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THE EFFECT OF SALT AND PYROPHOSPHATE ON THE STRUCTURE OF MEAT

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Abstract

Our objective was to determine whether or not salt and pyrophosphate have the same effect on the structure of pieces of meat as they have on isolated myofibrils. Blocks of pig *M. longissimus dorsi* were incubated in solutions of sodium chloride at pH 5.5 or sodium chloride plus sodium pyrophosphate at pH 5.5 or 8.0. The blocks were obtained from fresh (24h post-mortem) or aged (72h post-mortem) muscle and incubated for 5 or 24h with minimal agitation. There was considerable uptake of water by the tissue especially at the higher pH and longer times.

Electron microscopy of the meat incubated in salt plus pyrophosphate at pH 8.0 revealed complete or nearly complete extraction of the A-band to a depth of at least one fibre from the surface. In meat incubated in salt plus pyrophosphate at pH 5.5 the extraction of the A-band was less complete and appeared to occur only near the surface. In salt alone no extraction of the A-band occurred.

Swelling of myofibrils close to the surface could be detected either by a reduction of density or by a greater separation of filaments.

Break-up of the Z-line, probably due to mechanical disruption imposed by swelling of myofibrils, was a common feature of the salt treatments. Mitochondria near the surface were grossly swollen, especially with salt plus pyrophosphate at pH 8.0.

At low pH amorphous material was observed inside and outside the cell membrane, but at high pH filamentous material was present in these areas.

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Key Words: Muscle, meat, water uptake, myosin, salt and pyrophosphate, polyphosphate, electron microscopy.

Introduction

Sodium chloride has a widespread use in the manufacture of meat products. It has been used as a preservative to facilitate long-term storage and it imparts a characteristic flavour that remains greatly in demand. The addition of sodium chloride results in an increase in the water-holding capacity of meat, the uptake of additional water causing swelling of the tissue. In the presence of polyphosphate this occurs at a lower concentration of sodium chloride. The presence of sodium chloride, especially in combination with polyphosphates, also assists the retention of water during cooking. The literature on the water-holding properties of meat has been reviewed by Hamm (1960, 1975, 1981) and Offer and Trinick (1983).

Mechanical agitation, such as massaging or tumbling, of meat pieces or particles in the presence of sodium chloride and polyphosphate gives rise to the formation of a sticky exudate on the surface (Theno et al., 1978a,b,c). This exudate contains myofibrillar proteins, in particular myosin, and is responsible for the binding together of meat pieces in cooked meat products. The conditions under which these proteins are extracted are thus of considerable importance.

Recent work by Offer and Trinick (1983) describes the behaviour of single myofibrils from rabbit *M. psoas* in different concentrations of sodium chloride in the presence or absence of sodium pyrophosphate at pH 5.5. They observed that myofibrils swell very substantially in salt solutions resembling those used in meat processing, and concluded that the myofibrils were the major sites of water uptake in meat. In the absence of pyrophosphate a concentration of 0.8M sodium chloride was required for swelling. Under these conditions there was partial extraction of myosin from the centre of the A-band. In the presence of pyrophosphate, a much lower concentration of sodium chloride (0.4 M) was required for swelling and extraction of the A-band was complete or nearly complete. Removal of the A-band progressed from the ends towards the central M-line.

The findings of Offer and Trinick (1983) are valuable in suggesting the mechanism by which water is taken up by meat. However, it will be appreciated that in such experiments the myofibrils were exposed to very large volumes of solutions and therefore any solubilized protein, such as myosin, would be removed from the vicinity of the myofibril. Hence, any tendency for the myofibril to disaggregate under the salt conditions would be exacerbated. Furthermore, since myofibrils are sub-cellular structures, no cell membrane or endomysium was present to act as a barrier to the diffusion of solutes or a constraint on swelling of the myofibril. Finally, the experiments of Offer and Trinick were performed at room temperature, rather than the low temperature at which meat products are commonly manufactured.

Commercial practice is concerned with bulk tissue with a consequential restriction of access to those elements that are below the surface. In order to obtain information about the fate of muscle cells in whole tissue when treated with salt, a model system consisting of blocks of meat of standard dimensions may be used. Such a system will provide information about the depth of penetration of the salt solution used, the extent to which swelling of myofibrils occurs, the extent to which proteins, particularly the myosin of the A-band, are solubilized and any other structural changes that occur. It is particularly important to know the extent of extraction of the A-band under such conditions since myosin is a better binder of meat pieces than actomyosin (Macfarlane et al., 1977), and myosin extraction was a feature in the observations made by Offer and Trinick (1983).

Lewis and Jewell (1972, 1975) examined the surface of blocks of pig muscle soaked in solutions of sodium chloride with or without tripolyphosphate. Using light microscopy they detected little or no change in structure compared with fresh, untreated meat. Changes were observed using electron microscopy, however. In meat treated with salt plus polyphosphate these consisted mainly of disorganization of filaments in the H-zone, an observation which did not seem to fit with those made on extracted myofibrils by Offer and Trinick (1983). A re-examination of the structural changes produced by salt and polyphosphate was therefore desirable, particularly when using well defined ionic conditions and meat of well defined post-mortem history. In this paper we shall describe the changes observed in blocks of porcine *M. longissimus dorsi* exposed to a limited volume of salt solution, with and without polyphosphate, at a pH (5.5) close to the final pH of the muscle in rigor. Since brines of alkaline pH are frequently used in commercial meat processing the effect of a similar solution at a high pH (8.0) was also observed. The length of time for which the tissue was exposed to these solutions was

varied, as was the post-mortem age of the tissue prior to incubation. In order to avoid the complications produced by mechanical damage to the exposed surfaces of the blocks of tissue, minimal agitation was applied, serving only to mix the solution surrounding the tissue. The following report should be regarded as a preliminary communication, as a full analysis of the often quite complicated structural changes has yet to be made. In this study we have concentrated on the effect on fibres at the surface of the block of tissue, although we have attempted to see whether the structural changes altered with depth.

Materials and Methods

The *M. longissimus dorsi* from one side of a commercial hybrid pig (86.5 kg carcass weight) was used for these experiments. The animal was electrically stunned and slaughtered by sticking. Blood drainage occurred rapidly as the carcass was suspended head downwards, from the Achilles tendon of one leg. In order to limit shortening of the fibres of the muscle to be used, the hind leg on the side from which the *M. longissimus dorsi* would be removed was tied at approximately 90° to the spine.

After slaughter the carcass was held for 5 h at ambient temperature (18°C) and then transferred to a chill room at 0°C. The *M. longissimus dorsi* was removed from the 'stretched' side 24 h after slaughter. The muscle was divided longitudinally; one half, designated 'fresh', was processed immediately while the other half was wrapped and held for a further 48 h at 4°C and was designated 'aged'. These post-slaughter holding periods are comparable with current commercial practice in the United Kingdom.

Samples of approximately 1 g were removed for pH determination. These were homogenised using a laboratory mixer-emulsifier (Silverman Machines Ltd., Chesham, Bucks., UK), in 10 cm³ of a solution containing 150 mM potassium chloride and 5 mM iodoacetic acid neutralised to pH 7.0. The pH of each extract was determined with a PHM63 Digital pH meter (Radiometer, Copenhagen, Denmark) using a combined glass electrode (Russell pH Ltd., Auchtermuchty, Scotland). The final pH was 5.4.

Blocks of tissue

Six blocks of tissue, each measuring 3 x 2 x 1 cm were cut from the fresh or aged portion of the *M. longissimus dorsi* so that the longitudinal orientation of the fibres coincided with the long (3 cm) axis of the tissue block. Each block was weighed after removal of superficial moisture by gentle blotting until there was no further sign of moisture transfer on clean absorbent paper. These blocks were incubated in the salt solutions described below before preparation

for microscopical examination. Additional blocks of tissue were removed from the fresh or aged portion of the muscle but were not incubated in the salt solutions. These were fixed immediately and served as controls.

Salt solutions

Three solutions were used for incubation of the tissue blocks, as follows:

- A. 0.6 M sodium chloride (3.5%)
10 mM sodium acetate
1 mM magnesium chloride
adjusted to pH 5.5 with hydrochloric acid
 - B. Solution A plus
10 mM tetra sodium pyrophosphate
adjusted to pH 5.5 with hydrochloric acid
 - C. Solution A plus
10 mM tetra sodium pyrophosphate
adjusted to pH 8.0 with hydrochloric acid
- The solutions were stored at 4°C.

The concentration of sodium chloride shown above was chosen on the basis of experiments with myofibrils (Offer and Trinick, 1983). In the presence of pyrophosphate this concentration would be expected to be amply sufficient to cause swelling and A-band extraction. In the absence of pyrophosphate it would be on the threshold required for swelling. It should also be borne in mind that dilution of the irrigating solution will occur on incubation.

Incubation of tissue blocks

Each block of tissue was placed in a glass specimen tube 25 mm in diameter, 18 cm³ of one of the solutions, A, B or C were added to give a tissue to fluid volume ratio of 1:3. The tissue was supported on a stainless steel spring so that the entire block was surrounded by salt solution which, under gentle agitation, was able to circulate freely. The loaded tubes were placed radially in a rack inclined at 45° to the vertical and revolving at 30 rpm at 4°C for 5 or 24 h. The tubes were sealed to prevent loss of fluid during inversion or by evaporation.

When the incubation period was complete, the tissue blocks were removed from the specimen tubes, drained into the tubes and gently blotted prior to weighing. From the weights obtained before and after incubation, the gross weight gain was determined.

Analysis of solutions after incubation

Protein The biuret method of Gornall et al (1949) was used to measure the protein content of the solutions.

Chloride After dilution with deionised water the chloride content of each solution was determined. A Radiometer CMT 10 chloride titrator was used to make these measurements.

Preparation for microscopy

On completion of the incubation period samples measuring approximately 5 x 5 x 2 mm were removed from one end of the block of tissue as shown in Figure 1. One 5 mm dimension coincided with the longitudinal axis of the fibres. The samples were fixed in 2.5% glutaraldehyde buffered with 0.1M sodium

cacodylate at pH 6.8 in accordance with the common practice of using fixatives in the region of neutrality. After 2h fixation the samples were trimmed to form smaller blocks, approximately 5 x 2 x 2 mm, which were returned to the fixative for a further 22h. Thus the final samples incorporated parts of two of the faces of the original block of tissue - one in longitudinal orientation, the other being transverse. Dehydration through graded alcohols was followed by impregnation and embedding in LR white acrylic resin - medium grade (London Resin Co., Basingstoke, Hants., UK) in polypropylene capsules (TAAB, Reading, Berks., UK). The embeddings were polymerised at 60°C for 24 h.

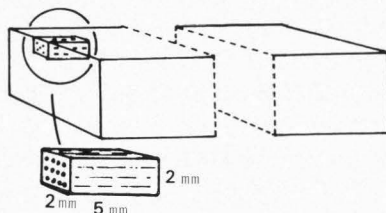


Fig. 1 Diagram showing sub-division of tissue block.

Sections, 30-50 nm thick, were cut using a glass or diamond knife on an LKBIII ultramicrotome. The sections were stained with uranyl acetate and lead citrate and examined in a Philips EM 400T electron microscope.

Results

Changes in the block of tissue

Change in weight An increase in the weight of the blocks of meat after incubation in the salt solutions was detected under all the conditions applied. The increases, expressed as a percentage of the initial weight, are listed in Table 1.

Table 1. Percentage increase in weight of blocks of meat following incubation.

Post mortem age of tissue (hrs)	Incubation time at 4°C (hrs)	% Increase in weight		
		NaCl pH 5.5	NaCl plus pyrophosphate pH 5.5	NaCl plus pyrophosphate pH 8.0
24	5	5.7	9.3	12.1
	24	14.5	16.2	20.0
72	5	7.4	11.6	10.7
	24	18.0	23.2	22.1

Table 2. Concentration of protein and chloride following incubation in different media.

Post mortem age of tissue (hours)	Incubation time at 4°C (hours)	Incubating Medium					
		NaCl pH 5.5		NaCl plus pyrophosphate pH 5.5		NaCl plus pyrophosphate pH 8.0	
		Protein mg/cm ³	Chloride M	Protein mg/cm ³	Chloride M	Protein mg/cm ³	Chloride M
24	5	7.1	0.52	8.6	0.56	12.5	0.53
	24	15.8	0.58	15.5	0.53	22.5	0.49
72	5	11.3	0.49	9.1	0.49	9.3	0.54
	24	15.1	0.49	15.7	-	19.8	0.43

Table 3. The incidence of various structural alterations following incubation in different media containing salt and pyrophosphate.

Incubating medium	NaCl pH 5.5		NaCl + pyrophosphate pH 5.5		NaCl + pyrophosphate pH 8.0	
	24	72	24	72	24	72
Post mortem age (hours)	24	72	24	72	24	72
Incubation time at 4°C (hrs)	5 24	5 24	5 24	5 24	5 24	5 24
Marked swelling of peripheral myofibrils	+	+	+	+		
Partial extraction of A-band to stubs						+
Total extraction of A-band: loss of M-line						+
Z-line break-up	+	+	(+)	(+)		+
Region of low density close to Z-line		+				+
Filamentous deposit of extracted protein near to cell membrane						+
Mitochondrial swelling	+		+	+	+	+

+ Definite change in structure observed

(+) The Z-line shows reduced electron density but is still intact.

Since the salt concentration of the brine was low (about 3.5% sodium chloride) the increases in weight observed must almost all be due to uptake of water by the blocks of meat. The increases are in some cases substantial and indicate considerable swelling of the tissue. The data show, predictably, that there was a greater increase in weight following long (24 h) incubation than after short (5 h) incubation, irrespective of the composition of the incubating medium. The gain in weight was greater in the presence of pyrophosphate than in its absence. Fresh tissue showed a greater weight increase at high pH than at low pH, whereas in aged tissue there was very little difference. Since only one block of tissue was available for each treatment replication of data was not possible in the experiments described.

Therefore no statistical significance is claimed for the differences observed. They do, however, indicate a trend.

Changes in appearance After incubation for 5 h the blocks of tissue became paler and more translucent to a depth of about 1 mm from the surface. This increased to 2-3 mm after 24 h incubation but the boundary of the

translucent zone was not well defined. There were no apparent differences in the effects on appearance caused by the different incubating media. Although the translucent zone presumably corresponds to a region in which marked structural changes, especially swelling, are occurring, the relationship between this and the depth of penetration of sodium chloride into the tissue remains to be explored. Zones of 1 or 2 mm in depth from the surface would occupy fractions of 0.33 or 0.59 respectively of the total volume of the meat blocks. If these zones represent regions where swelling has occurred then the water uptake in these zones is as much as 16-37%, depending on conditions.

Changes in the salt solutions

Appearance The solution at high pH became straw-coloured after incubation, due, presumably, to the extraction of myoglobin. The solution remained clear. The low pH solutions varied in their ultimate appearance. Short incubation of fresh or aged tissue gave rise to a clear straw-coloured solution, but after long incubation the solution became cloudy; a flocculent precipitate was eventually formed.

Analysis of the salt solutions. The total protein and chloride concentrations in each solution after incubation are listed in Table 2. This shows that a long incubation results, as expected, in an increase in protein extracted, compared with the short incubation. The presence of pyrophosphate at pH 5.5 had no effect on the amount of protein extracted, but at the higher pH there was increased extraction in the fresh tissue.

The amount of protein extracted from the blocks of meat was considerable; 23% of the total protein of the block of fresh meat was extracted in sodium chloride at pH 5.5 in 24 h at 4°C, and in the presence of pyrophosphate at pH 8.038% was extracted. The values for aged tissue also fell within this range. After incubation, the chloride ion concentration in the brine fell substantially (Table 2).

As explained above we know that there was net water uptake by the meat blocks. The decrease in chloride ion concentration must therefore be due to uptake of this ion beyond that taken up with the water. If all the water originally in the meat blocks (assumed to be 75% by weight) became equilibrated with the external salt solution, the concentration of chloride in the medium would fall to 3/3.75 of the original, that is 0.48 M. Although for each condition of incubation in Table 2 only one block of meat was used, and confirmation of these data is necessary, there is a strong suggestion that chloride ions have penetrated much or all of the water in the meat.

Changes in structure

The various structural changes and their observed incidence are listed in Table 3. As this is a preliminary report of work in progress, it will be appreciated that more examples of the phenomena listed may be revealed by more extensive examination.

In both fresh and aged controls Z-lines were observed as continuous electron-dense structures with thin filaments attached to them (Fig. 2). Nine of the twelve treatments produced break-up of the Z-line along its length, as illustrated in Fig. 3. This phenomenon was detected at a distance of at least 0.15 mm beneath the surface of the tissue block. A similar but less definite change in appearance was observed in two other treatments, with the Z-lines being less dense than in the controls. Change in Z-line structure was the most common of the phenomena related to the action of salt with or without pyrophosphate at high or low pH.

In these experiments we were concerned to determine whether there was any evidence of swelling in the myofibrils. It was of great interest, therefore, to discover that frequently there was a loss of density in these structures at the surface. In fresh tissue incubated in sodium chloride at low pH, myofibrils at the edge of the block showed considerably reduced density (Fig. 4). The appearance of myofibrils situated more deeply in the tissue resembled that of the controls.

The plane of the section illustrated in Fig. 4 was perpendicular to the exposed surface of the block of tissue. Since loss of density in the appearance of myofibrils is a feature which was commonly observed, it is important that possible interpretations are considered. First, the A-band is intact but increased separation between the filaments due to swelling has occurred. Such a reduction in packing density would give rise to loss of density in the electron image. The second possible interpretation is that the thick filaments of the A-band have been extracted and the spaces between the thin filaments are more clearly seen. This will also have the effect of reducing the density of the image. In Fig. 4 the reduced density of the uppermost myofibril is likely to be due to the first of these possibilities, since even with isolated myofibrils, this concentration of sodium chloride does not extract the A-band (Offer and Trinick, 1983).

Swelling was also observed in myofibrils in which the long axes were perpendicular to an exposed surface of a treated block of tissue. Fig. 5 illustrates this phenomenon. The exposed surface is represented by the cut ends of the myofibrils on the right hand side. Here the myofibrils have a lower density, the filaments being more clearly visible with greater separation between them, than those in the sarcomeres in the left half of the field.

It was of special interest to determine the extent to which the A-band was extracted. The removal of myosin from the A-band was observed in fresh and aged samples incubated in the presence of salt and pyrophosphate at high pH. Under these conditions extraction occurred at the ends of the A-band, leaving shortened filaments with the M-line conspicuously visible. Figs. 6 and 7 illustrate an example of these changes in myofibrils from aged meat incubated in salt and pyrophosphate at high pH. Trinick and Cooper (1980) described a similar effect with thick filaments of rabbit *M. Psoas* exposed to increasing concentrations of potassium chloride. In our experiments shortened filaments were found to vary in length from about 1.0 μm down to 0.2 μm , the lower end of the range corresponding to the stubs described by Trinick and Cooper (1980). In Fig. 6 thick filament stubs and M-line are clearly visible, while in Fig. 7 a further loss of A-band material has resulted in a reduction in the length of the stubs. The M-line, however, is still preserved. Further removal of A-band material, including the M-line is shown in Fig. 8 which depicts strings of I-bands in which the thin filaments are somewhat irregularly arranged. Fig. 9, which is a view of many myofibrils at low magnification, shows that the extent of extraction of A-band material, that is whether the extraction was complete or produced stubs, is a variable phenomenon with the distribution of shortened thick filaments being random. In the area

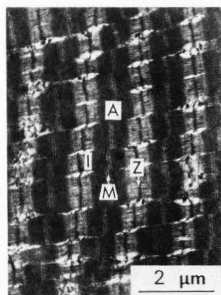


Fig. 2 Fresh muscle - control. A : A-band; I : I-band; Z : Z-line; M : M-line.

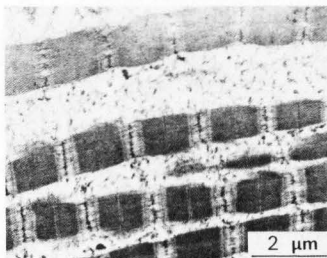


Fig. 4 Fresh muscle - incubated in sodium chloride, pH 5.5 for 24h. The myofibril at the top of the micrograph is adjacent to the cell membrane. Note the reduced density in this myofibril and the break-up of Z-lines.

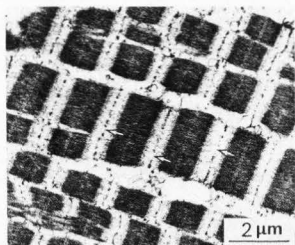


Fig. 3 Fresh muscle-incubated in sodium chloride, pH 5.5 for 5h. Note the break up of Z-lines (arrows).

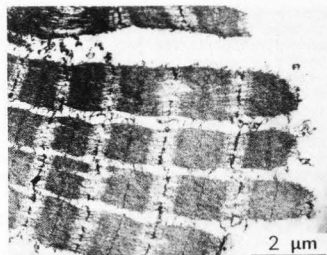


Fig. 5 Aged muscle - incubated in sodium chloride, pH 5.5 for 5h. Myofibrils near exposed surface of meat (right hand edge) show loss of density, probably due to swelling.

illustrated it appears that A-bands have been completely extracted from most sarcomeres. Extraction of the A-band was detected at a distance from the surface of the tissue of at least the width of a fibre after 5 h incubation in salt and pyrophosphate at pH 8.0.

With salt plus pyrophosphate at pH 5.5 extraction of A-band material still occurred (Fig. 10). In the top right of the figure there is an area at the surface where the myofibrillar structure seems to have been completely eroded. In the sarcomeres in the left hand part of the figure however, the extraction of the A-band is incomplete and the shortened A-bands are wider than seen in Figs. 6 and 7 and have very irregular edges. Examination at low magnification suggested that extraction of the A-band occurred in myofibrils close to the surface of the meat block but could not be detected at greater depths. This point requires further investigation because, particularly in shortened sarcomeres, it is not always possible to tell whether thick filaments are present or absent. With salt alone no extraction of the A-band was seen, even at the surface of the meat blocks.

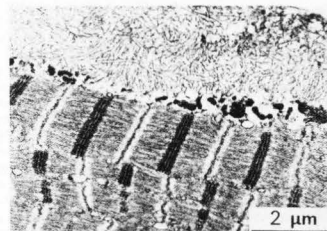


Fig. 6 Aged muscle - incubated in sodium chloride plus pyrophosphate, pH 8.0 for 5h. Thick filaments are reduced in length but myofibrils are still recognisable. Beneath the cell membrane (top) is array of dissociated filaments with occasional collagen fibril.

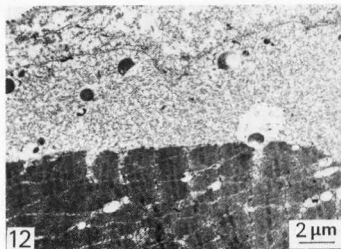
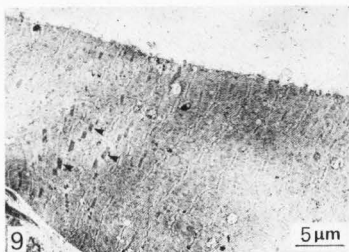
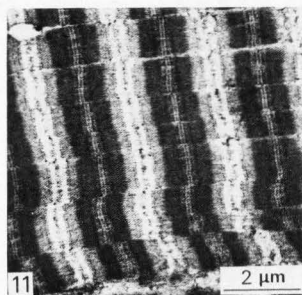
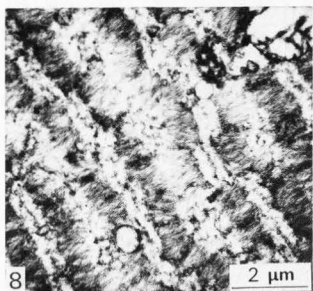
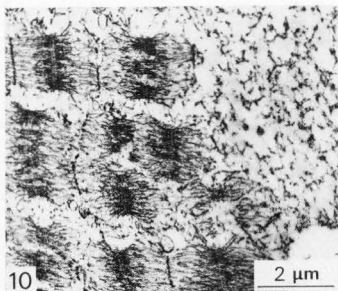
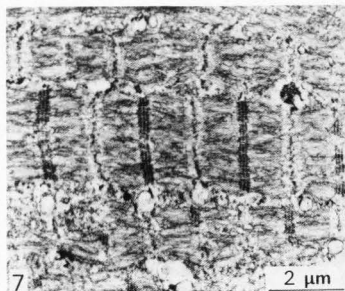


Fig. 7 Same sample as Fig. 6. Myofibrils have undergone further extraction to form shorter thick filaments - 'stubs'.

Fig. 8 Fresh muscle - incubated in sodium chloride plus pyrophosphate, pH 8.0 for 24h. Note the loss of thick filaments and M-line. Z-line is indistinct with detachment of thin filaments.

Fig. 9 Aged muscle - incubated in sodium chloride plus pyrophosphate, pH 8.0 for 5h showing scattered distribution of 'stubs' (arrows).

Fig. 10 Aged muscle - incubated in sodium chloride plus pyrophosphate, pH 5.5 for 5h. Note reduction in length of thick filaments and disorganised structure on the right.

Fig. 11 Fresh muscle - incubated in sodium chloride, pH 5.5 for 24h. Note the regions of low density between broken Z-lines and I-bands.

Fig. 12 Fresh muscle - incubated in sodium chloride plus pyrophosphate, pH 5.5 for 5h showing accumulation of amorphous material near the cell membrane. A similar but less extensive deposit was observed in the control.

In normal vertebrate skeletal muscle there is a continuity between the thin filaments of the I-band and the Z-line, as illustrated in Fig. 2. A region of very low density, of width 0.2 μm on either side of the Z-line, was commonly observed in fresh meat incubated for 24 h in sodium chloride at low pH (Fig. 11) and also in fresh and aged meat incubated in salt and pyrophosphate at high pH (Figs. 6 and 8). The area in Fig. 4, also illustrating fresh meat incubated for 24 h in sodium chloride at low pH, did not show this phenomenon. Occasional filamentous material could be seen in the region of low density. A similar phenomenon was illustrated by Walcott and Ridgway (1967) in Fig. 1 of their paper describing the ultrastructure of myosin-extracted striated muscle fibres. No discussion of the nature of this region was offered by Walcott and Ridgway (1967).

A possible explanation of this phenomenon is the weakening of the attachment of thin filaments to the Z-line, resulting eventually in the detachment of the thin filaments. This explanation is suggested by the appearance shown in Fig. 8 where a clear demarcation may be observed between the thin filaments and the region of low density adjacent to the rather poorly defined Z-line. However it is difficult to explain why the regions should be of constant width. Alternatively, it is possible that thin filaments are still anchored in the Z-line but loss of density is due to the selective extraction of part of a third kind of filament (Wang, 1982) in this region.

In view of the presence of a sticky exudate on the surface of salt-treated meat subject to mechanical agitation, it was of interest to determine whether any material was present close to the sarcolemma. Amorphous material, often in large quantities, was observed inside as well as outside the cell membrane in fresh tissue incubated in salt and pyrophosphate at low pH (Fig. 12). Similar material was also seen in the controls but it was present in smaller amounts; none was observed after incubation in salt only at low pH.

Incubation at high pH favoured the release of structures from myofibrils nearest to the exposed surface of the block of tissue. These structures retained a morphology resembling that of thick filaments (Figs. 6, 13 and 14). In Fig. 13 it can be seen that there is a gradient of structural change from the left to the right of the micrograph. Compact structure is maintained in the deep myofibrils. Nearer the surface there is increasing evidence of swelling, with greater separation between the filaments. At the surface some filaments are no longer part of the organized structure. In another area a considerable disarray of filaments was observed at the end of a muscle fibre in a block of tissue incubated in salt and pyrophosphate at high pH (Fig. 14). This is an interesting contrast with the area shown in

Fig. 6 in which the same treatment but for shorter time results in a sharp demarcation between myofibrils exhibiting A-band extraction, producing short lengths of thick filaments, and a clearly localized region containing dissociated filaments in disarray.

A common phenomenon in these experiments was the appearance of large vacuoles close to the surface of the meat blocks, especially those incubated in salt and pyrophosphate. Closer examination showed that the vacuoles were grossly swollen mitochondria since cristae could be recognized. Some were swollen to the point of rupture (Fig. 15), having increased in diameter nearly twofold. The swelling was greatest in areas of tissue nearest to the exposed surface of the block of meat; further in towards the centre of the meat block the mitochondria were not so enlarged (Fig. 16). Swelling was, however, detected to a depth of about 1 mm from the surface of the tissue. Swollen mitochondria were present in all treatments containing pyrophosphate and also in fresh tissue incubated with salt only for 24 h, but none were observed in the controls. Our colleague, Dr K.S. Cheah, (personal communication) has also observed the phenomenon in bacon.

Discussion

Extraction of A-band

Our objective in examining the structure of meat blocks incubated in salt with or without pyrophosphate was to determine whether the structural changes observed with isolated myofibrils also occurred with pieces of meat.

We have shown that with salt plus pyrophosphate at high pH the A-band is either completely extracted or nearly completely extracted to at least the depth of a fibre. At lower pH with salt plus pyrophosphate the A-band was only partially extracted near the surface of the meat and extraction did not occur at deeper levels. In the absence of pyrophosphate no extraction of the A-band could be observed. These observations are broadly consistent with those of Offer and Trinick (1983), made on isolated myofibrils. They reported that in the presence of pyrophosphate at pH 5.5, 0.4 M NaCl was sufficient to extract protein from the end of the A-band. The extraction was either complete or nearly complete leaving a region close to the M-line. In the absence of pyrophosphate the A-band was either not extracted or extracted very incompletely at its centre, a substantially higher concentration of NaCl (0.6 - 0.8 M) being required to effect extraction. Pyrophosphate was presumed to act as an analogue of ATP and by dissociation of actomyosin to allow the thick filaments to depolymerise at a lower salt concentration.

It may be readily understood why there should be differences between the present results and those of Offer and Trinick (1983)

with regard to the degree of extraction of the A-band. In the experiments of Offer and Trinick the myofibrils were treated with a large volume of salt solution so that if there was any appreciable dissociation of thick filaments, the myosin molecules would be removed and eventually the thick filaments would be extracted. In the present experiments the volume of brine was limited so that only if the position of equilibrium lay strongly in favour of dissociation would extraction be observed. It should also be noted that the salt concentration fell during the incubation to a level below the threshold required for extraction even in the myofibril experiments.

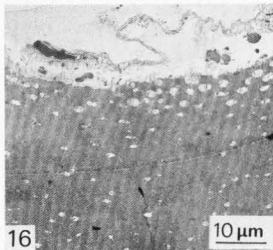
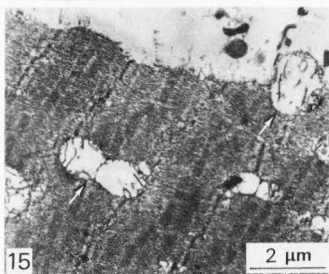
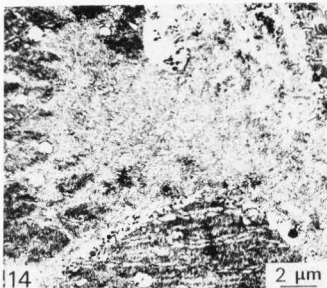
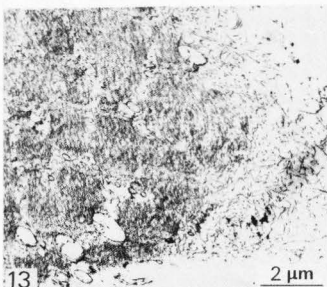
The difference between the depths to which extraction occurred in salt plus pyrophosphate at high and low pH may be explained in terms of the highly co-operative equilibrium that exists between myosin molecules and filaments (Josephs and Harrington, 1966). Below a certain critical protein concentration, 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. The extent of depolymerisation of thick filaments is thus highly dependent on the protein concentration; salt conditions effecting complete depolymerisation in a dilute suspension of myofibrils may achieve only a small degree of depolymerisation in a more concentrated system. When salt diffuses into the meat block the thick filaments will tend to depolymerise. The myosin molecules produced will diffuse out into the salt solution and a gradient of concentration of myosin molecules will be established in the block. But as myosin molecules diffuse away from the surface of the meat they will be

Fig. 13 Aged muscle - incubated in sodium chloride plus pyrophosphate, pH 8.0 for 24h. Structures resembling thick filaments have been released from peripheral myofibrils.

Fig. 14 Aged muscle - incubated in sodium chloride plus pyrophosphate, pH 8.0 for 24 h. Sarcomeres at the ends of myofibrils have been destroyed leaving filaments in disarray.

Fig. 15 Fresh muscle - incubated in sodium chloride plus pyrophosphate, pH 5.5 for 5h. Note swollen mitochondria (arrows).

Fig. 16 Fresh muscle - incubated in sodium chloride plus pyrophosphate, pH 5.5 for 5h. Swollen mitochondria were observed in peripheral fibres in nearly all treatments (see Table 3).



replenished by depolymerisation of more thick filaments; this reduces the depth over which the gradient of myosin molecules occurs. The effect is that the myosin molecule concentration is maintained constant at the critical concentration over a substantial part of the meat block and only in a region near the surface does the myosin molecule concentration fall below this level: in this region the thick filaments must all be depolymerised. The extent of this region will be dependent on the equilibrium constant for the depolymerisation and will therefore be highly dependent on the ionic conditions. Since the thick filament is more easily depolymerised at higher pH (Josephs and Harrington, 1966) the greater depth to which the A-band is extracted can be qualitatively explained.

Extraction of myosin from the A-band depends not only on the depolymerisation of the thick filament but on dissociation of myosin from the actin filaments. It is therefore of interest that despite the very high local concentration of actin filaments present in the meat blocks, extraction of the ends of the A-band to give stubs occurred under ionic conditions not greatly different from those used by Trinick and Cooper (1980) who were examining the depolymerisation of individual thick filaments.

Swelling

The most important observation from the work on isolated myofibrils (Offer and Trinick 1983) was that the myofibrils swelled very substantially either in salt alone or with pyrophosphate. It is technically much more difficult to detect swelling in the meat blocks. In part this is because there is shrinkage of specimens during the preparation for microscopy; in part it is because the longitudinal sections sample the hexagonal lattice randomly rather than along a fixed crystallographic plane so that interfibrillar spacing cannot be measured; in part it is because in the meat blocks one cannot observe a myofibril before, as well as after, the salt incubation. Nevertheless, we have observed a reduction in density in myofibrils very close to the surface (Fig. 4) which was probably due to swelling, and in other specimens we have observed a large spacing between filaments in such peripheral myofibrils (Figs. 13,15). There are strong grounds for supposing that moderate swelling occurred at much greater depths. The fall in concentration of the salt solutions suggests that the sodium chloride penetrated a substantial part of the meat blocks. Furthermore, the blocks exhibited a zone, extending a substantial distance (1-2 mm) from the surface, that was markedly more translucent than the remainder of the block. In view of the substantial water uptake of the blocks, resulting in increases in weight of up to 23%, the most reasonable explanation is that the translucent zone is a region where the muscle fibres, and presumably the constituent myofibrils, have swollen to a

maximum extent of 37%. This water uptake could be accounted for by only a 17% increase in the filament lattice spacing in this zone which we could not expect to detect in the present work.

Changes in Z-line

Offer and Trinick (1983) noted in their experiments on myofibrils that as a result of salt treatment the Z-line of most myofibrils swelled as much as the A-band and became very weak. In some myofibrillar preparations, however, although the A-band swelled, the Z-line did not and the myofibrils were pinched-in at each Z-line. Such observations suggest that the Z-line tends to resist myofibrillar expansion. It seems likely that the fragmentation of the Z-line we have observed is a structural change imposed on the Z-line when it is forced to swell in response to the swelling of the rest of the myofibril. It remains a possibility, however, that the fragmentation is due to partial extraction of the Z-line material.

Mitochondrial swelling

We observed very marked swelling of the mitochondria, particularly in the presence of pyrophosphate at pH 8. We are not aware of this phenomenon being described before and it raises the question of whether such swelling contributes substantially to water uptake. The swelling was marked only near the surface of the meat blocks and even in examples such as is shown in Fig. 16 the fraction of the area occupied by sectioned mitochondria is only of the order of 5%. It therefore seems unlikely that more than a small fraction of the water uptake could be due to this source.

Material near cell membrane

In commercial practice massaging and tumbling are processes used in the manufacture of meat products. A sticky exudate is formed on the surface of the meat pieces that serves to bind them together. It was therefore of interest that we observed material deposited inside as well as outside the cell membrane under most treatments. It is of particular interest and possible practical importance that the morphology of extracted material was influenced by pH. At low pH this material was amorphous but we observed filaments after incubation in salt plus pyrophosphate at high pH. These latter conditions would be expected to be those showing the best binding between meat pieces according to the experience of our own and other laboratories. The nature of these filaments awaits investigation.

Comparison with previous work

Our results may be compared with those of Lewis and Jewell (1975) who studied the effect of incubating meat blocks with salt and/or polyphosphate although there are some differences in the treatments. Lewis and Jewell mainly employed brine to meat ratios of 1:1; they used tripolyphosphate rather than pyrophosphate and the pH of their brine was not defined. In 4% salt alone they observed extraction of the A-band and commented on a loosening of structure. Our results are thus

very similar. With 4% salt plus 1% tripolyphosphate they concluded that the largest change was in the H-zone where the filaments became disorganised. This conclusion disagrees with our observations, where in the presence of pyrophosphate the A-band was depolymerised from its ends. However, their Figure 5 shows myofibrils in which the thick filaments have been extracted leaving strings of I-bands. This result, rather than their conclusion, is in agreement with our own results.

Future objectives

The observations we have recorded here represent only the start of an investigation of the effect of salt on meat structure. In future work we plan to examine more fully the changes in structure between the exposed surface and the deeper parts of the tissue in order to establish the relation between the gradients of sodium chloride concentration, of pyrophosphate concentration, of A-band extraction, of Z-line fragmentation and of swelling. We intend to determine the extent of swelling of the filament lattices at various depths by examination of transverse sections cut from a transverse slice through the centre of the block. Finally we intend to explore the relation between the deposits of material at the surface of the block and the sticky exudate.

Acknowledgements

The authors are grateful to D.J. Restall for drawing their attention to swollen vacuoles which he observed by light microscopy in salt-treated muscle. They also wish to acknowledge the skilled technical assistance of A. Cousins, Anne Phillips and R. Angell in the preparation of material for electron microscopy and in chemical analysis. The authors wish to thank Dr P.J. Knight for discussion and helpful comment.

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Discussion with Reviewers

R.G. Cassens: Do the authors believe that the solutions used preclude completely the action of proteolytic enzymes?

S.H. Cohen: Could you very briefly discuss the roles of CAF (calcium-activated factor) and cathepsin enzymes in what might be a synergistic action with salt and pyrophosphate on myofibrillar degradation?

Authors: We would certainly not suppose that proteolysis was absent in our experiments. There are two stages at which proteolytic activity may occur. The first is during the post-slaughter period, before incubation of the meat pieces in brine. The second is during the incubation period. In our controls with no salt treatment the Z-line was intact after 24 h, although a small amount of degradation occurred after 72 h. Therefore the very considerable fragmentation of the Z-line which we observe must arise during the salt treatment. This fragmentation could result from (a) mechanical damage due to swelling of the myofibrils, (b) extraction of Z-line material by salt, (c) enhanced proteolytic activity in the presence of salt as the Reviewers suggest. However, for the following reasons we think (c) is unlikely.

First, the calcium-activated neutral proteinase (CANP or CAF) is active in the pH range 6.5 - 8.0 but pyrophosphate acts as a calcium binding agent and therefore the activity of this proteinase would be expected to be much reduced.

Second, cathepsins are active below pH 5.5, cathepsin's B and D degrading myosin at pH 5.5. Lysosomes are stable at high salt concentrations (personal communication - Dr D.J. Etherington). Therefore, under the high salt conditions used in this experiment it is unlikely that there will be more lysosomal damage than in the untreated controls and the level of released catheptic activity would not be expected to be elevated.

R.G. Cassens: I have noted that the authors plan considerable future work but no consideration is to be given to nitrite. This is rather surprising since nitrite is very often used in combination with salt in the meat industry. What is the rationale?

Authors: Nitrite is added to meat products in order to enhance flavour and colour and to minimise microbiological activity. It has been reported by Fox et al. (1980) that nitrite in the presence of sodium chloride brings about changes in the interfibre spaces, particularly involving the endomyosium. Our present concern is with water uptake and extraction of protein. So far as we are aware, nitrite is not involved in these processes. Our aim is to work with model systems and we prefer to keep the conditions as simple as possible.

Ref: Fox Jay B Jr, Rorer FP, Fiddler RN, Carroll RJ, Wasserman AE (1980) J. Food Sci.

45, 1056-1057, 1059.

S.H. Cohen: Have you ever employed laser diffraction to help in the study of this problem? Do you think the uniformity of first order band width might be used as an indicator of how effective the treatment is?

Authors: Laser diffraction is normally used to measure sarcomere length, that is the periodicity along the fibre axis. If as a result of the salt treatment, this periodicity is disturbed, then the layer lines will appear less sharp. However, we think that this disturbance is unlikely since in the myofibril experiments of Offer and Trinick (1983) swelling of the myofibrils took place at constant sarcomere length.

It might conceivably be possible to use light diffraction to measure the average width of myofibrils, but the method has not been well worked out. Extraction of protein from part of the sarcomere, such as the A-band, will alter the variation of scattering along the sarcomere and hence change the relative intensities of the layer lines. Again however, the method remains to be explored.

J.J. Smith: Are structural changes or is myoglobin extraction responsible for translucent zones?

Authors: The translucent zones are paler in colour and scatter light to a lesser extent than the rest of the meat. Solutions of soluble proteins (including myoglobin) do not scatter light very much and we would not expect extraction of such proteins to cause an appreciable reduction in light scattering. Extraction of myoglobin would, however, account for the pale colour. We think it is likely that the diminished scattering is due to structural changes, for example myofibrillar swelling. This is an important problem which we are investigating.

J.J. Smith: Why was an attempt made to limit fibre shortening? Other variables such as pH, salt solution, volume and extraction temperature were chosen to be similar to commercial meat processing.

Authors: It is difficult to determine the fate of thin filaments if they are completely overlapped by thick filaments, as will occur in shortened fibres. Our aim in limiting fibre shortening was to minimise this overlap.

D.F. Lewis: Do you recommend sodium acetate and magnesium chloride as useful additives to curing brines?

Authors: No. They were included in these experiments to provide a comparison with the experiments of Offer and Trinick (1983) on myofibrils. We hope it will be appreciated that our experiments were designed to investigate the structural changes occurring on salt treatment and even though they resemble commercial conditions much more

closely than did the myofibril experiments, they differ from commercial conditions in using a much greater brine to meat ratio (3:1).

Magnesium chloride was included in the brine to avoid excessive dilution of the magnesium ions in the meat. Acetate was included as a buffer.

D.F. Lewis: Do you consider it a possibility that the region of low staining density on either side of the Z-line, described in several of your micrographs, represents loss of thin filaments, these remaining intact where they overlap with thick filaments in the A-band?

Authors: The production of the regions of low staining intensity is something of a puzzle and our current interpretation of these regions can only be tentative. Figure 11 shows these regions well. The dark staining bands are of a similar width to the A-bands of untreated meat (Figure 2) and we therefore assume that they are in fact A-bands. If this is correct the regions of medium staining intensity between these dark bands and the regions of low staining intensity are presumably regions of non-overlapped thin filaments. The reviewer suggests that the lightly stained regions represent regions where the thin filaments have been extracted. While we cannot exclude this interpretation, we do not favour it for several reasons. Firstly, it is not clear why the entire length of the thin filaments not overlapped by thick filaments is not extracted. In Figure 8 all the A-band is removed; there is a gap on either side of the Z-line but clear arrays of unextracted thin filaments may be seen. Secondly, using SDS polyacrylamide gel electrophoresis on supernatant and residue of extracted myofibrils exposed to 0.6M sodium chloride at pH 5.5, Offer and Trinick (1983) did not detect any loss of actin from the residue, nor its presence in the supernatant.

D.F. Lewis: Figure 7 shows some myofibrils where the M-line and pseudo H-zone have been largely extracted as seen, for example in the upper and lower myofibrils in the micrograph. In these myofibrils the filaments that are seen occupy the region between the H-zone and the I-band. In untreated meat this region contains both actin and myosin filaments. The range of thicknesses of the filaments in this region is consistent with both actin and myosin still being present. Why do you not even consider the possibility that both types of filament are present?

Authors: The evidence of SDS gel electrophoresis indicates that salt plus pyrophosphate readily extracts myosin. The greatly reduced density of the region that contained the A-band in Figure 7 also shows that most of the myosin in these myofibrils has been extracted. We suggest therefore that

the residual filaments are thin actin-containing filaments and that these may be clumped, as described by Lewis and Jewell (1975), thus presenting a greater thickness than that associated with discreet thin filaments. But we would not want to discount the possibility that some myosin remains in molecular form attached to actin filaments.

D.F. Lewis: Some of the "dissociated fibres" on the right hand side of Figure 6 show distinct collagen-type banding (view at X20 to see). This indicates that they are outside the cell membrane. Is it possible that the other "dissociated fibres" are derived from connective tissue proteins?

Authors: Dr Lewis is right to point out that in the region shown in Figure 6 there are a few collagen fibres. Some are in longitudinal orientation and a few are transverse. Those displayed in longitudinal section exhibit a very fine banding pattern, characteristic of collagen. These fibres are of uniform width with a sharp outline and resemble endomyosial collagen. The majority of the filaments in this region are less wide, have a frayed outline and do not exhibit periodicity so we do not think they are collagen. It is likely that in rigor the cell membrane is ruptured. Therefore it is not surprising to find extracellular collagen mixed with filaments of myofibrillar origin.

D.F. Lewis: Sarcoplasmic proteins constitute around 30% of the proteins in the meat cells. What effects do you think your treatments have on sarcoplasmic proteins?

Authors: Sarcoplasmic proteins are soluble in water or dilute salt solutions. They represent a complex mixture of about fifty components, many of which are glycolytic enzymes (Lawrie, 1979). It is likely that much of this material is washed out of the muscle fibres, as indicated by the presence of myoglobin in the brine after extraction. At least one sarcoplasmic protein (phosphofructokinase) precipitates at pH 5.5 but the amount of this protein is not large and precipitation would already have occurred before the salt treatment. Ref: Lawrie R.A. (1979) Meat Science, Pergamon Press, Oxford, 3rd Edn. Chapter 4.

D.F. Lewis: Siegal, Theno and Schmidt (1978) examined the composition of exudate from tumbled hams using brines containing 3% salt with and without 0.5% of a commercial polyphosphate preparation. They reported similar proportions of myosin and actin in the exudate and found little change in these values in the presence of polyphosphate. If your interpretation of your micrographs is correct would you not have expected them to have found an increased proportion of myosin when phosphate was present in the brine?

Ref: Siegal DG, Theno DM and Schmidt GR (1978) J. Food Sci. 43, 327-330.

Authors: In their experiments Siegal et al centrifuged the sticky exudate and washed the pellet several times. Unfortunately, they discarded the supernatants and the material they examined by SDS gel electrophoresis was therefore not the soluble proteins present in the sticky exudate but the insoluble residue. Not surprisingly, therefore, this material contained a high proportion of actin. This result is misleading in terms of observed structural changes. If the soluble proteins in the supernatant had been subjected to analysis we would have expected a higher proportion of myosin and a lower proportion of actin to be detected.

D.F. Lewis: To what factors do you attribute the difference in structural behaviour between your 24 h and 72 h post-mortem meat? In particular did you notice a pH change or any signs of autolysis in the unsoaked meats?

Authors: In our observations the differences in structural behaviour between 24 h and 72 h post-mortem meat are linked with the pH of the incubating medium. As shown in Table 3 total extraction of A-band material occurs only in 72 h post-mortem meat at pH 5.5 whereas this effect is observed in both 24 h and 72 h post-mortem meat at pH 8.0. We have already discussed the possible role of cathepsins and CAF in the changes described but further observations need to be made in order to obtain a fuller understanding of the mechanisms involved.

We did not observe any substantial signs of autolysis in the unsoaked meats and we do not have any data on changes in pH in the brine or the meat.

D.F. Lewis: Lewis and Jewell (1975) described work with constant ionic strength systems but varying the proportions of polyphosphate and chloride. In your work the total ionic strength of each system is different. What effect do you think this has on the meat structure?

Authors: It has long been known (Bendall, 1954; Hamm, 1960) that both polyphosphate and chloride ions have specific effects. In such a system the total ionic strength is of lesser importance than the individual concentration of these ions.

From the pH values of pyrophosphoric acid at high ionic strength (van den Oord and Wesdorp, 1978) we can calculate that for the medium with pyrophosphate at pH 5.5 the total chloride concentration was 0.62M and the ionic strength $\mu = 0.68$. For the medium with pyrophosphate at pH 8.0 the corresponding values were 0.60 M and 0.71, and for the medium without pyrophosphate, 0.60 M and 0.61. Therefore, in any case, the difference in chloride concentration and ionic strength between these media was small.

Ref: Bendall, JR (1954) J. Sci. Fd Agric. 5, 468-475. The swelling effect of polyphosphates on lean meat.

van den Oord AHA, Wesdorp JJ. (1978) Proc. 24th European Meeting of Meat Research Workers, Kulmbach, Paper D13. The specific effect of pyrophosphate on protein solubility of meat.

D.F. Lewis: Lewis and Jewell (1975) described work with animals of different ages (from 18 to 24 weeks) of different breed and sex and at post-mortem storage times of 24 and 48 h. Their main finding was that after heating meat soaked in salt and polyphosphate, the region of the sarcomere that was least dispersed was the region where the A and I band overlap. They noted some variability in the behaviour of the M-lines between different samples. As a result of your studies on meat of a "well defined age", which factors do you consider contribute most to the variation in M-line behaviour?

Authors: This question raises a number of issues. First, our phrase "well defined age" refers to the post-mortem history of the meat rather than the physiological age of the animal. Second, we also observed variations in the behaviour of M-lines in our samples from a single animal. In order, therefore, to determine whether this variation depends on age, sex or breed we would need to examine many more samples.

D.F. Lewis: Regarding your analysis of the incubating media, a) does the precipitated protein in the brine contribute to the protein level measured by the biuret method or is only soluble protein measured? b) do the calculations on theoretical chloride levels include any allowance for chloride added as hydrochloric acid to adjust the pH? c) is chloride bound to the precipitated protein in the brine included in the measured chloride value?

Authors: In removing aliquots for measuring protein levels the incubating medium was well mixed. It is therefore to be assumed that any precipitated or suspended protein in the sample analysed will contribute to the measured protein concentration.

With regard to the chloride levels expected for complete equilibration, the additional chloride contributed by the hydrochloric acid required for neutralising the pyrophosphate is, as mentioned above, very small (0.012 M for the pH 5.5 medium, 0.001 M for the pH 8.0 medium). Thus the calculated chloride concentration of 0.48 M expected for complete equilibration with the medium lacking pyrophosphate would be essentially unchanged with the pyrophosphate-containing medium. In this medium the chloride concentration would be 0.49 M at pH 5.5 and 0.48 M at pH 8.0.