SESSION 3 - PROGRESS IN THE BIOCHEMISTRY, PHYSIOLOGY AND STRUCTURE OF MEAT

Progress in the biochemistry, physiology and structure of meat

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This is a particularly exciting time to review the advances that have been made in this area of meat science, many of which have important implications for our understanding of the eating quality of meat. With some notable exceptions, there was perhaps a tendency in the past for meat scientists either to concentrate at the fundamental end of the research spectrum or at the applied end. What we have been seeing over the last few years is a growing tendency for this gap to be bridged. Increasingly therefore, the fundamental mechanisms responsible for the important phenomena in meat science are being tackled and clear hypotheses formulated.

I believe one of the reasons for the advances is the growing realisation of the importance that a structural approach - not excluding observation with the naked eye - can make to meat science. If we know the structure of the system we are dealing with, we will know the spatial relationship of its components. Such knowledge sets many constraints on how the system could behave, for example what components a given component could interact with, and aids our thinking by eliminating other possibilities. Observation may also directly tell us where a process is occurring. Our mental processes are helped because most of us think in pictures and we can translate our micrographs into simple diagrams. In this respect we are of course at an enormous advantage compared with other food scientists because we are dealing in muscle with such a highly ordered system. It is a healthy state that the barrier that once divided muscle and meat science is now truly down.

In this review I shall be highlighting some areas where I think substantial progress has been made. The choice is necessarily a personal one and limits of space preclude me from covering all the topics that merit attention.

The structural basis of texture

Texture is the most important aspect of the eating quality of meat but in the past it has been understood only empirically. Recently however, very considerable advances in our understanding of the subject have been achieved.

When meat is chewed in the mouth, it is broken up into smaller pieces. If difficulty is experienced, we describe the meat as tough; if it is easy, the meat is described as tender. The mechanical forces acting on the meat are complex, and are likely to include shear, compressive and tensile forces (see for example Davey and Winger, 1980). To investigate the mechanism of fracture, it is desirable initially to simplify the forces acting on the meat. In particular, the application of tensile forces to materials provides the results which are most amenable to analysis. Carroll et al. (1978) and Purslow (1984) have therefore observed the way in which the components of meat behave when meat is pulled apart by tensile forces and the way in which it is ultimately fractured. Carroll et al. made the pioneering observations of structural changes using both light and electron microscopy but an analysis of the events was not attempted. Purslow has provided a very full description of the changes at the visual level and related these to the mechanical properties of the meat. He analysed the results in terms of fracture mechanics.

When a transverse slice of cooked meat is pulled apart by forces perpendicular to the fibre direction (as may easily be done on a dinner plate), gaps initially develop throughout the slice in regions at the boundaries between one fibre bundle and another (Purslow, 1984). As more force is applied and the slice is stretched, these gaps widen. Some of these gaps then connect up along boundaries between fibre bundles, the crack being bridged at intervals by thin strands of connective tissue. Ultimately fracture occurs along this surface. The fibre bundles remain essentially intact and are not broken into fibres. It is as yet unclear whether the fracture surface lies within the perimysium (the sheath surrounding each fibre bundle) or between the perimysium and endomysium (the sheath surrounding each fibre) as Carroll et al. (1978) have proposed. In either event it is connective tissue that is severed. Since a structure fractures at its weakest point, these experiments identify the connective tissue between fibre bundles as the weakest part of the structure. This idea takes some getting used to as we are accustomed to think of connective tissue as being tough. However, firstly we must remember we are talking of cooked meat and therefore gelatinised connective tissue. Secondly, it is only a plane within the perimysium plus endomysium that is structurally weak; the remainder could be strong.

When a strip of cooked meat is pulled apart by forces along the direction of the fibres, the fibres extend. (For a detailed description of the changes at the sarcomere level see Locker et al. 1977 and Locker and Wild, 1982a,). As more force is applied, the fibre bundles separate from one another and eventually snap across independently of one another (Purslow, 1984). The fracture surface therefore does not simply pass straight across the meat. Rather, much of this surface does not lie in a transverse plane but runs along the boundaries between fibre bundles. Thus the fracture surface may pass between two fibre bundles, then pass fairly cleanly across one of them, then run longitudinally between this fibre bundle and another and so on, with the result that it is stepped irregularly up and down the fibre axis. The axial position within the sarcomere of the fractures across the fibre bundles was not examined by Carroll et al. (1978) or Purslow (1984) but is likely to be in the I-bands (Locker and Leet, 1976b; Locker and Wild, 1982a,). This type of fracture behaviour Purslow points out is typical of composite materials made of strong rod-like elements embedded in a weaker matrix and confirms the view that in cooked meat the muscle fibres are the stronger element.

The strength in the longitudinally applied stress experiments is very substantially greater than for the transversely applied stress experiments. This is due to the fact that fracture across fibre bundles is much more difficult than between fibre bundles. This puts on a rational basis the long-standing practice of carving meat across the grain so that the eater, either with his knife or his teeth, has only to fracture the meat along the fibre direction. In general, however, depending on the shape and size of the meat piece, the ease of fracture will depend both on the strength of the connective tissue and of the fibres. It remains to be explained how variations in the thickness and the network geometry of the perimysium in different cuts of meat affect the strength of fracture and whether conditioning decreases toughness only by its effect on weakening the fibres or also acts on the connective tissue thereby decreasing the adhesion between bundles.

Contribution of collagen to texture

Collagen is the major structural protein of connective tissue. Its biochemical properties influence the strength of the connective tissue and hence the texture of meat. Although the quantity of collagen in a muscle partly determines its toughness (Bransfield, 1977), an equally important factor is the chemical nature of the covalent cross-links between collagen molecules (Bailey, 1972; Shimokomaki et al., 1972; Bailey and Sims, 1976; Bailey, 1979). In young animals these cross-links are a mixture of the keto-imine type and the aldimine type. Both can be detected by introduction of a radioactive label through reduction with borohydride. Keto-imine cross-links predominate over aldimine cross-links, but the ratio is twice as high in the tougher muscles as the tender (Light and Bailey, 1983; Light, personal communication). In older animals these bivalent cross-links are converted to a non-reducible cross-link, the mature cross-link. These are probably trivalent and bind three collagen chains together (Light and Bailey, 1979, 1980a,b; Bailey, et al., 1980). The chemical nature of these mature cross-links has been controversial (for reviews see Tanzer, 1976 and Light and Bailey, 1980c). No single novel component has yet been unequivocally shown to fulfil the role of the mature cross-links in collagen.

By digestion of mature collagen with proteolytic enzymes or cyanogen bromide, it has been possible to isolate high molecular weight polymers containing the mature cross-link (Light and Bailey, 1980a,b). Although the chemical nature of the mature cross-link is not yet certain, a compound has been isolated from acid hydrolysates of these high molecular weight collagen peptides. It is not found in peptides from soluble collagen nor is it associated with uncross-linked collagen fibres and it accumulates in collagen during maturation. For these reasons this compound is thought to be the elusive mature cross-link and is being chemically characterised (Light and Bailey, 1981). Another compound, pyridinoline, has been shown to cross-link cartilage and bone in mature tissue but its role in muscle collagen is not known (Fujimoto et al., 1977; Robins, 1983).

If collagen molecules in solution are heated, they denature to gelatin and in doing so shrink to about a quarter of their former length. Collagen molecules in fibre form also shrink, albeit at a higher temperature. Such a

system is capable of developing considerable tension (Mohr and Wendall, 1969; Shimokomaki et al., 1972; Allain et al., 1978), but only if there is mechanical integrity brought about through covalent cross-links. A collagen matrix which is not cross-linked or which is stabilised by heat-labile aldimine cross-links dissolves forming gelatin on heating and exerts no tension. The keto-imine cross-links are, however, thermally stable and confer stability on the matrix. On heating, most of the collagen does not dissolve, and tension is exerted, the magnitude depending on the number of these cross-links (Allain et al., 1978). The mature cross-link, being multivalent, confers even greater stability on the matrix so that greater tensions are exerted.

Because the isolation of mechanically intact intramuscular connective tissue is relatively difficult, most studies on the thermal contraction behaviour of collagen have used tendon or skin. Recently, however, it has been demonstrated that empty endomysial sheaths shrink both longitudinally and transversely at temperatures greater than 51°C (Light and Bailey, 1983; Light, personal communication).

The texture of meat may depend on the nature of the cross-links for two reasons. Firstly, although we do not know the precise site of fracture when fibre bundles are separated, it seems likely that toughness will depend on the mechanical strength of the heat-denatured, gelatinous fibres of the perimysium or those linking the perimysium and endomysium (Light and Bailey, 1983). This strength will be greatest in the connective tissue of meat from older animals, causing it to be particularly tough. In younger animals the strength will depend on the source of the muscle, being greater in muscles with a higher proportion of keto-imine cross-links. We might expect the toughness to depend on the nature of the cross-links also because these will influence the magnitude of the thermal shrinkage of the meat and hence the number of fibres per unit cross-sectional area.

It is as yet unclear whether the tenderising that occurs on prolonged stewing of meat is due to the slow breakdown of the keto-imine and mature cross-links or to hydrolysis of a small number of collagen peptide bonds.

Water-holding

The water content of meat is important both economically and because it affects consumer acceptability. Water is lost from meat not only by evaporation but by the formation of drip from the cut ends. On cooking very substantial losses occur. By contrast, in meat processing, when meat pieces are treated with salt sometimes with polyphosphate added, gains of weight up to 40% occur. This water uptake is accompanied by the formation of a sticky exudate on the surface of the meat pieces which acts to bind them together on cooking.

Hamm (1960, 1975, 1984) has laid the foundation to our understanding of water-holding. He has pointed out that water bound to the surface of proteins is too small to contribute appreciably to the losses and gains of water from meat. He has also emphasised the importance of myofibrillar proteins in determining water-holding. Likening meat to a polyelectrolyte gel, he showed that the observed effect of pH on water-holding could be explained by the change of charge on the proteins with pH. At the isoelectric pH (~5) the proteins would have zero net charge but at lower or higher pH's the proteins would have a positive or negative net charge and repel one another causing expansion. He made the important suggestion that swelling would be resisted by the links between myosin and actin in the rigor state. Hamm also suggested that the increased water holding in the presence of sodium chloride was due to the binding of Cl^- ions to the myofibrillar proteins, thereby increasing their negative charge (at pH's >5).

While Hamm's work has been seminal to our understanding of the physical chemistry of water-holding, it was not directed at structural questions, particularly what exactly happened to myofibrils either when water was taken up by meat as in processing, or when water was lost. For example Fig. 16.1 of Hamm (1975) would suggest that when water was taken up by meat, the organisation of the myofibril was lost.

Fortunately it is possible by phase-contrast light microscopy to see directly whether salt solutions alter the volume of myofibrils and cause structural changes including extraction of proteins (Hanson and Huxley, 1955). The advantage of this technique is that the myofibrils remain hydrated and there are no artefacts of specimen preservation such as occur with electron microscopy. Offer and Trinick (1983) demonstrated that when rabbit psoas myofibrils were irrigated with salt solutions resembling those used in meat processing, they swelled transversely. The volume increase, although variable, was typically ~100%. This degree of swelling is more than enough to account for the water uptake in meat processing. The results therefore suggest very strongly indeed that myofibrils are the site of water retention in meat processing.

With salt alone at pH 5.5, swelling started at 0.6 M NaCl and was not complete until 1 M NaCl. The swelling was accompanied by partial extraction of the A-band at its centre. In the presence of 10 mM pyrophosphate, swelling occurred at much lower concentrations of NaCl (0.4 M) and extraction of the A-band, was complete (or nearly so) beginning from its ends. The swelling was highly co-operative: no swelling occurred with even prolonged irrigation with 0.3 M NaCl plus pyrophosphate. The maximum swelling of myofibrils at high salt concentrations is unaffected by the presence of pyrophosphate (Offer and Trinick, 1983; Knight and Parsons, 1984). In all cases, even where the expansion of the myofibril was large and there was extraction of the A-band, the myofibril did not lose its structural integrity and arrays of regularly spaced I-bands remained.

Although all rabbit psoas myofibrils examined swelled in salt, there was substantial variation especially between preparations in the degree of swelling, in the salt concentration required for extraction and in the pattern of extraction. Very large differences in the behaviour of beef myofibrils within a preparation has been observed (Knight and Parsons, 1984). This is of interest as such variation may be related to the suitability of meat for processing. Among the variations seen by Offer and Trinick (1983) was that sometimes the Z-line expanded as much as the A-band but in other cases it did not expand at all so that the I-band was flared on either side of the Z-line. This suggests that the Z-line could act as an important constraint to swelling. The swelling of the A-band often exceeded that of the I-band and it seemed possible that only the A-band actively swelled while the I-band followed passively. However, strings of I-bands, obtained by extraction of myofibrils, swelled reversibly in high salt concentrations, showing that both A- and I-bands actively swell.

With regard to the mechanism of swelling, the pioneering work of G.F. Elliott and his co-workers (see Elliott, 1963) has led us to appreciate that the size of the filament lattice depends on a balance between electrostatic repulsive forces between the filaments and attractive forces. The latter are likely to be provided principally by transverse structural constraints such as the attached cross-bridges present in rigor, the Z-line and the M-line. An expansion of the filament lattice, such as occurs in high salt concentrations, could therefore come about either if the electrostatic repulsive forces increased or if the constraining forces diminished or to a combination of these.

Considering first the repulsive forces, Offer and Trinick (1983) followed Hamm (1960) in suggesting that Cl^- ions might bind to the filaments, thereby increasing the negative charge on the filaments. The difficulty with this suggestion is firstly that above a certain charge density on the filaments, probably already present in the untreated muscle, the repulsive force is not increased when a rise in charge density occurs (Millman and Nickel, 1980). Secondly, increasing the concentration of sodium chloride would be expected greatly to increase the screening of the charges (Ledward, 1983; Offer, 1984). It appears that the only way the repulsive forces could be increased would be if the radius of the charges around the filament axis were increased since the repulsive forces are extraordinarily sensitive to this parameter (Millman and Nickel, 1980; Elliott and Bartels, 1982; Millman et al., 1983). It is therefore unclear at present whether or not increases in electrostatic forces are in part responsible for the swelling. The fact that the swelling at high salt concentrations is highly co-operative suggests that removal of transverse structural constraints plays a major role in causing swelling. Offer and Trinick (1983) pointed out that co-operative removal of the cross-bridge constraint could occur either by dissociation of myosin heads from actin (such as would occur in the combined presence of pyrophosphate and high concentrations of chloride) or by depolymerisation of thick filaments (such as would occur in the presence of chloride alone at higher concentrations). The effect of salt and pyrophosphate on the structure of the other potential candidates, the Z-line and M-line, is unknown.

The work described above was done with myofibrils. Complementary studies

have been independently carried out on fibres by Wilding et al. (1984). They found that after about 20 hours post-mortem at 4°C, rabbit longissimus dorsi fibres swelled two or three times in diameter when immersed in hypertonic salt solutions. Very importantly they were able to demonstrate by X-ray diffraction (a non-destructive technique) that the spacing of the myofibrillar filament lattice did indeed increase. One of the most interesting aspects of their work was that the amount of swelling was strongly dependent on the time post-mortem. The swelling showed a peak at 18-35 hours post-mortem, but at times less or greater than this, there was little or no swelling. However, at such times if the endomysium were locally nicked, the fibres swelled out showing that the fibre contents were still capable of swelling. Wilding et al. concluded that the endomysial sheath around a fibre acts as a restraint to swelling and that the amount of swelling with hypertonic salt solutions depends on a balance of the propensity of myofibrils to swell and the constraint of the endomysium. It thus appears that the suitability of meat for processing may depend both on the state of the myofibrils and on the state of the endomysium.

The mechanism of drip formation

The experiments just described clearly suggest that transverse expansion of the filament lattice of myofibrils is the cause of water uptake in meat processing. The question now arises of whether the converse occurs when water is lost from meat, particularly in the formation of drip and cooking loss; that is, does the filament lattice shrink?

Considering first the changes occurring on rigor development, there are two causes of myofibrillar shrinkage, the fall in pH from about 7 to about 5.5 due to glycolysis and the depletion of ATP resulting in the attachment of myosin heads to actin. It has been known for some time since the work of G.F. Elliott and his colleagues that the spacing of the hexagonal lattice changes with a variety of conditions, particularly with pH. Rome (1968) showed in muscle fibres lacking a membrane that a fall in pH from 7 to about 5.5 causes about a 10% reduction in the spacing (corresponding to a 28% reduction in myofibrillar volume).

Using fibres lacking a membrane, the effects on lattice spacing of cross-bridge attachment have now been demonstrated (Matsubara et al., 1984). At a sarcomere length of 2.2 μm there is at constant pH a reduction of about 12% in the filament lattice spacing when relaxed muscle goes into rigor. At longer sarcomere lengths the reduction is smaller, and at a sarcomere length where the thick and thin filaments no longer overlap, there is no change in spacing, as would be expected. Although both studies have been on model systems lacking membranes, one would expect these two factors to apply in intact muscle and for there to be a substantial shrinkage, perhaps as much as 40% of the volume of the myofibrils and hence of fibres, after rigor.

Weffron and Hegarty (1974) indeed showed that, some time after rigor, isolated unfixed fibres shrink by about 15% in diameter. Histological transverse sections of muscle confirmed this shrinkage and revealed a large increase in the extracellular space. Offer et al. (1984) using a silver stain to show up the endomysium have clarified this further and have shown that in the rigor state fibres are shrunken away from their endomysial sheaths leaving water-filled gaps. Sometimes these gaps are all the way round the fibre, sometimes only a part of the way. There is also shrinkage of fibre bundles away from the perimysium so there are substantial gaps around the perimysium often very much wider than the perimysium itself. These features may be seen also in the micrographs of Paul (1965). One should caution that there is always a worry about shrinkage in the preparation of material for microscopy but the X-ray diffraction results provide grounds for thinking that the histological pictures do provide some semblance of the truth.

NMR studies on muscle should give information on the water domains. In the relaxed state the spin-spin relaxation of water protons could be approximately fitted by a single exponential but in rigor there was in addition a slower component (Hazelwood et al., 1969; Belton et al., 1972; Pearson et al., 1974). The classical interpretation for multi-component spin-spin relaxation is that the components arise from domains physically separated from one another. Lillford et al., (1979, 1983) and Wynne-Jones et al. (1981) have pointed out, however, that it is not necessary for the domains to be separated by a physical barrier such as a membrane and that a sample will give rise to two components if there is structural heterogeneity over a distance at least as great as the diffusion distance of water on the NMR time-scale. Microheterogeneity down to ~10 μm can be detected. Further, relaxation is most appropriately described by a continuous distribution of relaxation times rather than by a small number of discrete processes. Lillford et al. (1980) concluded that in the rigor state in beef longissimus dorsi muscle 94% of the water was intracellular and 6% extracellular. It is however unclear whether the histological pictures of rigor and the NMR spectra are really in conflict since it is possible, for example, that the water in the relatively small gaps between fibres contributes by NMR to the "intracellular domain", yet collectively makes a large contribution to the extracellular space.

With regard to drip formation, there are three main questions: (1) from what water compartment of the meat does the water come? (2) down what channels does it flow? (3) what is the nature of the force driving the drip out?

Penny (1975) suggested that drip came from the extracellular water compartment, enlarged after rigor as a result of fibre shrinkage. If after rigor development the cell membrane is ruptured or becomes leaky, the sarcoplasmic proteins would be present in the extracellular space, explaining why drip consists of an aqueous solution of sarcoplasmic proteins including myoglobin (Howard et al., 1960). Currie and Wolfe (1980, 1983) have attempted to measure by the use of inulin the extracellular space of muscle as a function of time post-mortem. They found that the space increased very greatly at or after rigor but they considered that the very high value reached (70% of the available water) was probably due to disruption of the cell membrane.

With regard to the channels, it has been known for some time that drip occurs at the cut ends of the meat. Since the percentage drip decreases as the thickness of slice increases, it is reasonable to suppose that drip flows along channels parallel to the fibres (Howard 1956; Taylor and Dant, 1971). Preliminary observation suggests that drip at the cut surfaces of meat oozes preferentially from the boundary between fibre bundles (particularly the coarser bundles), rather than from the boundaries between fibres; that is it oozes from the region close to the perimysium (Davies, Brown and Offer, unpublished results)). This can be rationalised on the basis that for a fixed pressure difference the flow rate along a channel is very sensitive indeed to its width; for flow along a rigid cylindrical capillary, the Poiseuille equation shows it depends on the fourth power of its radius. Secondly, surface tension forces which oppose the exudation would decrease with the width of the channel. Hence, for both reasons we can see why drip would be expected to travel preferentially along the gaps between one fibre bundle and its neighbour, which, if the histological results are not an artefact of preparation, are the widest channels in the meat. This would explain why the total amount of drip from a muscle is very substantially less than most estimates of the amount of extracellular space.

With regard to the source of the pressure that forces the drip out to the cut ends, there appear to be only two logical possibilities; gravity or an internal pressure generated by the shrinking of some structure. If the sealed top of a vertical capillary filled with water is opened, the water will run at least partly out, depending on the magnitude of the surface tension forces. If only gravity were effective in producing drip, we would expect drip to appear only on the lower surface of a piece of meat and that none would appear on the upper surface. In practice drip is formed on both upper and lower surfaces (Davies, Brown and Offer, unpublished results). However, we find that more drip is formed on the lower than on the upper surface and Howard (1956) observed that more drip is formed when the fibres are vertical rather than horizontal. These results suggest that gravity does make a contribution, but that an internal pressure is also generated.

The only plausible structure that could exert an internal pressure is the network of connective tissue. Offer et al. (1984) proposed that in living muscle the endomysium and the perimysium might be stretched by the fibres they contain. Correspondingly they would exert a pressure on their contents. If the fibres shrink on reaching the rigor state, the endomysium would exert its pressure on the water in the gap between it and the fibre, and the perimysium on the water in the gap between it and the fibre bundle. For reasons explained above, if the latter gap is wider, it will be the preferential route for drip to be squeezed out to the cut ends.

Cooking loss

When meat is cooked, it shrinks extruding fluid rapidly. It seems to be

generally agreed that in intact pieces of meat shortening of the muscle fibres, with accompanying reduction in sarcomere length, occurs at temperatures above 60°C (Paul, 1965; Giles, 1969; Dubé et al., 1972; Davey and Gilbert, 1974a; Locker and Daines, 1974; Bouton et al., 1974, 1976a; Hegarty and Allen, 1975). What is less clear is the change in dimension of the meat transverse to the fibre axis as a function of temperature. For example Davey and Gilbert (1974a) find no change in the cross-sectional area on cooking, Bouton et al. (1976b) a substantial shrinkage and Locker and Daines (1976) a reduction in one transverse dimension and an increase in the other in several muscles.

To aid investigation of the mechanism of cooking loss, the system has been simplified by determining the extent of transverse and longitudinal shrinkage when strips of muscle, fibre bundles and single fibres are heated. With such small systems, temperature equilibrium is rapid and the time course of shrinkage can therefore be studied. Strips or fibre bundles shrink both transversely and longitudinally when heated at pH 5.5, the reduction in length being reflected in a reduction of sarcomere length (Giles, 1969; Bendall and Restall, 1983). The transverse shrinkage starts at about 40°C and becomes more rapid and more extensive with rise of temperature. At 60°C the initial rate corresponds to a 31% volume reduction per minute and the final extent to a volume reduction of 36% (Bendall and Restall, 1983). Longitudinal shrinkage by contrast does not start until the temperature exceeds 60°C. It is rapid and the final length simply depends on the temperature, 80% of the initial length at 70°C and 72% at 90°C. The total volume shrinkage in the temperature range 70° - 90°C from the combined effects of transverse and longitudinal shrinkage is about 60%. Thus these smaller systems simulate the shrinkage shown with large joints.

It is also agreed that single fibres shrink transversely when heated at pH 5.5 (Hostetler and Landmann, 1968; Bendall and Restall, 1983), although at higher pH there is no shrinkage (Aronson, 1966). As with fibre bundles the shrinkage starts at about 40°C and becomes more rapid with rise of temperature. At 60°C the initial rate of shrinkage (40% of the initial volume per minute) and the total extent of shrinkage (50% reduction in volume) are maximal. However, there is disagreement on whether or not at higher temperatures single fibres shrink longitudinally like fibre bundles, Hostetler and Landmann providing evidence that they do, Bendall and Restall that they do not. One possible reason that the latter authors did not observe longitudinal shrinkage is if adhesion of the fibres to the microscope slide was too great to allow shortening. Jeacocke, Restall and Offer (unpublished experiments) therefore suspended single fibres vertically with a small weight at the lower end. Such fibres shorten very substantially on heating, the degree of shortening increasing with temperature and reaching about 50% at 80°C. Using apparatus that allows a rapid rise in temperature, we have shown that longitudinal shortening is very rapid indeed: shortening is largely complete within about 2 seconds of reaching the final temperature. On cooking the stiffness of the fibre diminishes greatly and it is now readily and reversibly extensible to double its length.

The transverse and longitudinal shrinkage of fibre bundles and fibres might occur either by active shrinkage of the fibres themselves or be imposed on them by the connective tissue that surrounds them (the perimysium and endomysium in the case of bundles, the endomysium alone in the case of fibres). It should be emphasised that in the above experiments these sheaths are too thin to be seen. Observation of both components would be worthwhile. One way of determining whether the shrinkage of fibres is active or passive is to examine the effect of heat on myofibrils, which of course lack connective tissue. Isolated myofibrils shrink transversely at temperatures above 40°C and longitudinally at temperatures above 60°C (Aronson, 1965; Offer et al., 1984). This clearly suggests that the fibre shrinkage is active.

Another way of determining whether fibre shrinkage is active or passive is to examine histological preparations of cooked meat. Transverse sections show that the fibres have shrunk away from the endomysium substantially more than in the uncooked rigor state (Paul, 1965; Locker and Daines, 1974a,b; Schmidt and Parrish, 1971; Offer et al., 1984). Thus the gaps between the muscle fibres and their endomysial sheaths present in rigor are considerably increased. This appearance is not much changed in the temperature range 50 to 80°C. This clearly shows that the fibres actively shrink transversely on cooking and not because they are compressed. The gap between the perimysium and fibre bundles seen in the rigor uncooked state remains and indeed often appears to be enlarged (Offer and Restall unpublished results) suggesting that the perimysium is not compressing fibres together. Longitudinal sections confirm that the gaps between fibres and endomysium run long distances along the fibre axis, although at intervals the fibre and its sheath make contact.

We are therefore faced with the fact that in meat we have two components that are both capable of thermal shrinkage; the connective tissue network and also the myofibrils of the fibres. With regard to transverse shrinkage, the evidence is that even in the uncooked rigor state the fibres are not filling the endomysium, nor the bundles filling the perimysium. Moreover transverse shrinkage of the fibres starts at temperatures (~40°C) very much below the thermal shrinkage temperature of the connective tissue. Hence, on heating when the fibres shrink still further, the space between them and their endomysial sheaths becomes even greater. Electron micrographs of cooked meat show that the cell membranes are grossly disrupted, part staying with the fibre and part with the endomysium (Locker and Daines, 1974b). It is therefore understandable that the aqueous solution in the gaps will contain sarcoplasmic proteins at a high concentration.

It needs to be emphasised that transverse shrinkage of fibres per se will not lead to transverse shrinkage of the meat as a whole. The repeating unit that makes up the fibre bundle is the endomysial sheath and its contents, not the fibre. The mere transfer of water from fibres into the gaps between them and their endomysial sheaths will not of itself lead to a change in volume of the meat. Only if the aqueous solution in those gaps escapes to the outside will the meat as a whole shrink transversely. It is therefore not surprising that there should be considerable variation between authors on whether meat shrinks transversely on cooking; the extent of shrinkage will presumably depend on the geometry of the specimen. The quantitative differences between the transverse shrinkage observed in fibres and fibre bundles observed by Bendall and Restall (1983) can be explained on this basis.

As explained above, there appear to be occasional contacts between even shrunken fibres and their endomysial sheaths in the cooked state. Hence the connective tissue and the fibres remain coupled together: if one shrinks longitudinally, they will both shrink longitudinally. The temperatures at which they individually shrink are similar. It has already been pointed out that the endomysium starts shrinking at temperatures above 51°C (Light and Bailey, 1983; Light personal communication) and intramuscular collagen (probably consisting largely of perimysium) shrinks longitudinally at 64°C (Mohr and Bendall, 1969). Although collagen makes up only a small percentage of the total protein in meat, collagen fibres can exert very large forces on thermal shrinkage. At present, therefore, it is unclear whether connective tissue or myofibrils dominate the longitudinal shrinkage of meat.

It was shown by Locker and Daines (1974) that cooking loss increases as the distance between the cut ends is reduced. This suggested that the fluid travels in a direction parallel to the fibres. We have supposed that in uncooked meat drip travels along the annular spaces between fibres and their endomysial sheaths (Offer et al., 1984) but perhaps even more along the spaces between fibre bundles and their perimysial sheaths (see above). Fluid lost on cooking may pass down the same channels (Locker and Daines, 1974a,b; Offer et al., 1984).

The following picture of the changes occurring on cooking can be suggested. At 50°C the sizes of the sub-endomysial and the sub-perimysial channels increase due to fibre shrinkage. This together with the lowered viscosity of water would greatly augment the flow of drip to the cut ends. Transverse shrinkage of the meat will take place in parallel with this loss. At higher temperatures the collagen of first the endomysium and then the perimysium will denature thereby greatly increasing the pressure driving fluid out to the cut ends, so the rate of weight loss will be increased. At this or some higher temperature, the connective tissue network and the muscle fibres will co-operatively shrink longitudinally, the extent of shrinkage increasing with temperature. The relation between transverse and longitudinal shrinkage of muscle fibres and the two phases of toughness development (Davey and Gilbert, 1974a) remains to be explored.

It used to be supposed that the proteins of the myofibril (loosely referred to as actomyosin) denatured at about 50°C, and collagen at about 65°C. Differential scanning calorimetry enables the thermal transition temperatures of the major proteins in meat to be determined. Post-rigor rabbit muscle gave transitions at 60°C, 67° and 80°C ascribed to the denaturation of myosin, sarcoplasmic proteins and actin respectively (Wright et al., 1977). The high transition for actin should make us cautious in attributing shrinkage of meat at high temperature necessarily to collagen and may be related to the

longitudinal shrinking of myofibrils. In a more recent study on purified myosin, Wright and Wilding (1984) have shown that myosin does not denature at a single temperature. It exhibits in general three transitions, the temperatures of which depend on pH and ionic strength. The three transitions appear to be associated with discrete regions of the myosin molecule. At pH 5.5 and low ionic strength, conditions which resemble meat, only two transitions with midpoints at 54°C and 67°C are seen.

Titin and the gap filaments

The sliding filament hypothesis of Huxley and Hanson (1954) and Huxley and Niedergerke (1954) considered the contractile apparatus of muscle to consist of two types of filament, thick and thin. But even at that time there were already indications that a third type of filament might be present. Myofibrils from which the thick filaments have been extracted can be stretched and shorten when released (Hanson and Huxley, 1955). Some structure must therefore extend at least across the H-zone and be extensible. Moreover, if both the thick and thin filaments were extracted from myofibrils, the remaining Z-lines were not liberated into solution but remained in an ordered array. This suggests that the additional set of filaments might join neighbouring Z-lines.

Huxley and Peachey (1961) noticed fine filaments crossing the gap between A- and I- bands in muscle stretched beyond the point of overlap. These gap filaments have been extensively studied (Sjöstrand, 1962; Carlsen et al., 1965; McNeill and Hoyle, 1967; Locker and Leet, 1975, 1976a,b). Their diameter appears rather variable but is about 30 to 40 Å. Locker and Leet (1975, 1976a) have shown that they are very extensible and can bridge gaps as great as 2 μm. The gap filaments appear to be continuous with the ends of the thick filaments(Sjöstrand, 1962; Carlsen et al.; 1965, Locker and Leet, 1975, 1976a). Sometimes there appeared to be the same number of gap filaments as thick filaments (Locker and Leet, 1975) and sometimes only about half as many (Locker and Leet, 1976a). In the latter case no systematic spatial relationship between gap and thick filaments was observed (for example every other thick filament being continuous with a gap filament). It seems possible that the fewer gap filaments seen in the latter case arises either because of imperfect preservation or to proteolysis. Locker and Leet (1976a) also showed that if the myosin of the A-band in stretched muscles were completely extracted, the gap filaments could be seen to span the entire space between neighbouring I-bands. This would suggest that the gap filaments occupy the entire length of the A-band as well as the space beyond. In extracted muscle in which the thick filaments had been greatly truncated but not completely removed, the gap filaments appeared to be continuous with these structures. This suggests that the gap filaments act as a core to the thick filament backbone.

Unfortunately it has not proved possible to see where the gap filaments are anchored at the other end, but they appear to pass between thin filaments. Locker and Leet (1976a,b) have produced evidence that gap filaments are continuous through the Z-line but it is of an indirect nature and cannot be considered definitive. They have interpreted their results to mean that each gap filament forms the core of two thick filaments in neighbouring sarcomeres and is continuous through the Z-line between them. An alternative, which I find more attractive, is that a gap filament forms the core of only one thick filament and connects with both Z-lines on either side of it. This would explain the results of Hanson and Huxley (1955) discussed above. The presence of an elastic filament linking thick filaments to the Z-line would account for the resting tension in muscle and would explain why A-bands are centrally positioned within a sarcomere, contrary to what would be expected from the relationship between isometric tension and sarcomere length (Wang, 1982b; Magid, 1983). Although the evidence for models in which the gap filaments act as a core to the thick filament is persuasive, it is not decisive and other models have been proposed. Wang (1982) has provided evidence that the gap filaments might pass along the surface of the thick filaments. McNeill and Hoyle (1967) observed super-thin filaments in the A-band (including the H-zone) as well as in the gap region in a variety of muscles. In the A-band they were parallel to, but lay in between, the thick filaments. It is clear that more work will be required to define the arrangement of the gap filaments in the sarcomere. Whatever their location one might expect that they would obey the same symmetry elements as govern the rest of the sarcomere, that is to reverse polarity on either side of the M-line and Z-line.

The picture of which proteins might form gap filaments is becoming clearer. Maruyama and his co-workers obtained a preparation by rigorously extracting muscle with solvents including acetic acid, KI, alkali and phenol (Maruyama, 1976; Maruyama et al., 1977, 1980, 1981). The residue, termed connectin, had elastic properties but, because of its insolubility in benign aqueous solvents, could not be purified and fully characterised. Some of the preparation, however, would dissolve in SDS but most of the dissolved protein was too high in molecular weight to enter polyacrylamide gels of normal concentration.

More recently Wang and his co-workers showed that myofibrils contained a substantial quantity of a protein termed titin because of its extraordinarily high molecular weight ($\sim 10^6$) observed on electrophoresis in SDS on polyacrylamide gels of low concentration (Wang et al., 1979; Wang 1982a,b). On such gels titin forms a closely-spaced doublet but the two bands have similar amino acid compositions and cross-react immunologically so they are clearly closely related. To purify titin, myofibrillar proteins solubilised in SDS were separated by gel filtration in SDS, titin being the first protein to be eluted. Titin, like connectin, can also form an elastic gel if the SDS is first removed (Wang and Ramirez-Mitchell, 1979). However, one would like to see proof that the elastic properties of titin and connectin are not merely a consequence of exposure of a protein to denaturing solvents.

It was shown that connectin preparations contain titin (Maruyama et al., 1981a) but other proteins are also present in substantial amounts (Wang, 1982b). The unsatisfactory nature of the original titin preparation (as with the connectin preparation) is that the protein is in the denatured form. Fortunately it has now proved possible to extract about half of the titin of myofibrils in the native state with high concentrations of salt (Kimura and Maruyama, 1983; Trinick et al., 1984; Wang et al., 1984). This has opened the way to a full characterisation of this remarkable protein. The sedimentation coefficient indicates it is a highly asymmetric molecule (Trinick et al., 1984; Maruyama et al., 1984). The chain weights of the two bands are 1.4×10^6 and 1.2×10^6 (Wang, 1982a; but see Maruyama et al., 1984). The native molecular weight at low concentration measured by sedimentation equilibrium is 2.7×10^6 (Maruyama et al., 1984) suggesting that the molecule contains two polypeptide chains. However, the plot of $\log_e$ concentration versus radial distance squared was curved indicating polydispersity. This might arise from an association equilibrium but further investigations will be required before we can define the size of the molecule and understand the precise nature of the polydispersity.

In the electron microscope titin molecules, revealed by metal shadowing after spraying, are seen as filaments about 40 Å wide frequently associated with large globular structures (Trinick et al., 1984; Maruyama et al., 1984; Wang et al., 1984). After layering on the mica substrate, however, the globular structures are absent suggesting that they are the product of shear (Trinick et al., 1984). The filaments are heterogeneous in length from 1000 Å to 10000 Å (Trinick et al., 1984; Maruyama et al., 1984; Wang et al., 1984). Maruyama et al. and Wang et al. found rather pronounced peaks in the length distribution histograms suggesting that the heterogeneity arises from end-to-end polymerisation of molecules about 1100 to 1500 Å long. Negative staining provides greater resolution than shadowing and a 40 Å periodicity along the length of the titin filaments has been revealed (Trinick et al., 1984, Wang et al., 1984). Using this technique, the diameter of the filaments is about 35 Å. This can be compared with the 30 to 40 Å diameter of gap filaments. It is clearly highly desirable that there should be complete consistency between the results of electron microscopy and sedimentation equilibrium.

It is of considerable interest that filaments with a very similar appearance to titin filaments can be seen in negatively stained thick filaments. These are either coiled up at the ends of the thick filaments or run alongside (Trinick et al., 1984). This suggests strongly that titin filaments are associated with thick filaments in vivo and are probably anchored to the ends of the thick filaments where they may form elastic connections to the Z-line. Proof that titin forms part or all of the gap filaments is, however, lacking at present although there is about enough titin to make up the gap filaments. Titin constitutes about 10% of the myofibrillar protein (Trinick et al., 1984) and is thus the third most abundant protein (Wang et al., 1979). Assuming that myosin constitutes 55% of the myofibrils (Huxley and Hanson, 1957), that the chain weight of titin is 1.4×10^6 and that there are 300 myosin molecules per thick filament, this amount of titin corresponds to 21 titin chains per thick filament. A cylindrical protein molecule of mass 1.4×10^6 daltons and diameter 35 Å would have a length of 1770 Å. Hence there is more than enough

titin in the myofibril to make one 35 μ filament spanning the entire length of the sarcomere for each thick filament.

The results of labelling myofibrils with fluorescent antibodies to titin have not given simple results (Wang et al., 1979; Maruyama et al., 1981a,b; Wang, 1982). The A-I junction is consistently labelled but sometimes the Z-line or M-line are labelled (occasionally brightly) and even the whole A-band may be labelled. The labelling pattern changes not only with sarcomere length but also depends on how the myofibrils were prepared. Thus it is unclear at present exactly where titin is located in the sarcomere.

Another puzzle is why only a fraction of the titin in a myofibril is soluble in salt solutions (Trinick et al., 1984; Wang et al., 1984)) and the relation between this soluble titin and the insoluble residue which forms a substantial part of connectin preparations. Nor is it clear what the relationship is between these two fractions and the two titin bands seen on SDS polyacrylamide gels. Titin is very susceptible to proteolytic degradation (Maruyama et al., 1981b; King et al., 1981; Wang, 1982b), the initial event according to Wang (1982) being the conversion of the higher molecular weight form to the lower. Wang et al. consider the latter to be the soluble form but this has not been agreed. Trinick et al. (1984) have suggested that in order for titin to be extracted from myofibrils, the titin filaments may have to be severed from their anchorages by proteolytic attack as well as the thick filament depolymerised. The heterogeneity of length of the titin filaments seen in the electron microscope could be explained if they were end-to-end polymers of titin molecules, the internal molecules intact, and the molecules at the ends proteolytically cleaved. Thus it is possible that no depolymerisation of the native titin filaments of the myofibril is involved in the extraction and purification of soluble titin.

Interest in the gap filaments by meat scientists has been aroused by the work of Locker's group (Locker et al., 1977; Locker and Wild, 1982a,b; Locker, 1982). When meat is cooked, the rigidity of rigor is replaced by extensibility. The thick filaments coagulate to give a seemingly amorphous A-band at about 50°C. Most of the filaments of the I-band disintegrate at about 70°C, and the remainder are thought to be gap filaments. Locker et al. (1977) and Locker and Wild (1982b) showed that if cooked meat were stretched, initially the extension is taken up entirely in the I-band but at 50% stretch the A-band also extends and it may now be seen to contain an array of fine filaments with fragments of coagulum attached. They presumed that these were gap filaments and concluded that, of the filaments present in uncooked meat, only the gap filaments survive cooking with any integrity. This means that the gap filaments are the only structure to resist extension; that is they are the sole contributor by the myofibrils to the tensile characteristics of cooked meat. It is interesting in this context that titin, like gap filaments, is so easily degraded by proteolytic enzymes and it is an attractive proposition that the tenderising of meat on ageing is due to proteolysis of this protein (Locker et al., 1977; King and Kurth, 1980; King et al 1981; King and Harris, 1982). However, the heat stability of gap filaments found by Locker's group needs to be reconciled with the lability of titin in meat found by King and his co-workers. They observed diminution in the amount of intact titin when unaged meat (or a homogenate) was heated at about 60°C. Since the decrease was inhibited by pepstatin, they attributed this to the action of cathepsins still active at this temperature. Degradation of titin was more complete at pH 5.5 than at higher pH's.

Cold-shortening

One of the principal causes of tough meat is the phenomenon of cold-shortening, first described by Locker and Hagyard (1963). This occurs if a carcass is cooled too quickly after slaughter. When certain muscles are cooled to temperatures below about 10°C, they contract. The contraction is fully reversible provided the ATP of the muscle is not greatly depleted: the muscle will relax again if the temperature is raised. The cold-induced contraction is slow and weak compared with a physiological contraction, the tension exerted being only about a thirtieth or so of that of a physiological isometric tension (Bendall, 1973). If the muscle is unloaded, the shortening can exceed 60% (Locker and Hagyard, 1963; Marsh and Leet, 1966). In a carcass, the extent of shortening may vary depending on the attachment to the skeleton. Even if a muscle is fixed at both ends, cold-shortening is possible over part of its length if this cools faster (Davey and Winger, 1980). For reasons that are not clear, if the shortening is about 40% the cooked meat is very tough but the toughness declines very steeply with greater shortening (Marsh and Leet, 1966; Davey and Gilbert, 1975).

One of the main questions was the cause of this temperature-induced shortening. It was suggested that, like a normal, physiological contraction it was triggered by calcium ions (Bendall, 1964; Hill, 1972; Bendall, 1973). Davey and Gilbert (1974) concluded from a study of the settling behaviour at 5 and 15°C of fibre fragments in a range of Ca^{2+} concentrations that cold-induced shortening was indeed caused by an elevation of Ca^{2+} concentration. Until recently, however, direct proof of a rise in Ca^{2+} concentration was lacking.

Jeacocke (1984a,b) has studied this question by injection of the calcium-sensitive dye arsenazo III into muscle fibres. This dye changes colour when it binds Ca^{2+} ions and so by monitoring the absorption spectrum of the muscle fibre, the time course of the intracellular Ca^{2+} concentration can be followed. Jeacocke found that when the muscle fibre was cooled to 3°C, there was indeed a rise in the Ca^{2+} concentration to the order of micromolar.

It remains to be explained what is the source of this Ca^{2+} and why only certain muscles are vulnerable to cold-shortening. Red muscles are much more susceptible than white muscles (Bendall, 1966, 1973; Hill, 1972; Davey and Gilbert, 1974). For example, rabbit psoas muscle does not cold-shorten, whereas rabbit semitendinosus does. Nor within a muscle do all the fibres behave in the same way: only a minority shorten to the full extent, the rest being thrown passively into folds (Voyle, 1969). It seems probable that at least part of the explanation is that lowered temperatures reduce the activity of the Ca^{2+} pump of the sarcoplasmic reticulum (Martonosi and Feretos, 1964; Inesi et al., 1973). The fact that the sarcoplasmic reticulum in slow twitch fibres is relatively poorly developed would account for their special vulnerability (Bendall, 1973; Davey and Gilbert, 1974). However, because oxygen suppresses cold-shortening, it has been proposed that calcium released from mitochondria under anaerobic conditions may also play a part (Ruegg and Marsh, 1975; Cornforth et al., 1980). It seems more likely that mitochondria alter the Ca^{2+} binding properties of the sarcoplasmic reticulum by their effect on the relative concentrations of ATP, ADP and P_i (Jeacocke personal communication).

The onset of rigor

After slaughter, the ATPases of the muscle cell remain active. For some time the ATP concentration is maintained partly by the conversion of glycogen to lactic acid and partly by consumption of the creatine phosphate stores (see Bendall, 1973 for a review). Only when the creatine phosphate stores are nearing exhaustion does the ATP concentration start to fall. There is then a gradual increase in the stiffness of the muscle over a period of an hour or two as the ATP concentration falls to zero.

What remained a puzzle was that work done on actin and myosin in solution showed that very low concentrations of ATP suffice to dissociate actomyosin. This is also true of fibres in which the membranes have been removed. One might therefore have expected that a muscle would remain relaxed until the ATP concentration was very low and only then would a rapid onset of rigor occur, unlike the gradual onset of rigor experimentally observed.

This problem has been tackled recently by Jeacocke (1984a,c). He has shown that the onset of rigor in a single muscle fibre (with intact membrane) is in fact rather rapid, much more rapid than in a bundle of fibres. The onset of rigor in different single fibres occurred at a wide range of times post-mortem, but in each case once rigor started to occur, it was complete soon after.

Jeacocke concluded therefore that the slow onset of rigor in a muscle is due to the heterogeneous nature of the fibres it contains. The cause of this heterogeneity remains to be elucidated but could well be due to differences in metabolism between different fibre types making up the muscle.

While on the subject of rigor development, I should like to draw attention to the remarkable advances that have been made in following muscle metabolism by the use of phosphorus nuclear magnetic resonance (see Gadian, 1984 for a review). This is a non-destructive technique and it is possible to follow in an intact muscle the time course of changes in the concentration of the principal phosphorus-containing components, creatine phosphate, ATP and inorganic phosphate. It is therefore most helpful in the study of the energetics of muscle cells. Because the resonance frequency of inorganic

phosphate depends on the relative proportions of $H_2PO_4^-$ and HPO_4^{2-}, it is also possible to follow changes in intracellular pH. The results from NMR are broadly consistent with those obtained previously by chemical methods, although NMR indicates a substantially lower level of inorganic phosphate (and by calculation also the free ADP level) in relaxed muscle. As yet it has been very little applied to the development of rigor in different muscles, and to the effect of electrical stimulation.

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