use of SDS to dissolve the supernatant volume used to measure protein extraction. However, even though the tips of the micropipettes that were used to transfer the supernatants to the volumetric flasks where they were mixed with SDS, were repeatedly washed in the SDS the supernatants were to be dissolved, the viscosity of the supernatants made them form a layer in the walls of the micropipette tips. This will have introduced errors in the measurements made.

In NaCl alone at a concentration of 0.1 M NaCl myofibrils with intact sarcomeres were observed, but images showing a densely packed, disorganised mass of myofibrils were also found (Fig. 3.9). However, these images looked denser than similar images at higher NaCl concentrations identified as type B areas in this experiment and Experiment 2. This also supports the suggestion made in Experiment 1 that compression of myofibrils between a cover slip and a slide was partially responsible for the images which suggested that the structure of myofibrils was crushed. At a NaCl concentration of 0.4 M fields with myofibrils with intact sarcomeres and B type areas, as those found in 0.4 M NaCl alone in Experiment 2, were observed. After that NaCl concentration no myofibrils could be found and A and B type areas similar to those found in Experiment 2 were observed.

In NaCl plus pyrophosphate (Fig. 3.10) at a NaCl concentration of 0.2 M NaCl, B type areas and myofibrils with intact sarcomeres could be found in the same microscopic fields. This suggests once more, as in NaCl alone that compression of myofibrils did produce in Experiments 1 and 2 the images of disordered myofibrillar structures even at concentrations where extraction of the A-band or weakening of the Z-lines have not been reported under these same ionic conditions (see Offer & Trinick, 1983, section 1.8). At 0.4 M NaCl myofibrils

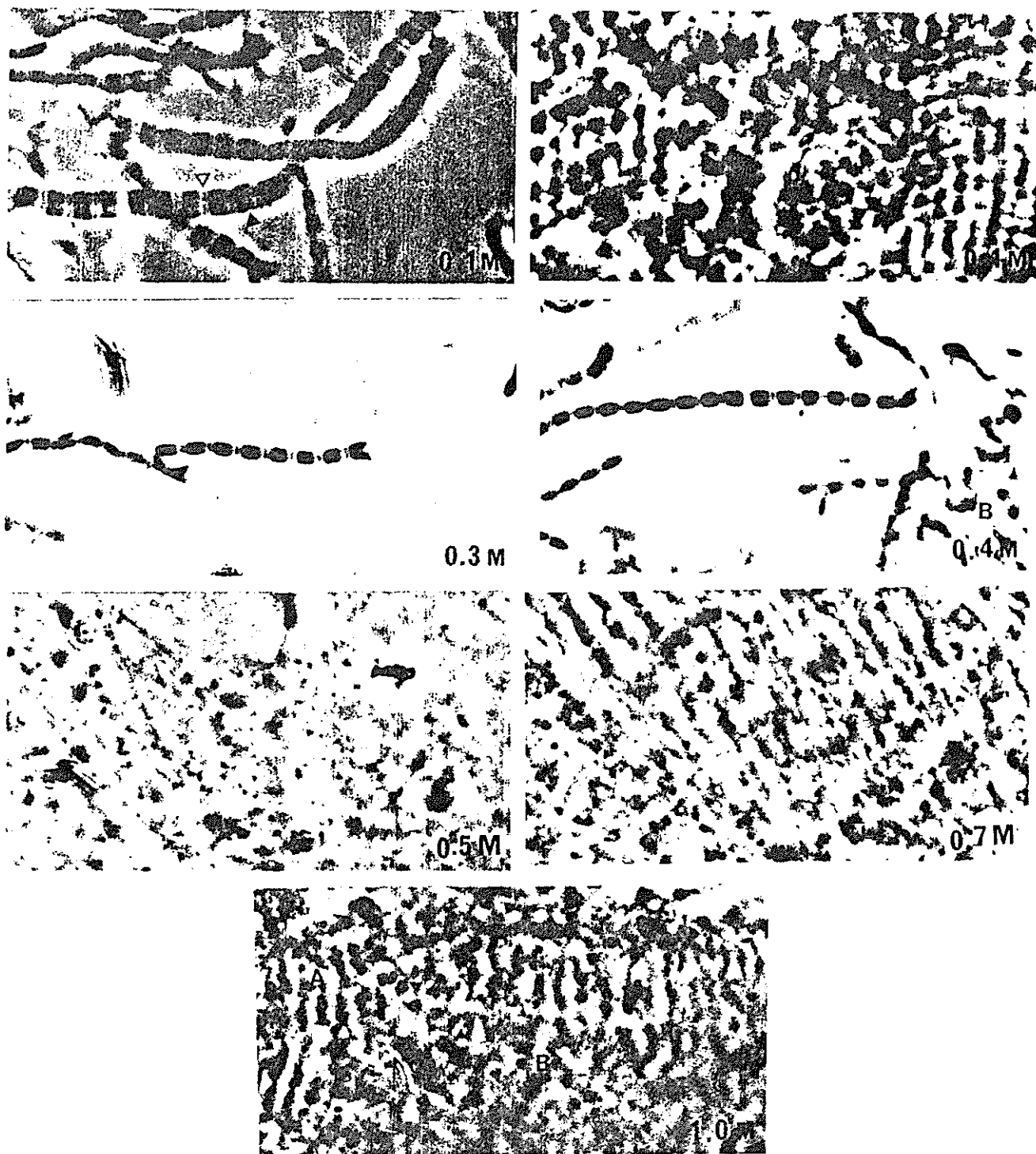


Fig. 3.9. Experiment 3. Phase-contrast light micrographs showing the effect of 0.1, 0.3, 0.4, 0.5, 0.7 or 1.0 M NaCl and 1 mM MgCl_2 , 20 mM imidazole chloride at pH 6.0 on the structure of myofibrils in a suspension with a myofibrillar protein concentration of 8.5 mg.ml, after an incubation period of 180 min at 4°C. In 0.1: Δ intact Z-line, \blacktriangle intact A-band. In 0.4 and 1.0 M: B represents area showing a packed mass of myofibrils with a disorganised structure. In 1.0: A represents area showing mass of myofibrils showing a disorganised structure but where traces of a band pattern were observed. Magnification 1814 x.

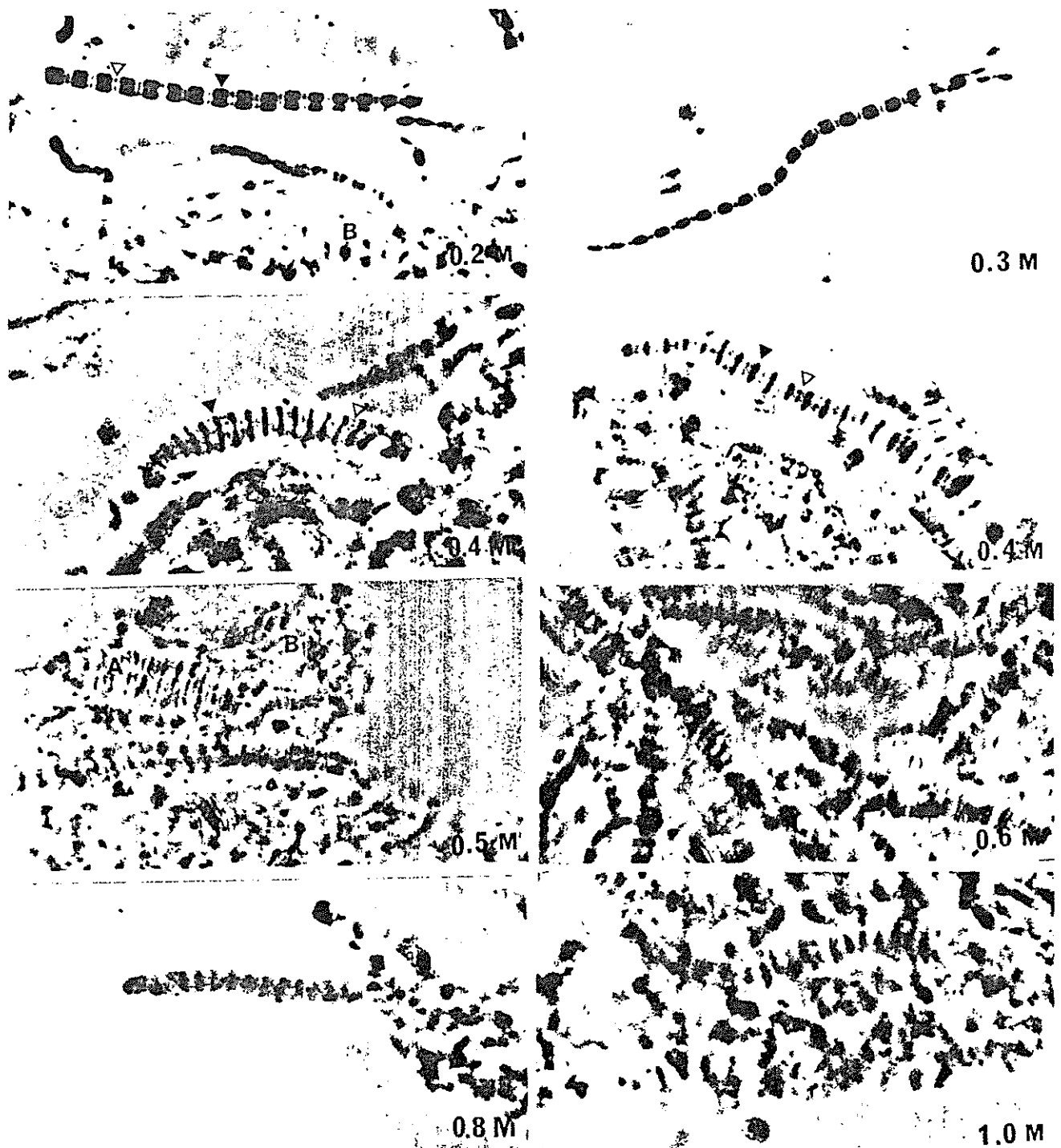


Fig. 3.10 Experiment 3. Phase-contrast light micrograph showing the effect of 0.2, 0.3, 0.4, 0.5, 0.6, 0.8 and 1.0 M NaCl plus 1 mM pyrophosphate, 1 mM MgCl_2 , 20 mM imidazole chloride at pH 6.0 on myofibrils in a suspension, with a myofibrillar protein concentration of 8.5 mg.ml^{-1} , after an incubation period of 180 min at 4°C. In 0.2 M: Δ intact Z-line, \blacktriangle intact A-band B area showing disordered myofibrillar structures. In 0.4 M: Δ traces of Z-line and \blacktriangle suggestion of A-band which having been extracted from its ends. In 0.5 M: A traces of band pattern and B mass of myofibrils in disordered state. Magnification 1814 x.

where the A-band had been extracted from its ends and the Z-line had become fainter were observed along with B type areas similar to those observed in Experiment 4 and in this experiment in NaCl alone at the same NaCl concentration. In 0.5 M NaCl A type areas suggesting that the A-band had been extracted from its ends and that Z-lines had become weaker could be observed along with B type areas. After that NaCl concentration only A and B type areas were the main feature observed but myofibrils with disorganised sarcomeres like the one in Fig. 3.10 - 0.8 M were also seen. The defined images of changes in structure of myofibrils treated with NaCl plus pyrophosphate that were found in this experiment may have been possible because a lower myofibrillar concentration was used in this experiment, making it possible to isolate individual myofibrils.

If the swelling index for NaCl alone is studied (Fig. 3.11 (a)), it is seen that the myofibrillar pellet increased in weight even at the lowest salt concentrations and rose to a maximum in the 0.7 M NaCl concentration before falling. In 0.4 M NaCl a first increase in the swelling index was observed to accompany the signs of a disorganisation of the structure of the myofibrils. In the presence of 1 mM pyrophosphate a peak value for swelling was registered, once more, at 0.4 M NaCl but swelling was greatly depressed by pyrophosphate when compared with swelling in NaCl alone. In the presence of pyrophosphate the peak in swelling at a NaCl concentration of 0.4 M is seen to have accompanied by an extraction of the A-band from its ends as found in other experimental work (Offer & Trinick, 1983; Parsons & Knight, 1990).

In NaCl alone (Fig. 3.11 (b)) protein extraction rose at 0.4 M to a value representing about 17 % of the initial protein concentration present in the system, this value was almost doubled in increasing the NaCl concentration to 0.5 M. From then on it increased progressively to a maximum of 61% in 1.0 M NaCl. In NaCl plus 1 mM pyrophosphate myofibrillar protein extraction increased steeply between the 0.3 and 0.4 M concentrations, reaching a maximum of about 70 % of the myofibrillar protein at 0.8 M, and is kept near that value in the next concentrations of salt. In both NaCl alone and NaCl plus pyrophosphate a rise in protein

EXPERIMENT 3

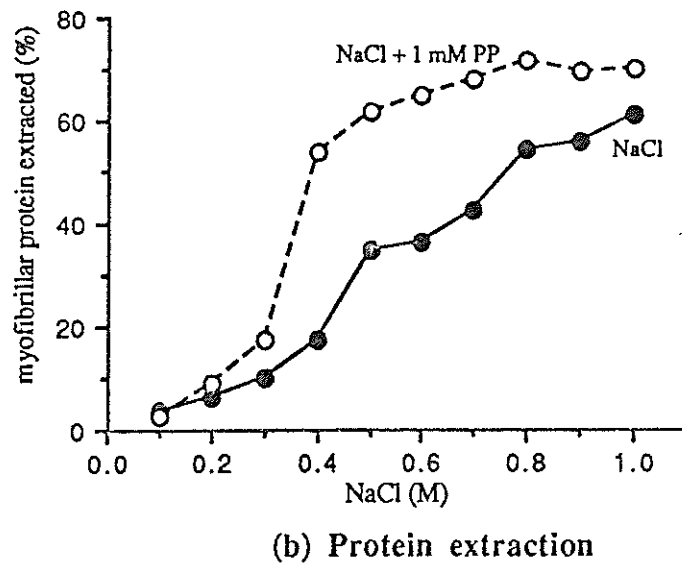
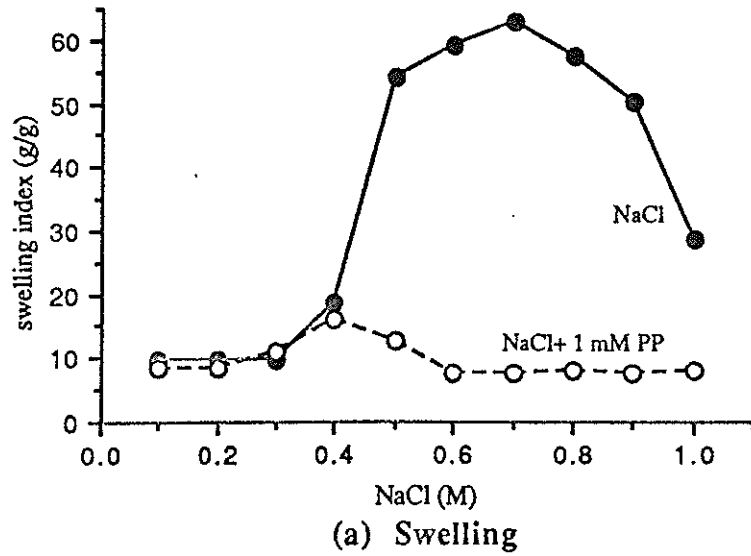


Fig. 3.11. Effect of NaCl and NaCl plus 1 mM pyrophosphate (PP) on (a) swelling of myofibrils and (b) extraction of protein at pH 6.0 and a myofibrillar protein concentration of 11.7 mg.ml^{-1} . The incubation period was 180 min and centrifugation was for 60 min at $9000 \times g$.

extraction is observed to have accompanied the changes in the structure of the myofibrils.

The relationship between protein solubilisation and swelling (Fig. 3.12 (a) & (b)) in NaCl alone was similar to that in Experiment 2 up to 0.7 M NaCl where 40% of the protein was extracted, but swelling decreased after that level of extraction of myofibrillar protein. A difference was found between swelling in the presence of pyrophosphate in Experiments 2 and 3. In Experiment 3 swelling only fell after 50% of the myofibrillar protein had been extracted, while in the previous experiment swelling fell earlier at an extraction of about 40% of the myofibrillar protein. The disorganisation of the myofibrillar structure in NaCl alone and an extraction of the A-band from its ends in NaCl plus pyrophosphate are indicated to be related both with swelling and protein extraction.

3.1.3. Swelling, protein solubilisation and changes in structure of myofibrils treated with NaCl or NaCl plus 10 mM pyrophosphate

The concentration of polyphosphate used is critical in promoting both the depolymerisation of the thick filament and the dissociation of actomyosin. From Experiments 2 and 3 it was concluded that 1 mM pyrophosphate associated with 0.4 M NaCl, under the conditions used did not produce greater swelling than NaCl alone at the same NaCl concentration. This contrasted with the results of Offer & Trinick (1983) in which myofibrils swelled more in 0.4 M NaCl plus 10 mM pyrophosphate than in 0.4 M NaCl alone. The pyrophosphate concentration was therefore raised to 10 mM maintaining the other reagents in the same concentration as before. In this experiment the myofibrillar protein concentration in the incubations was 4 mg.ml⁻¹.

The images of the myofibrils after the incubation period are shown in Figs. 3.13 & 3.14. Even though focussing of the images was not possible it was observed that up to a NaCl concentration of 0.4 M no change was found in the structure of myofibrils in NaCl alone. At

EXERIMENT 3

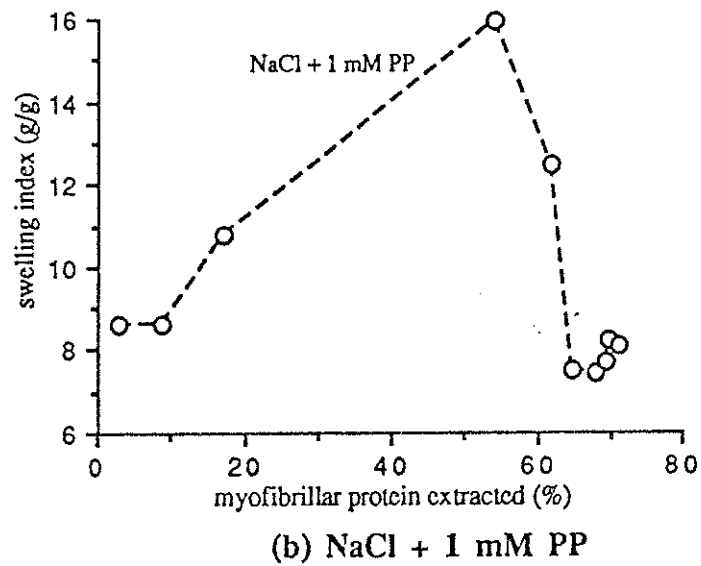
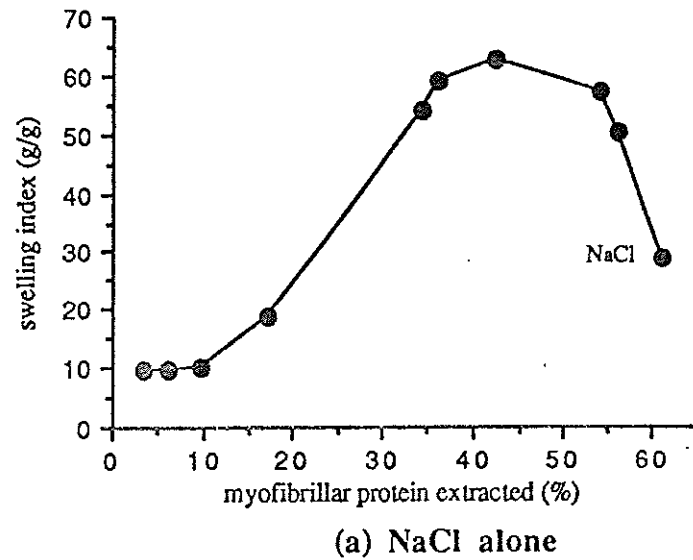


Fig. 3.12. Effect of protein extraction on swelling of myofibrils treated (a) with NaCl alone and (b) with NaCl plus 1 mM pyrophosphate (PP). The incubation period was 180 min at $\sim 4^{\circ}\text{C}$ and centrifugation was for 60 min at $9000 \times g$. The initial myofibrillar protein concentration was 8.5 mg.ml^{-1} .

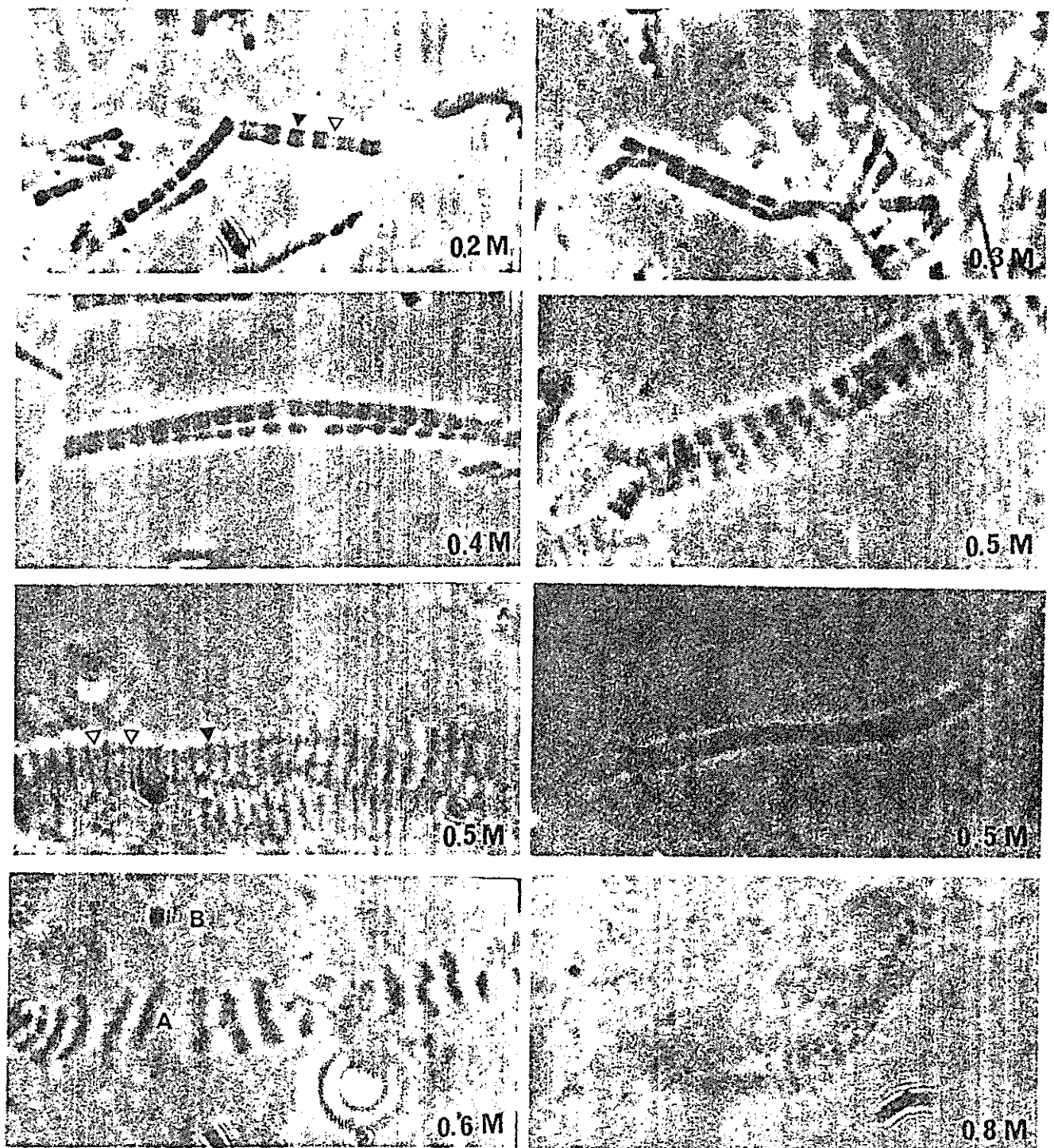


Fig. 3.13 Experiment 4. Phase-contrast light micrographs showing the effect of 0.2, 0.3, 0.4, 0.5, 0.6 and 0.8 M NaCl, 1 mM MgCl₂, 20 mM imidazole chloride at pH 6.0 on myofibrils in a suspension, with a myofibrillar protein concentration of 4.1 mg.ml⁻¹, after an incubation period of 180 min at 4°C. In 0.2 M: Δ intact Z-line and \blacktriangle intact A-band. In 0.5 M: possible traces of A-band (\blacktriangle) and Z-line (Δ). In 0.6 M: A area suggesting traces of band pattern and B area similar to areas B in other experiments. Magnification 1814 x.

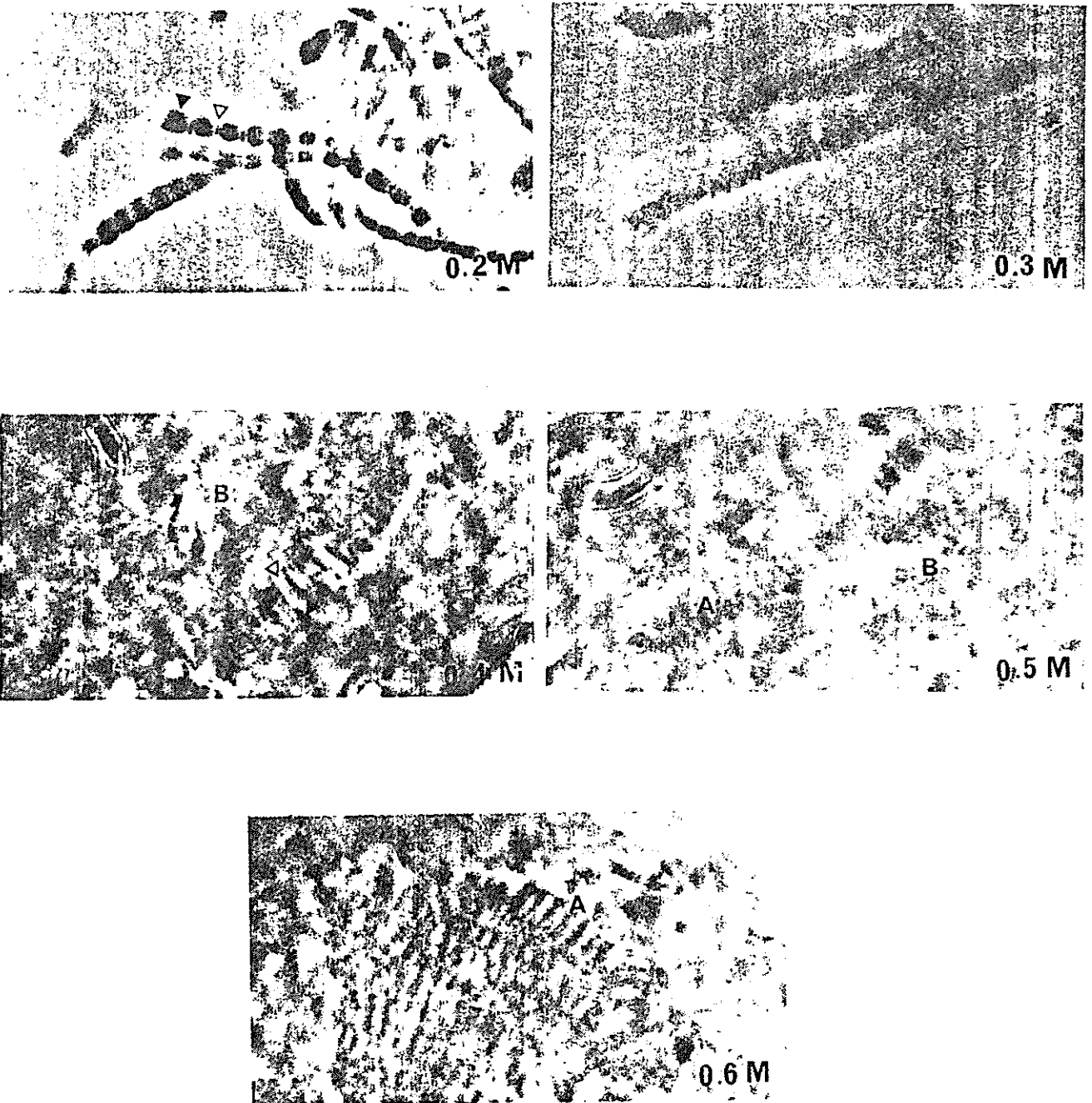


Fig. 3.14 Experiment 4. Phase-contrast light micrographs showing the effect of 0.2, 0.3, 0.4, 0.5 and 0.6 M NaCl, 10 mM pyrophosphate, 1 mM MgCl_2 , 20 mM imidazole chloride at pH 6.0 on myofibrils in a suspension, with a myofibrillar protein concentration of 4.1 mg.ml^{-1} , after an incubation period of 180 min at 4°C . In 0.2 M: Δ intact Z-line and \blacktriangle intact A-band. In 0.4 M: possible traces of A-band (\blacktriangle) and Z-line (Δ), and disordered mass of myofibrils. In 0.5 M: A area showing traces of band pattern and B disordered mass of myofibrils. In 0.6 M: A as in 0.5 M. Magnification 1814 x.

0.5 M NaCl however, faint images of extraction of the A-band were found. In some myofibrils the traces of the A-band were thicker (Fig. 3.13 - 0.5 M (a)) than in others (Fig. 3.13 - 0.5 M (b)) where the type of extraction seemed to be similar to that occurring in NaCl plus 1 mM pyrophosphate in 0.4 M NaCl in Experiment 3. From and including a concentration of 0.6 M NaCl, A and B type images were observed.

In NaCl plus 10 mM pyrophosphate, unlike in all previous experiments, images of a disorganised myofibrillar structure suggesting the extraction of the A-band were already visible in 0.3 M NaCl like in Experiment 2. At a NaCl concentration of 0.4 M faint images suggesting the extraction of the A-band from its ends were observed surrounded by a B type background. From and including 0.5 M NaCl on only A and B type areas were found in the microscopic fields.

Swelling (Fig. 3.15 (a)) was highest in 0.6 M NaCl alone and in 0.3 M NaCl when 10 mM pyrophosphate were present. In NaCl alone a sharp rise in swelling occurred between 0.4 and 0.5 M, with swelling having about the same value in the first three salt concentrations and increasing slightly in 0.4 M. With 10 mM pyrophosphate present, swelling values are about 28% lower than those in salt alone in the 0.1 and 0.2 M NaCl concentrations, with a maximum value, 32% higher than the same value in salt alone, being found in 0.3 M NaCl. From this concentration on, swelling steadily decreases becoming uniform and slightly above that found in 0.1 and 0.2 M NaCl from 0.6 - 1 M NaCl. Thus, at NaCl concentrations above 0.4 M 10 mM pyrophosphate caused a marked inhibition of swelling.

In NaCl alone myofibrillar protein extraction (Fig. 3.15 (b)) was low up to and including 0.4 M NaCl but, sharply increased in 0.5 M, after which it rose in a progressive manner up to 1.0 M where about 88% of the protein initially present in the system was extracted. The extraction of protein rose first from 0.1 to 0.2 M NaCl and then from 0.3 to 0.4 M NaCl. Above this concentration, it rose progressively to a maximum of 4.0 mg.ml⁻¹ in 1.0 M NaCl, which

EXPERIMENT 4

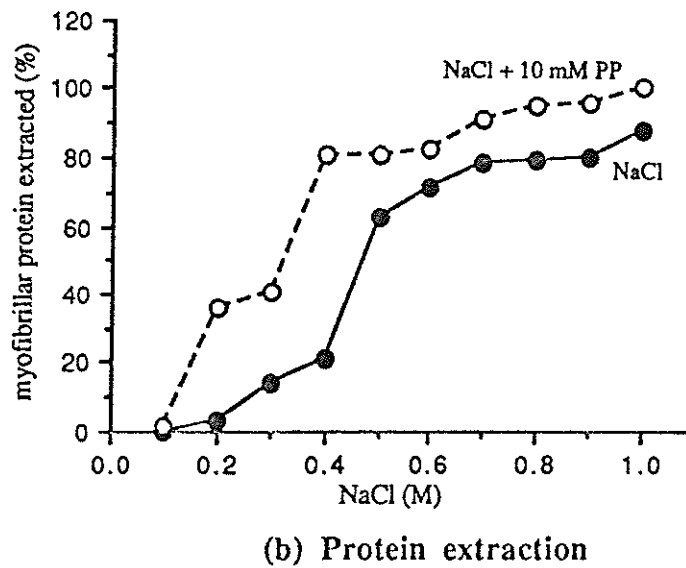
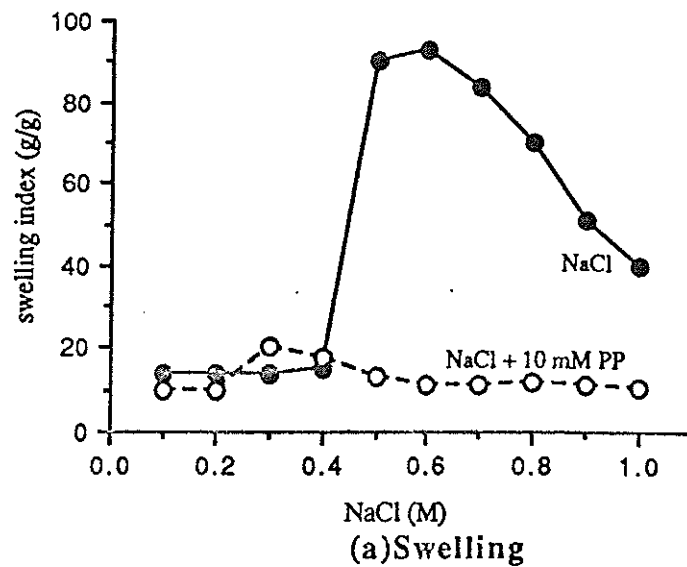


Fig. 3.15. Effect of NaCl and NaCl plus 10 mM pyrophosphate (PP) on (a) swelling of myofibrils and (b) extraction of protein, at pH 6.0 and a myofibrillar protein concentration of 4.1 mg.ml^{-1} . The incubation period was 180 min and centrifugation was for 60 min at $9000 \times g$.

represents about 100% of the protein of the myofibril being extracted. Too much reliance should not be placed on this figure since the extinction coefficient of myofibrillar protein was used to calculate the protein concentration in the supernatants but myosin was probably the main protein extracted (Bendall, 1954; Jolley & Savage, 1985). There was also a deficiency in the technique used to measure protein extraction as found in Experiment 3.

The relationship between swelling and protein extraction is shown in Fig. 3.16 (a) & (b) and is seen not to have differed from that observed in the previous experiments, except for the percentage of protein that was extracted before swelling was depressed in NaCl alone. In Experiment 2 swelling was maintained at its maximum level when about 40% of the myofibrillar protein had been extracted and in Experiment 3 swelling decreased after that level. While in Experiment 4 when 40% of the myofibrillar protein was extracted swelling was still rising and only fell after 71% of the protein was extracted.

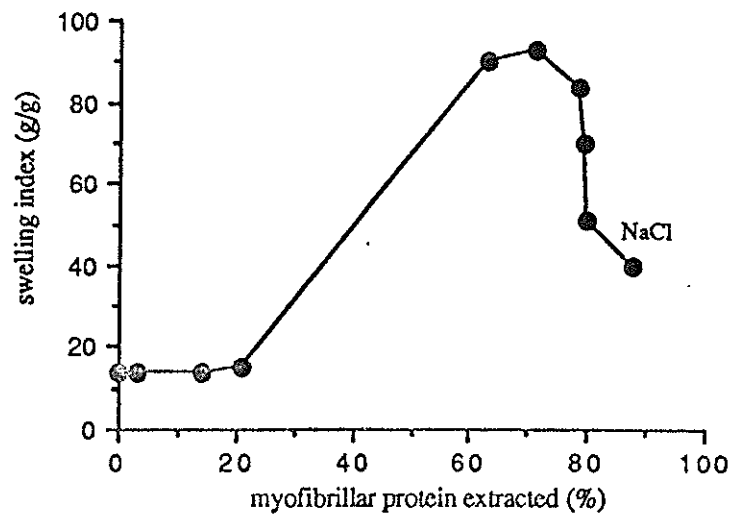
The values of the pH of the supernatants in both salt and salt plus pyrophosphate are shown in Table 3.3. In NaCl the deviations from 6.0 were small but with pyrophosphate there was a consistent trend towards higher pH values as the NaCl concentration increased.

The results of this experiment show that at NaCl concentrations greater than 0.4 M the presence of 10 mM pyrophosphate did not produce more swelling than in salt alone, on the contrary it strongly depressed it. Maximum swelling in the presence of NaCl plus pyrophosphate, however, occurred in 0.3 M NaCl where it was, as above indicated (Fig. 3.15 (a)), noticeably (48%) higher than in salt alone. This fact was not observed in the presence of salt plus 1 mM pyrophosphate in Experiments 2 and 3 where maximum swelling only occurred in 0.4 M NaCl and was at that point lower than in NaCl alone. Its occurrence indicates that there is a certain range of salt concentrations at which the presence of a certain concentration of pyrophosphate may induce greater swelling than that observed in salt alone. Furthermore it is seen that using 0.3 M (1.8%) NaCl associated with 10 mM (0.22%) pyrophosphate on

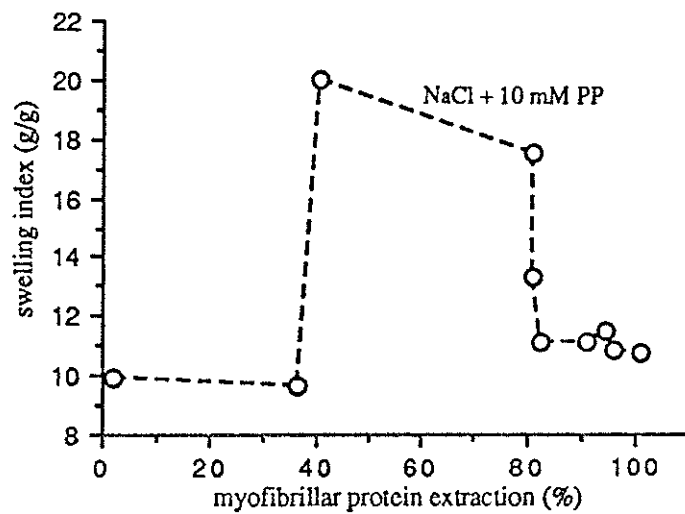
Table 3.3. Experiment 4. pH of supernatants at 5°C in NaCl and NaCl plus 10 mM pyrophosphate (PP)

NaCl (M)	pH	
	NaCl	NaCl + PP
0.1	6.00	5.91
0.2	5.93	5.91
0.3	6.02	5.91
0.4	6.01	5.92
0.5	6.02	5.96
0.6	5.99	5.98
0.7	5.97	6.01
0.8	5.98	6.04
0.9	5.97	6.11
1.0	5.97	6.11

EXPERIMENT 4



(a) NaCl alone



(b) NaCl + 10 mM PP

Fig. 3.16. Effect of protein extraction on swelling of myofibrils treated (a) with NaCl alone and (b) with NaCl plus 10 mM pyrophosphate (PP). The incubation period was 180 min at $\sim 4^{\circ}\text{C}$ and centrifugation was for 60 min at $9000 \times g$. The initial myofibrillar protein concentration was 4.1 mg.ml^{-1} .

myofibrils at pH 6.0 has an effect very similar to that produced by 0.17 M (1%) NaCl plus 11.3 mM (0.25%) pyrophosphate on mince at pH 5.7 (Bendall, 1954), where swelling in the presence of 1% NaCl alone was 31% less. It is also interesting to notice that images of disorganised structures of myofibrils and extraction of the A-band, in both NaCl alone and in NaCl plus pyrophosphate, were closely followed by changes in swelling and protein extraction.

3.2. Experiments with mince

3.2.1. Swelling and protein solubilisation in mince treated with 1% NaCl or 1% NaCl plus 0.25 or 0.5% pyrophosphate

The above established similarity in the effect of 0.3 M NaCl, 10 mM pyrophosphate, 1 mM $MgCl_2$, 20 mM imidazole chloride, at pH 6.0 on swelling of myofibrils pellet and that observed by Bendall (1954) on mince led to a repetition of the experimental approach used by that author to confirm whether those results were reproducible. Mince from back and hind-leg rabbit muscles refrigerated for 24 h at 0°C was used in this repetition, on a smaller scale, of the Bendall (1954) experiment, not only in terms of the volume of the total system, but also in the number of samples used in each treatment. A known weight of mince was treated with salt alone or salt plus two different pyrophosphate concentrations, and controls were established by treating mince with distilled water. After incubation, centrifugation and removal of the supernatant, swelling was measured by the amount of brine the mince pellet had taken up and protein in the supernatant was used as a measure of the amount of myofibrillar protein that had been extracted. The pH of the supernatants was also measured.

The measurement of the pH of the supernatants was made at 5 and 20°C with the intention not

only of checking if any changes had occurred during incubation, but also to find out if the pH values measured differed from the average value (5.7) measured by Bendall (1954). The pH of the supernatants (Table 3.4) at 5 and 20°C was higher in all treatments than in the experiments made by Bendall (1954). The smallest difference found was in 1% NaCl, at 20°C, where there was a 0.26 difference in pH value between this experiment and the experiments of Bendall (1954). No difference between the pH of the different treatments, measured at both temperatures, was found that might influence the result of the action of the additives used in Experiment 5.

Swelling results are shown in Fig. 3.17 (a) calculated using a swelling index calculated as in the myofibrillar experiments and in Fig. 3.17 (b) compared with those of Bendall (1954) using the method he used. The results of swelling expressed in the second way showed that addition of water to the mince provoked an average increase of 5.6% (SD 4.0%) in its volume. A concentration of 1% NaCl increased the volume of the raw meat, on average, by 21% (SD 5.1%). The association of 0.25% pyrophosphate and 1% NaCl produced a 48% (SD 3.3%) average increase in the pellet volume, and 0.5% pyrophosphate plus 1% NaCl increased this value to 59.5% (SD 2.9%). These results are very similar to those obtained by Bendall (1954): water - 7%; 1% NaCl - 20%; 1% NaCl, 0.25% pyrophosphate - 51%; 1% NaCl, 0.5% pyrophosphate - 64%. If swelling is calculated using the same method that was used in the myofibrillar experiments it is seen that in 0.17 M (1%) NaCl plus 11.3 mM (0.25%) pyrophosphate the swelling index was 66% greater than the swelling index of myofibrils in 0.2 M NaCl plus 10 mM pyrophosphate (Fig. 3.15 (a)). This may indicate that the 1.3 mM pyrophosphate difference between the two systems was responsible for this greater swelling in mince, that there was less protein extracted from the mince than from myofibrils or that endogenous ions influenced the results. If the difference in protein extraction was responsible for this difference in swelling, that would confirm the suggestion that swelling is dependant on the amount of myofibrillar protein that is present in meat (Offer

Table 3.4. Experiment 5. pH, at (a) 5 and (b) 20°C , of supernatants from mince treated with water, 1%NaCl and 1%NaCl plus 0.2 and 0.5% pyrophosphate

tube	treatment							
	water		1% NaCl		1% NaCl + 0.25% PP		1% NaCl + 0.5% PP	
	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)
1	6.08	5.95	6.08	6.04	6.18	6.07	6.11	5.90
2	6.16	6.01	6.03	5.93	6.16	6.09	6.11	5.90
3	6.16	5.99	6.05	5.94	6.20	6.17	6.13	6.00
4	6.08	6.00	6.08	5.94	6.21	6.14	6.12	6.00
5	6.13	5.91	6.09	5.96	6.21	6.11	6.11	5.97
mean	6.12	5.97	6.07	5.96	6.19	6.12	6.12	5.98
SD	0.04	0.04	0.02	0.04	0.02	0.04	0.01	0.02

EXPERIMENT 5

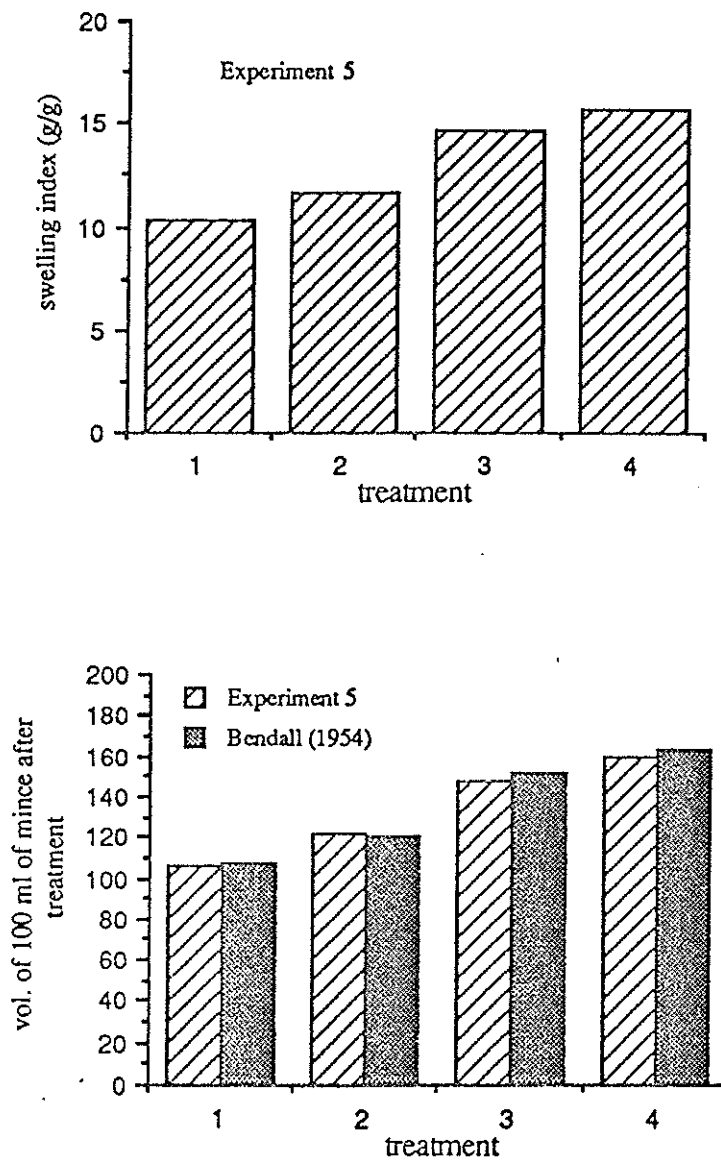


Fig. 3.17. Swelling of mince using the method used by Bendall (1954).

Treatments: (1) effect of distilled water

(2). " of 1% NaCl

(3). " of 1% NaCl + 0.25% pyrophosphate

(4). " of 1% NaCl + 0.5% pyrophosphate

- (a) swelling in Experiment 5 calculated through a swelling index, as in experiments with myofibrils (b) results of swelling in Experiment 5 calculated according to Bendall (1954) and comparison between results of that author and Experiment 5. The incubation period was 24 h and centrifugation was for 30 min at 1200 x g.

& Knight, 1988). Unfortunately the results of protein extraction in this experiment were not reliable for the reasons stated ahead and the difference in centrifugal speed used in the two experiments - 9000 x g in myofibrils and 1200 x g in mince - do not permit a reliable comparison.

The protein concentration in the supernatants of the 1% NaCl, 0.5% pyrophosphate treatment (calculated from the absorbance at 280 nm) was, on average, 6.5 g.100 ml⁻¹. In the Bendall (1954) experiments, calculated from the increment of the refractive index of the supernatants, this value was found to be 5.5 g.100 ml⁻¹. The measurement of protein extraction, however, cannot be correct since the calculated values for myofibrillar protein present in the supernatants largely exceeded the initial amount of protein calculated to be there. Not only because sarcoplasmic was also present in the supernatants but, also due to the fact (as referred in Experiment 4) that the myofibrillar protein extinction coefficient value was used, and predictably myosin was the myofibrillar protein that was solubilised in greater quantities, which having a lower extinction coefficient will have affected the calculation of myofibrillar protein by excess. The presence of nucleotides, which were shown to be present by the higher values of the absorbance values at 260 nm than at 280 nm, also make the protein measurements unreliable. The pH of the supernatants was measured at 5 and 20°C and the results shown in Table 3.4. do not indicate its variation, in any treatment, and when compared between treatments, to be enough to influence the action of the additives.

The mixtures in the different tubes were observed during and at the end of the incubation period. The contents in the NaCl and NaCl plus the 0.25% pyrophosphate concentration were firm when the tubes were slightly agitated or inclined. The contents of the tubes where 1% NaCl, 0.5% pyrophosphate was present were submitted to the same mechanical actions and appeared to be very viscous and lacking coherence, sliding on the walls of the tubes. After centrifugation and removal of the supernatants, attempts to remove the minced pellets

from the centrifuge tubes showed the pellets in the NaCl and NaCl plus 0.25% pyrophosphate to be firmer and more difficult to remove than those in NaCl plus 0.5% pyrophosphate. Even after removal the firmer pellets where 0.25% pyrophosphate was present still kept their form and were difficult to break, while the ones in which 0.5% pyrophosphate was present lost their form and were very viscous.

It is concluded that the results were very similar to those of Bendall (1954) and that 0.25 and 0.5% pyrophosphate at a 1% NaCl concentration does enhance water uptake and protein solubilisation.

3.2.2. Swelling and protein solubilisation in washed mince treated with NaCl or NaCl plus 10 mM pyrophosphate

The confirmation of the Bendall (1954) results reported above led to this experiment under controlled conditions, so that the enhancement of water uptake and protein solubilisation could only be attributed to the presence of polyphosphates as in the myofibrillar experiments and endogenous ions, nucleotides or sarcoplasmic proteins would not be interfering factors. Even though (1) the physical state of the mince would still influence the penetration of salts and the diffusion of protein out of the meat would be slowed; (2) water and protein could still be held in between the mince particles and (3) the myofibrillar protein concentration would be much higher. At the same time the wider range of NaCl concentrations, as used in the myofibrillar experiments, would permit to confirm that the reason why polyphosphates did not - in the experimental work done by various authors - produce greater swelling and protein solubilisation than NaCl alone, was because this effect is only observed under certain ionic conditions which they failed to study. The same steps as in the myofibrillar experiments were followed except that muscle was used in the form of mince which was washed twice in 0.1 M NaCl, 1 mM MgCl₂, 20 mM imidazole chloride, pH 6.0. Absorbancy of the supernatants

measured after the first wash revealed the presence of nucleotides which were not present in the supernatant after the second wash.

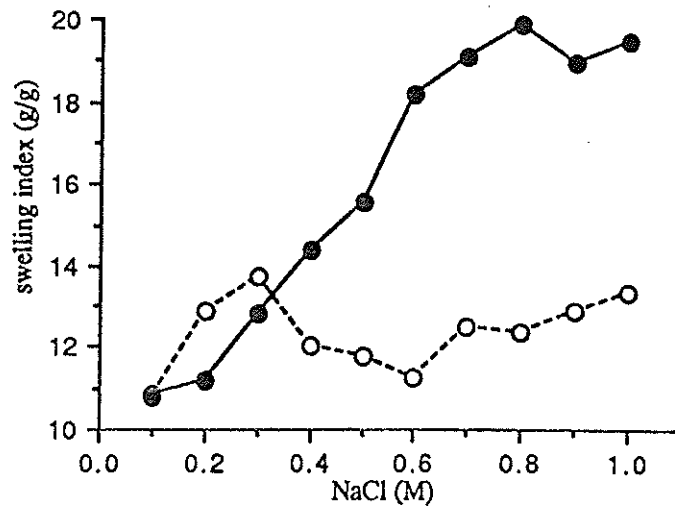
The pH of the supernatants (Table 3.5) after treatment with NaCl or NaCl plus pyrophosphate did not show any significant change to have occurred that could alter the results of swelling and protein extraction. In NaCl alone, it is seen (Fig. 3.18 (a)) that the swelling index rose in successive steps from 0.2 M NaCl up to a maximum in 0.8 M of NaCl, decreasing slightly in 0.9 and 1.0 M NaCl. The supernatant volumes were very small at NaCl concentrations of 0.7 M and above, and swelling results in those salt concentrations were probably underestimated by the insufficient volume of brine available to be taken up by the mince in the 1:1 brine-to-mince volume ratio used. In the presence of 10 mM pyrophosphate, the swelling index (Fig. 3.18 (a)) showed a peak value at a concentration of 0.3 M NaCl, falling to a minimum in 0.6 M NaCl and rising once more at a concentration of 1.0 M to a value slightly lower (3%) than that previously registered in 0.3 M NaCl. Swelling was depressed by the presence of pyrophosphate at NaCl concentrations above 0.3 M and higher values than those observed in salt alone only occurred in 0.2 and 0.3 M NaCl. In 0.2 M NaCl, 10 mM pyrophosphate the swelling was 22% higher than in salt alone if swelling is calculated as in Bendall (1954), as the volume of 100 ml of mince after treatment (Fig. 3.19).

Extraction of myofibrillar protein (Fig. 3.18 (b)) in the presence of salt alone was lower than in the presence of salt plus 10 mM pyrophosphate, and rising consistently from 0.1 to 0.9 M NaCl. Protein extraction in NaCl alone rose progressively to a maximum of about 50% in the two last salt concentrations. Protein extraction in the presence of 10 mM pyrophosphate rose abruptly from the 0.2 to 0.3 M NaCl concentration, probably due to scattering and from then on progressively rose to its maximum in 1.0 M NaCl where 76% of the myofibrillar protein initially present in the system was found in the supernatant.

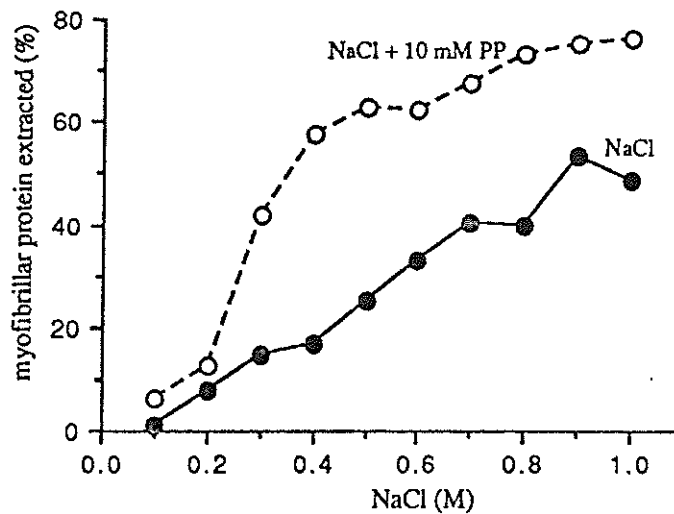
Table 3.5. Experiment 6. pH of supernatants, at 5°C, in NaCl and NaCl plus 10 mM pyrophosphate (PP)

NaCl (M)	pH	
	NaCl	NaCl + PP
0.1	6.13	6.02
0.2	6.16	6.02
0.3	6.07	6.02
0.4	6.10	6.00
0.5	6.05	6.01
0.6	6.06	6.00
0.7	6.07	6.03
0.8	6.01	6.04
0.9	6.07	6.09
1.0	6.05	6.09

EXPERIMENT 6



(a) Swelling



(b) Protein extraction

Fig. 3.18. Effect of NaCl and NaCl plus 10 mM pyrophosphate (PP) on (a) swelling of washed mince and (b) on extraction of protein from washed mince, at pH 6.0 and a final myofibrillar protein concentration calculated to be of 50 mg.ml⁻¹. The incubation period was 24 h and centrifugation was for 30 min at 1200 x g.

EXPERIMENT 6

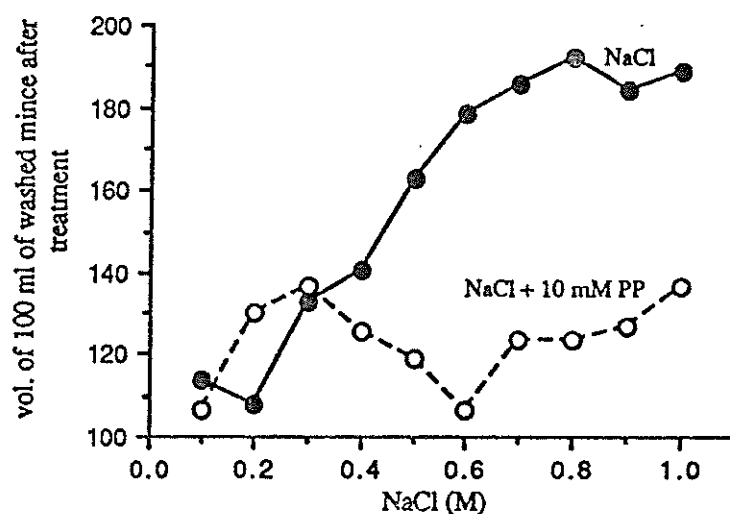
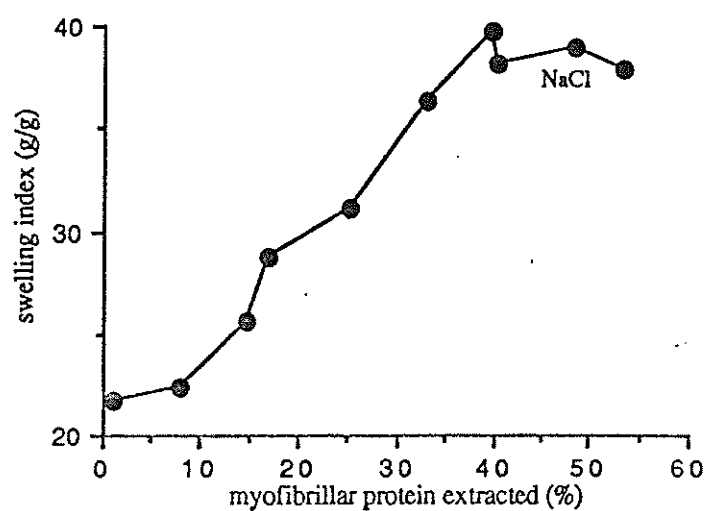


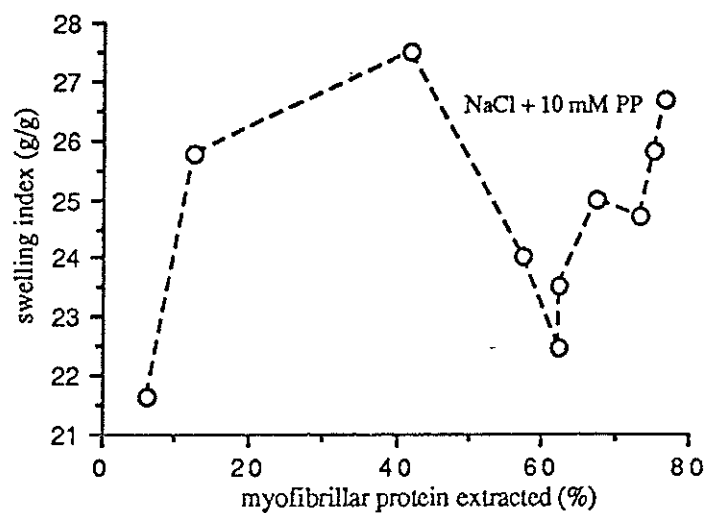
Fig. 3 19. Swelling of washed mince calculated according to Bendall (1954). In NaCl and NaCl plus 10 mM pyrophosphate, at pH 6.0 and a myofibrillar protein concentration calculated to be of 50 mg.ml⁻¹. The incubation period was 24 h and centrifugation was for 30 min at 1200 x g. The difference in shape between curves in Fig. 3.18 and 3.19 is due to in Fig. 3.19 swelling having been calculated as the amount of fluid taken up by the mince and in Fig. the swelling index having been calculated using the weight of the myofibrillar pellet.

The peak registered in the swelling index in washed mince in the 0.2-0.3 M NaCl concentration range when 10 mM pyrophosphate was present corresponds to the NaCl and pyrophosphate concentration at which Bendall (1954) observed that the presence of pyrophosphate increased swelling of mince. The result of swelling of washed mince in 0.2 M (1.2%) NaCl plus 10 mM (0.22%) pyrophosphate, centrifuged at 1200 x g for 30 min resembled that of Bendall (1954) in 1% NaCl (0.17 M), 0.25% (11.3 M) pyrophosphate and of Experiment 4 in which myofibrils were incubated in 0.3 M NaCl, 10 mM pyrophosphate, but were centrifuged at 9000 x g for 60 min. Swelling, calculated using the index used in the experiments with myofibrils, was seen to be very similar in 0.2 M NaCl plus 10 mM pyrophosphate in Experiment 6 and 0.17 M NaCl plus 11.3 mM pyrophosphate in Experiment 5. If swelling in NaCl plus pyrophosphate in Experiments 5 and 6 are compared at the 0.17 M NaCl and 0.2 M NaCl concentration, in each experiment, using the method Bendall (1954) used (Figs. 3.17 (b) & 3.19) the volume of the washed mince is seen to have increased less than in unwashed mince. It can be concluded that the elimination of endogenous ions, nucleotides and sarcoplasmic proteins from the mince or the fact there was 1.3 mM pyrophosphate in the Bendall (1954) experiments may have produced this difference in swelling at those close NaCl concentrations. As to the swelling throughout the range of NaCl concentrations it can be seen that the swelling index in salt alone (Fig. 3.18 (a)) did not decrease in the last salt concentrations above 0.5-0.7 M NaCl like in the myofibrillar experiments (2, 3 and 4) (Figs. 3.7 (a), 3.11 (a) & 3.15 (a)). This probably occurred because maximum protein extraction is maintained at levels which in Experiments 2, 3 and 4 correspond to the highest swelling registered. While in salt plus pyrophosphate (Fig. 3.18 (a)) the swelling index, as stated above, unlike in Experiments 2, 3 and 4, and like in Experiment 1 is seen to increase after having fallen to a value as low as that registered in 0.1 M NaCl. The line showing the relationship between protein extraction and swelling in salt plus pyrophosphate (Fig. 3.20 (b)) did, for that reason, not show the same shape as those of Experiments 2 and 3 and 4. Swelling at the highest levels of protein extraction (about 76%)

EXPERIMENT 6



(a) NaCl alone



(b) NaCl + 10 mM PP

Fig. 3.20. Effect of protein extraction on swelling of washed mince treated (a) with NaCl alone and (b) with NaCl plus 10 mM pyrophosphate (PP). The incubation period was 180 min at 0°C and centrifugation was for 30 min at 1200 x g. Calculated myofibrillar protein concentration in the systems (see text): 50 mg.ml⁻¹.

rose again in 1.0 M NaCl to a value which is almost as high as that registered in 0.3 M NaCl, unlike in Experiments 2, 3 and 4 where a depression is seen in such high values of extraction. The same relationship between the amount of protein solubilised and swelling as in the experiments with myofibrils was found when salt was used alone (Fig. 3.20 (a)). Swelling may have, therefore, been maintained at its highest level because maximum protein extraction was maintained at levels which in Experiments 2 and 3 correspond to the highest swelling registered. In salt plus pyrophosphate (Fig. 3.20 (b)) the swelling index is seen to increase up to the point where only 42% of the myofibrillar protein has been extracted. After that when the protein extracted rose to 57% (0.4 M NaCl), swelling steeply fell, but rose again in 0.6 M NaCl where 67% of the myofibrillar protein had been extracted. This differs (as mentioned above) from Experiments 2, 3 and 4, where when the protein extracted represented more than about 50% of the initial protein present in the myofibrillar suspension a steep fall in swelling occurred, without a rise at higher NaCl concentrations.

4. DISCUSSION

One of the aims of this thesis was to re-examine whether pyrophosphate, the active form of polyphosphates, had a greater effect than NaCl in both swelling and myofibrillar protein extraction on uncooked meat. A second aim was to explore the conditions of NaCl concentration at pH 6 required for pyrophosphate to increase or to decrease swelling. A third aim was to investigate the discrepancy between the results for swelling observed in mince (Bendall, 1954) and myofibrils (Offer & Trinick, 1983; Parsons & Knight, unpublished results), where NaCl plus pyrophosphate in mince increased water-holding more than NaCl alone and in the myofibrils NaCl plus pyrophosphate was seen to decrease swelling. A final aim was to explore the effect of protein solubilisation on swelling when NaCl or NaCl plus pyrophosphate are used. Protein solubilisation was in some cases seen to be accompanied by an increase in swelling (Callow, 1932; Bendall, 1954; Sherman, 1961; Hellendoorn, 1962; Hamm, 1979; Offer & Trinick, 1983) and in others it either had no influence in swelling (Hamm, 1979) or was seen to accompany a depression in swelling (Offer & Trinick, 1983; Parsons & Knight, unpublished results).

The approach chosen to achieve these objectives included: (1) the elimination of the possible effect of endogenous ions, sarcoplasmic proteins and nucleotides on the systems used so that any changes in water-holding capacity, protein solubilisation and in muscle structure could only be attributed to NaCl and pyrophosphate; (2) the use of a wide range of NaCl concentrations (0.1 to 1 M) assumed to be a wide enough range for the exhibition of the effects produced by NaCl and NaCl plus pyrophosphate, and at the same time confirm that the apparently contradictory results obtained by previous investigation was due to the use of a limited range of NaCl concentrations; (3) the use of 1 and 10 mM pyrophosphate to show the critical effect of its concentration on swelling and protein solubilisation; (4) using in some

controlling pH at a value of 6 so that the action of all additives could be compared under the same conditions, and the action of the additives could not be attributed to the changes in pH that they could promote; (6) an estimation of the weight of a myofibrillar or mince pellet incubated in NaCl or NaCl plus pyrophosphate to check whether the presence of pyrophosphate did induce more swelling or on the contrary depressed it; (7) the estimation of myofibrillar protein extraction by NaCl and NaCl plus pyrophosphate; (8) investigation by microscopy of structural changes in sarcomeric band pattern that accompany the action of NaCl and NaCl plus pyrophosphate and (9) the use of muscle in two different physical states not only to confirm whether the effects produced by pyrophosphate were reproducible in both, but also to compare the results in the myofibrillar system used in this project with the results of Bendall (1954) where 0.25 and 0.5% pyrophosphate plus NaCl produced more swelling and protein solubilisation than NaCl alone in raw mince.

4.1. Effect of pyrophosphate on swelling

The results showed that in NaCl plus pyrophosphate in Experiments 1, 2, 3, 4 and 6 swelling reached a maximum at 0.4 M NaCl when 1 mM pyrophosphate was used, and at 0.3 M NaCl when 10 mM pyrophosphate was used. In Experiment 1, using 1 mM pyrophosphate, it was also observed that swelling in the tubes with myofibrillar suspensions in concentrations up to and including 0.3 M NaCl swelling was greater than in NaCl alone at all concentrations. In Experiments 2 and 3 where 1 mM pyrophosphate was used this was not seen and swelling was only slightly greater than in NaCl alone in 0.3 M NaCl, while the maximum value of the swelling index at 0.4 M NaCl was lower than the same value in NaCl alone. However, when 10 mM pyrophosphate was used, in myofibrils and washed mince swelling was maximum in 0.3 M NaCl and the maximum difference between swelling in NaCl alone and NaCl plus pyrophosphate was in 0.3 and 0.2 M NaCl, respectively, for muscle used in the two different

physical states. The swelling index in myofibrils (Experiment 1) in NaCl plus 1 mM pyrophosphate and in washed mince (Experiment 6) in NaCl plus 10 mM pyrophosphate is seen to rise again after 0.6 M NaCl (see Figs. 3.4 & 3.18 (a)).

The maximum differences between swelling in NaCl alone and in NaCl plus pyrophosphate in the cases where the presence of pyrophosphate produced more swelling were: (a) in the presence of 1 mM pyrophosphate 33% more (Experiment 1 with myofibrils, in 0.3 M NaCl) (Fig. 3.4); (b) in 10 mM pyrophosphate 48% more (Experiment 4 with myofibrils, in 0.3 M NaCl) (Fig. 3.15 (a)) and 15% (with washed mince, in 0.2 M NaCl) (Fig. 3.18 (a)). In washed mince it can be added that if swelling is calculated using the same method as Bendall (1954) (Fig. 3.19) then 10 mM (0.22%) pyrophosphate plus 0.2 M (1.2%) NaCl produce 22% more swelling than NaCl alone. This effect is similar to that observed by Bendall (1954) using 1% (0.17 M) plus 0.25% (11.3 mM) or 0.5% (22.5 mM) pyrophosphate, where the swelling was 31 and 44%, respectively, higher than in 1% NaCl alone (Fig. 3.17 (b)). The difference at very similar salt and pyrophosphate concentrations between swelling in salt alone and in salt plus pyrophosphate can be attributed to the absence of endogenous ions which were removed by washing of the mince.

The discrepancy between swelling results registered in myofibrils (Offer & Trinick, 1983; Parsons & Knight, unpublished results) and mince (Bendall, 1954) was due to the fact that while the myofibrils were treated in a 0.1-1.0 M NaCl concentration range the mince was only treated with a 1% (0.17 M) salt concentration which lies in the limited range of NaCl concentration at which polyphosphates are indicated by this work to produce more swelling than NaCl. In both myofibrils and mince the NaCl and pyrophosphate concentrations at which the effect was observed are in the range commonly used under practical conditions in meat processing. The fact that there was similarity between results in different systems and the fact that this similarity was found when using salt and pyrophosphate at which they are used in the

industry (see Introduction Mahon *et al.*, 1971; Hughes, 1988), makes it possible to assume that the increase in water uptake when 10 mM pyrophosphate was present can be profitably used under practical conditions. In 1 mM pyrophosphate using a centrifugal speed of 3000 x g the increase on swelling is also suggested to be interesting but since myofibrils swollen in NaCl alone can not be pelleted, as found by Mitchell & Egelendsal (1989), a comparison with swelling in the presence of pyrophosphate can not be made at that centrifugal speed.

The explanation for the effect that pyrophosphate has on swelling in the range of NaCl concentrations between 0.1 and 0.4 M NaCl is not simple and can be attributed to the two actions pyrophosphate has been found to have on the thick filament: dissociation of actomyosin and depolymerisation of the thick filament (see Introduction). From the results obtained in these experiments and the explanation given for the mechanism of salt-induced swelling of myofibrils by Offer & Knight (1988), two possibilities for the mechanism of action of polyphosphates may be indicated. One possibility is that depolymerisation of the thick filament by NaCl at certain concentrations was assisted by pyrophosphate and so swelling increased initially as seen in all experiments, because the tails of the myosin molecules attached to the thin filament would have promoted entropic swelling pressure [see section 1.7, Offer & Knight (1988)]. At higher NaCl concentrations than 0.3 or 0.4 M dissociation of actomyosin promoted by pyrophosphate may have occurred and swelling was depressed because myosin molecules were no longer attached to actin in order to produce swelling. Swelling as observed in these experiments (Figs. 3.7 (a), 3.11 (a), 3.15 (a) & 3.18 (a)) would steeply fall and protein would be expected to be solubilised. The second possibility is that dissociation of actomyosin may have occurred first. Swelling initially seen in the presence of pyrophosphate would correspond to a gradual transition of the myofibrils from a rigor to a relaxed state. The depression of swelling at higher concentrations would correspond to a collapse of the myofibrillar structure due to a loss of the support provided by the thick filament which may have been depolymerised. Protein solubilisation in this state would also

be expected to be highly increased. It is considered that the second hypothesis for the mechanism of action of pyrophosphate is more viable. If the depolymerisation of the thick filament occurred first then swelling in the presence of pyrophosphate would be expected to have been nearly as great as that observed in NaCl alone at higher concentrations. This would be expected to happen at a stage where enough depolymerisation would have freed enough myosin molecules to promote swelling and dissociation of actomyosin was still starting to take off. But the results show that maximum swelling in NaCl plus pyrophosphate never achieved the same value as swelling in NaCl alone at the same level of protein extraction (Figs. 3.8 (a) & (b), 3.12 (a) & (b), 3.16 (a) & (b) and 3.20 (a) & (b)). In the second possible hypothesis, supposing that dissociation occurred first. As dissociation progressed, the myofibrils would be expected to be passing from a rigor to a relaxed state but the myosin molecules would still be polymerised in the form of a thick filament and so swelling would be the expression of the progressive breaking of the transverse constraint imposed by the cross-bridges to swelling. Depolymerisation of the thick filament occurring at an higher salt concentration would not have the same effect on swelling as in the first possibility, since the myosin molecules would no longer be anchored to the thin filament and able, therefore, to produce entropic swelling pressure. The action of pyrophosphate only corresponding to an elimination of the constraint imposed by cross-bridges to the expansion of the filament lattice would correspond to a low increase in myofibrillar volume of about 9% (Offer *et al.*, 1989). This is supported by the smaller swelling observed in the presence of 1 mM pyrophosphate in the 0.1-0.4 M NaCl concentration range when compared to that produced in the presence of 10 mM pyrophosphate. Which is thought to indicate that even though dissociation occurred first in both cases (1 and 10 mM pyrophosphate), some depolymerisation of the thick filament by NaCl was already taking place. The higher 10 mM pyrophosphate concentration would have co-operated with NaCl in producing depolymerisation of the thick filament at that stage where total dissociation of actomyosin had not yet been achieved and the number of attached myosin molecules was still high enough to promote some swelling. A concentration of 1 mM

pyrophosphate with NaCl in the 0.1-0.4 M NaCl concentration range may have been high enough to produce dissociation of actomyosin, but not depolymerisation of the thick filament and so swelling corresponded to the increase in volume of the myofibrils permitted by the expansion of the filament lattice by a reversal of rigor.

As mentioned earlier, unlike in the experiments where isolated myofibrils were progressively irrigated in higher NaCl concentrations in the presence of 10 mM pyrophosphate (Offer & Trinick, 1983; Parsons & Knight, unpublished) in Experiments 1 and 6, where the centrifugal speeds were respectively of 3000 and 1200 x g, a second increase in swelling was observed in 1.0 M NaCl plus 1 or 10 mM pyrophosphate. Knight & Parsons (unpublished) have observed that if the NaCl concentration is increased in a single step to 1.0 M, swelling is greater than if that same concentration is arrived at progressively. This is explained by Knight & Parsons (1988) on the basis that there are restraints to swelling, such as the Z-disc, which as already mentioned stop the myofibrils from swelling indefinitely. If the salt concentration is raised in small steps, the relatively small swelling forces produced cause an elastic expansion of the myofibril. The swelling force is reduced as myofibrillar proteins, especially myosin, are progressively lost making the swelling force stay relatively low as the salt concentration rises. As the myosin molecules needed to drive swelling entropically are no longer present in the sarcomere attached to the thin filament (Offer & Knight, 1988). By contrast, a single step to 1.0 M NaCl causes the sudden expression of a large swelling force and it is supposed that this is sufficient to cause rupture of some of the elastic restraints, so that a larger swelling is observed that is not reversed as the myofibrillar proteins are extracted. Further evidence supporting this point of view are the observations made by Offer & Trinick (1983) that in irrigated myofibrils when the Z-line remained strong, little expansion of the Z-line occurred, the swelling of the I-band and the edges of the A-band were therefore bowed and most importantly in such cases the swelling of the A-band was not great. This contrasted with other cases where the Z-line became weak or absent after salt treatment, the swelling of the I-band

was nearly as great as the A-band and in such cases the swelling of the A-band tended to be large. It was concluded by them that while swelling of the myofibrils occurs when the constraint of the cross-bridges is removed by salt treatment, the maximum extent of swelling may depend on the integrity of the Z-line. Signs of weakening of the Z-lines were observed in 0.4 M NaCl plus 1 mM pyrophosphate (see Fig. 3.10), but unfortunately images of the state of the Z-line above that concentration could not be obtained. Agreement is also found in the observations that in conditioned meat where the Z-line is lost water-holding capacity increased (Hamm, 1960) and also that the effect of NaCl alone and NaCl plus pyrophosphate on swelling of mince increased as the conditioning period increased (Swift & Ellis, 1956). Support is found in these facts and in the results obtained in this thesis to suggest that that even though pyrophosphate greatly influences the action of NaCl, the effect of pyrophosphate on the expression of swelling may also be reciprocally influenced by the concentration of NaCl, in the NaCl concentration range where NaCl plus pyrophosphate was seen to produce more swelling than NaCl alone.

4.2. Effect of pyrophosphate on protein solubilisation

In all experiments, under the conditions used, protein solubilisation was greater in NaCl plus pyrophosphate than in NaCl alone. The presence of 10 mM pyrophosphate produced an higher extraction of protein than 1 mM pyrophosphate. These results are in agreement with results found in homogenised beef [van den Oord & Wesdorp, 1978 (see Introduction); Hamm, 1979; Brebner, 1981] and pork muscle (van den Oord & Wesdorp, 1978), in myofibrils (Offer & Trinick, 1983), in blocks of pig meat (Voyle *et al.*, 1984) and in the manufacture of meat products (Jolley & Savage, 1985).

In Experiment 3 with myofibrils, the first experiment in which values for protein extraction were obtained at all NaCl concentrations, at 1.0 M NaCl only 9% more protein was extracted

in 1.0 M NaCl plus 1 mM pyrophosphate than 1.0 M NaCl alone (Fig.3.11 (b)). However, bigger differences of about 20 to 40% were registered at lower concentrations of NaCl showing, in that experiment, that the difference between protein solubilised in NaCl and in NaCl plus 1 mM pyrophosphate decreased as the salt concentration was increased. In Experiment 4 (Fig. 3.15 (b)) also with myofibrils, but using 10 mM pyrophosphate the same evolution in the difference between protein extraction in NaCl and NaCl plus pyrophosphate was not observed. Protein solubilisation in NaCl plus pyrophosphate was always greater than in NaCl and that difference was of between about 15 to 30% at some NaCl concentrations. In Experiment 6 (Fig. 18 (b)) with washed mince a similar pattern in the difference between protein solubilisation in salt and pyrophosphate was observed even though less protein was extracted in both treatments.

The differences pointed out are explained by the effect pyrophosphate has in promoting the dissociation of actomyosin (Weber & Portzehl, 1952). They are also explained by the knowledge that it assists NaCl in depolymerising the thick filament (Gränicher & Portzehl, 1952). Therefore, myosin molecules are dissociated from actin, and they are also free to diffuse away due to the depolymerisation of the thick filament. The images obtained by phase-contrast micrography even though in most cases only show a general disorganisation the structure of myofibrils in some cases did confirm the images obtained by Hanson & Huxley (1955), Offer & Trinick (1983), Parsons & Knight (1990) showing a greater extraction of the A-band than in NaCl alone.

4.3. Relationship between protein solubilisation and swelling

The solubilisation of protein in muscle systems in NaCl or NaCl plus pyrophosphate has been seen in some cases to be associated with an increase and in other cases with a decrease in

swelling. A positive correlation between protein solubilisation and swelling has been found in mince (Bendall, 1954), in homogenised muscle (Brebner, 1981), in meat pieces (Callow, 1932; Voyle *et al.*, 1984; Jolley & Savage, 1985) and Mitchell & Egelensal (1989) when salt is used alone or associated with polyphosphates. Such a correlation has been found to be negative in homogenised muscle (Hamm, 1979) when salt is used with pyrophosphate but positive when salt is used alone. It has also found to be positive in salt alone in myofibrils and negative when salt plus pyrophosphate are used (Offer & Trinick, 1983). In the present work it was found that swelling increased, in both salt and salt plus pyrophosphate, as protein was extracted up to a value between 41 to 54% in Experiments 2, 3 and 6 (see Figs. 3.8 (a) & (b), 3.12 (a) & (b), 3.16 (a) & (b) and 3.20 (a) & (b)). At myofibrillar protein extraction values above this percentage a fall in swelling was registered, which was abrupt in the presence of pyrophosphate. The percentage of myofibrillar protein appearing in the supernatant sharply increased when going from a 0.3 or 0.4 M NaCl (with 10 mM pyrophosphate) (Figs. 3.15 (b) & 3.18 (b)) or from 0.4 or 0.5 M NaCl (with 1 mM pyrophosphate) (Figs. 3.7 (b), 3.11 (b) & 3.15 (b)). The fall in volume of the myofibrillar pellets when protein was extracted is explained by the fact that there was a correspondent fall in the number of myosin molecules attached to the thin filament (Offer & Knight, 1988). Lying out of this tendency were the results of Experiment 4 in NaCl alone (Fig. 3.15 (b)), but this may have been due to the difficulties found in estimating protein in the supernatants. In this experiment swelling was still seen to increase when about 63% of the myofibrillar protein had already been extracted, and was only seen to fall after about 71% of this protein had been extracted. It is also important to mention that the images of the changes in the structure of myofibrils were seen to have changed and accompanied both swelling and protein extraction (see micrographs in Results). Traces of extraction of the A-band were found in some cases, especially in the presence of pyrophosphate. But, the images showing an almost generalised disorganisation of the structure of myofibrils were commonly observed to accompany an increase in protein extraction and in swelling of myofibrils.

The reason for the correlation between swelling and protein extraction not always being observed in different meat systems can be attributed to different factors. Callow (1931) observed, in meat pieces, that the diffusion of sodium chloride into the muscle was rapid, but the diffusion of proteins from the muscle was very slow. The observation made by Callow (1931) and the conclusion presented previously that swelling decreased because myosin molecules needed to drive swelling were extracted, fit with the suggestion that has been made (P.Lillford, personal communication, cited in Offer & Trinick, 1983) that in the processing of large pieces of meat the presence of the sarcolemma and endomysium may inhibit swelling of the myofibrils by impeding ingress of ions or by mechanical restraint. This makes it possible to think that if ions are not able to reach the myofibrils then proteins will also have difficulties in crossing these barriers. So the rate of diffusion of salt and pyrophosphate - needed to depolymerise the thick filament and dissociate actomyosin - into and the protein molecules solubilised in the opposite direction will depend on the integrity of the muscle structures and the thickness of meat pieces, and will account for the differences in protein extraction and the different pattern of swelling. This explains why in a homogenate, in myofibrils in a suspension or myofibrils irrigated in a glass slide - where the access of ions to the myofibrillar structure is readily achieved and protein is easily carried away from the system - swelling may be depressed at concentrations of NaCl alone or with pyrophosphate at which high quantities of myosin may be depolymerised and dissociated. This is also suggested if the swelling and protein extraction results (Figs. 3.7 (a) & (b), 3.11 (a) & (b), 3.15 (a) & (b) and 3.18 (a) & (b)) that were obtained in this work with muscle in different physical states, mince and myofibrils are compared. Another factor still influencing different correlations between swelling and protein extraction, found in different references is the concentration at which the additives are used. Just as a slow or hindered penetration of salt or polyphosphates does not make them available to exert their actions, a weak concentration of these even under good conditions of penetration will not produce the desired effect. The assumptions made are

supported by the suggestions made by Voyle *et al.* (1984) trying to explain the difference in results they then obtained by treating meat pieces with NaCl and NaCl plus pyrophosphate with those obtained by Offer & Trinick (1983) irrigating myofibrils with the same salts. They indicated, based on observations by Josephs & Harrington (1966) that under a certain critical protein concentration, which depends on the ionic conditions, myosin exists entirely in the form of molecules. Any myosin in excess of this critical concentration exists as filaments. In a meat system they supposed that the concentration of the myosin molecules is maintained at a high concentration over a substantial part of a meat piece and only in a region near the surface does the myosin molecule concentration fall. Therefore, it is possible to think that in the depth of the meat the thick filament is not depolymerised. At least as much as in the surface and so protein can not be extracted and swelling may be low. It may also be added to this suggestion that even if depolymerisation occurs, the permanence of the myosin molecules near the environment from where they were dissociated - because their diffusion is hindered - may make it possible for them to re-attach to the thin filament. A balance between attachment and detachment of myosin to actin may be found and only if the incubation period and the salt and pyrophosphate concentrations, pH value and mechanical action (tumbling and massaging) allow enough protein to flow out of the system will the quantity of molecules needed to promote swelling fall below the required level.

Swelling of myofibrils incubated in NaCl plus 1 mM pyrophosphate and centrifuged at 3000 x g for 60 min (Fig. 3.1), and swelling of washed mince incubated in NaCl plus 10 mM pyrophosphate and centrifuged at 1200 x g for 30 min (Fig. 3.18 (a)) is seen to increase after 0.6 M NaCl, as mentioned earlier. This increase in swelling apparently contradicts the suggestions expressed above but the suggested reasons for this increase have already been mentioned in section 4.1 of this discussion. However, more experimental data exists that may explain this fact. A study of the results of Offer & Trinick (1983) shows that in some of the myofibrils they irrigated 0.3 or 0.4 M NaCl, 10 mM sodium pyrophosphate, 1 mM MgCl₂, 10

mM sodium acetate, pH 5.5 - in which maximum swelling in the presence of pyrophosphate was registered - the Z-line is still detectable and gradually disappeared above 0.4 M NaCl. In 0.6 M NaCl, 1 mM MgCl₂, 10 mM sodium acetate, pH 5.5 - a salt concentration at which on average maximum swelling, in NaCl alone in the present work was registered - the Z-line was already no longer visible in their experiments. It, therefore, could no longer exert its constraint on swelling as they then explained.

4.4. Discrepancy between results in the literature and the present results for swelling of myofibrils in salt alone

The tendency observed for muscle in different physical states to increasingly swell in the presence of NaCl alone in the 0.1-1.0 M concentration range used in previous experimental work (Callow, 1932; Hamm, 1979; Offer & Trinick, 1983; Parsons & Knight, unpublished results) was also observed in the present work in washed mince centrifuged at a centrifugal speed of 1200 x g (Fig. 3.18 (a)). This tendency was not observed in the present experiments where myofibrils were centrifuged at 9000 x g (see Figs. 3.7 (a), 3.11 (a) & 3.15 (a)) and those of Mitchell & Egelensal (1989) under the same ionic and centrifuging conditions. An explanation for this can be found in the literature. It has been observed that the myofibrillar lattice is susceptible to osmotic compression (Millman, 1981). In the relaxed state, where the cross-bridges are unattached, the lattice is relatively compressible. At a pressure of 10⁵ Pa (produced osmotically) the interfilament distance in rigor has been observed to decrease by about 15% (Millman, 1981) corresponding to a volume reduction of 30%. In the relaxed state, where the cross-bridges are unattached Millman (1981) also observed that the lattice is relatively compressible while in the rigor state, where the cross-bridges are attached, it is markedly more rigid. It is concluded that cross-bridges are not only structural constraints to swelling (Offer & Trinick, 1983), but may under high pressures act to stop the filament lattice from collapsing.

The results of swelling in the present work seem to be related with those observations by Millman (1981) and Offer & Trinick (1983) and can be used to explain the difference in results obtained for swelling in the experiments in salt alone. The most relevant information comes from the fact that at 1200 x g (washed mince) and 3000 x g (myofibrils in Experiment 1) when pyrophosphate is associated with NaCl in a 1 and 10 mM concentration, respectively, there is swelling in the last NaCl concentrations which is not seen in the experiments in the presence of pyrophosphate where a centrifugal force of 9000 x g is used (see Figs. 3.4 & 3.18 (a) and 3.7 (a), 3.11 (a), 3.15 (a) & 3.18 (a)). The centrifugal speed of 9000 x g conditions corresponded to 88×10^7 Pa and so would be expected to produce greater collapsing of the myofibrillar structure than 1200 x g (1.2×10^8 Pa) or 3000 x g (3.0×10^8 Pa). The fact that swelling, in the experiments at 9000 x g, was greatly depressed in NaCl concentrations - with or without pyrophosphate - where a high percentage of myofibrillar protein was extracted clearly indicates the myofibrils to have changed from a rigor to a relaxed state. This change led to the loss of the mechanical support by the cross-bridges to compression exerted by the centrifugal force applied in the myofibrillar experiments. More evidence of this effect is seen in Experiments 2 and 3 in the presence of 1 mM pyrophosphate (Figs. 3.7 (a) & 3.11 (a)) where myofibrils in NaCl concentrations above and including 0.6 M NaCl are observed to shrink when compared with those treated with 0.1 to 0.3 M NaCl. Furthermore, in the case of the mince model the amount of protein extracted in 1.0 M NaCl alone is lower than that observed to be associated with a fall in swelling in the case of myofibrils, and that would be expected to reduce compression. Contradicting these assumptions are only the results of Experiment 4, as mentioned earlier (Fig. 3.15 (a)). If it is assumed that only after a certain level of protein extraction is compression of the filament evident, then the depressing effect in that experiment in salt alone should have started earlier than in the other experiments.

4.5. Possible future experimental work

The results obtained in this work indicate that in the presence of 10 mM pyrophosphate a concentration of 0.3 M NaCl in myofibrils and 0.2 M NaCl in mince is needed for the production of a useful effect on swelling in the range of practical concentrations used by the industry, *i.e.* polyphosphates up to 0.5% (Mahon *et al.*, 1971) and NaCl from 0.1 M (0.5%) to 0.9 M (5.0%) (Hughes, 1988). Even though the endogenous ions of the meat were present in his system, Bendall (1954) obtained similar swelling in mince using similar additive concentrations: 11.3 mM (0.25) or 22.5 mM (0.5%) pyrophosphate and 0.17 M (1%) NaCl. This together with the suggestions made in section 5.1 of this discussion makes it possible to think that other NaCl concentrations near these may have in conjunction with pyrophosphate, in concentrations around 10 to 20 mM, an effect in depolymerising of the thick filament, while the number of myosin molecules attached to the thin filament is still at a level which may promote swelling. With this end an experimental approach involving the use of a myofibrillar pellet under a range of NaCl concentrations between 0.2 and 0.3 M NaCl in 0.01 M increments in association with 10 mM pyrophosphate would in a first instance point out the best NaCl concentration at which pyrophosphate might show the most profitable co-operation with salt. Higher pyrophosphate concentrations could also be associated with salt so that the effect of pyrophosphate concentration at low NaCl concentrations could be studied in order to establish the best combination between these additives.

The idea suggested by the results of these experiments that the Z-line is still strong enough to hinder the expression of swelling in both in the A- and I-bands in the salt concentrations at which pyrophosphate in association with NaCl, produces more swelling than NaCl alone needs to be confirmed. This may be done by using NaCl concentrations between 0.4 and 0.7 M NaCl in 0.01 M increments, especially between 0.4 and 0.5 M, in conjunction with lower

pyrophosphate concentrations, since these are near those commonly used in the manufacture of meat products. By doing this, a balance may be established where the concentrations of NaCl and pyrophosphate are already producing weakening of the Z-line, but the level of protein extraction will still permit the production of entropic swelling pressure and increased water-holding.

Magnesium is necessary for pyrophosphate to promote the dissociation of actomyosin (Weber & Portzehl, 1952) but reduces the promotion of the depolymerisation of the shaft of the thick filament by pyrophosphate (Harrington & Himmelfarb, 1972). It would be expected that in the absence of Mg^{2+} swelling in the presence of NaCl plus pyrophosphate would be as great as in NaCl alone and would occur at an earlier NaCl concentration. This would be possible because: (1) extensive dissociation of the myosin molecules from the thin filament by pyrophosphate would not take place and (2) depolymerisation of the thick filament, even though reduced, would permit the attached myosin molecules to promote swelling pressure. A repetition of these experiments but in the absence of Mg^{2+} would therefore show whether the conclusions on the mechanism of action of polyphosphate, arrived at in this thesis, were correct.

Endogenous ions existing in meat (see section 1.8) will certainly also have an influence in the action of salt alone and when associated with polyphosphates. The 21% difference between swelling in mince in the presence of 1% (0.17 M) NaCl, 0.25% (11.3 mM) pyrophosphate and swelling in washed mince in the presence of 1.2% (0.2 M) NaCl, 0.22% (10 mM) pyrophosphate, (see Figs. 3.17 (a) & 3.18 (a)) suggests that the endogenous ions or other components of the meat that have been washed may be responsible for a significant amount of swelling. The inevitability of the presence in meat under practical circumstances of ions and other small molecules that may influence water-holding, indicates it to be advisable to study

pyrophosphate, (see Figs. 3.17 (a) & 3.18 (a)) suggests that the endogenous ions or other components of the meat that have been washed may be responsible for a significant amount of swelling. The inevitability of the presence in meat under practical circumstances of ions and other small molecules that may influence water-holding, indicates it to be advisable to study controlled and uncontrolled systems of muscle in different physical states in order to understand how far the influence of these molecules, even though possibly highly interactive, is important and whether a positive combination of their effects with salt plus pyrophosphate may be beneficial. With this aim washed mince and myofibrillar suspensions could be treated with the ions and other small molecules used separately and in different combinations, in the concentrations they are naturally found in meat.

Analysis of the proteins that were extracted from the myofibrils or from washed mince by SDS gel electrophoresis is additionally needed so that the solubilisation of myosin and its presence in the supernatants may be confirmed. The amount of myosin extracted could then be quantitatively related with its action in promoting entropic swelling pressure.

Lastly a comparison between the action of polyphosphates of different chain lengths and pyrophosphate could be made using myofibrils and washed mince to confirm not only these results obtained using pyrophosphate, the active form by which the effects of polyphosphates are produced, but also other results existing in the literature.

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