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# THE ROLE OF GAP FILAMENTS IN MUSCLE AND IN MEAT

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## Abstract

The neglected "gap filaments" are now beginning to receive close attention in several centers, in terms of organisation, composition and function. The author's model for their connections gave them a role as cores to A-filaments. This review attempts to weigh the implications of such a role, and relevant evidence, old and new. New ideas arising largely from PAGE* and immunochemical studies on candidate proteins, and from the developing concept of the cytoskeleton, are considered.

The author's theory of meat tenderness, based on G-filaments, has been tested by PAGE studies on changes in the large structural proteins (particularly titin and nebulin) during tenderising treatments. The results, together with those from parallel work elsewhere, are in some conflict with the theory.

*Polyacrylamide Gel Electrophoresis

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## GAP FILAMENTS IN MUSCLE

### Introduction: sliding filaments are not the whole story

In 1954, two classic papers in "Nature" laid down the sliding filament theory of muscular contraction, which proved to be a landmark in muscle biology. In one of these papers, H.E. Huxley and Hanson (1954) felt bound to discuss some observations which the theory could not explain. They noted that when the myosin was extracted from their myofibrils, the center of the sarcomere appeared empty under phase contrast. This was an illusion, since as the authors themselves said: "The ghost fibrils are however still structurally continuous; stretched fibrils shorten spontaneously to a little less than rest length during extraction --- they may be reversibly extended again --- with great ease, and it is apparent that only weak forces oppose such a stretch; the gap elongates in the process but the length of material from the Z-line to the gap remains constant ---". They concluded that the gap was bridged by an elastic component: "The S-filaments provide continuity between the set of actin filaments associated with one Z-line and that associated with the next ---". The S-filaments were last heard of when Huxley and Hanson (1957) assessed the "S-substance" in the gap as 3% of the myofibril by interference microscopy (note that the gap was only a third of the sarcomere, and if the S-filaments extended from one Z-line to the other they would amount to 9%). The definitive electron microscopy study of Huxley (1957) did not substantiate the presence of extra filaments, and S-filaments were shelved.

### The gap filaments and other "extra" filaments

The other paper associated with the sliding filament theory was the first to record gap filaments. A.F. Huxley and Peachey (1961) were investigating the ability of stretched fibres to contract. A micrograph of a fibril stretched well beyond the point where A-and I-filaments over-lap, led them to comment: "The suggestion of fine filaments connecting the ends of the two major sets of filaments may be a result of super-position in the section or may represent an additional component of the myofibril". So, in a sentence, they dismissed this important observation. It was Sjostrand (1962) who finally described and named the "gap

filaments". Carlsen et al. (1965) gave them further attention, but there the gap-filaments (G-filaments) rested, having no role in the new theory which was then stimulating muscle biology.

The idea of extra filaments received fresh attention with the "superthin" filaments (McNeill and Hoyle, 1967; Walcott and Ridgway, 1967). These have recently been discussed again (Hoyle, 1983) but their relationship, if any, to the G-filaments remains obscure.

The presence of residual filaments after extraction of actin and myosin has been recorded by Guba et al. (1968a), dos Remedios (1969) and by dos Remedios and Gilmour (1978). These "extra" filaments will be further discussed below.

In 1973, I found by chance that beef fibers could be stretched by 4-5 times (up to 12 μm) and that G-filaments showed to great advantage in such material. A series of subsequent papers described the morphology of the G-filaments in some detail (Locker and Leet, 1975, 1976a, 1976b). A model for their connections (Fig. 1) was proposed (Locker and Leet, 1976a). It was based on the curious behaviour of A-filaments at these extreme degrees of stretch (stretching and dislocation), counts on the sparse but well defined array of filaments in the gap, and the lack of strain in I-filaments (summarised in Locker, 1982a). According to this model each G-filament forms a core to an A-filament, emerging at one end only, passing independently of the I-filaments through the Z-line, between the I-filaments of the next sarcomere and into a second A-filament, to terminate as a second core. The symmetry is based on the Z-line, with half the A-filaments in any A-band linked to A-filaments in one adjacent sarcomere and half to the other side. This model may seem odd, but it appeared to offer the only explanation of the evidence. The author will be happy to see it modified if satisfactory alternative explanations emerge, as in fact seems inevitable. The model will be discussed further in relation to the difficulties it raises, and to newer evidence.

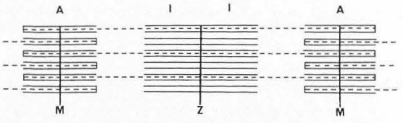


Figure 1. Model for the connections of G-filaments (dotted). Each acts as a core to two A-filaments in adjacent sarcomeres linking these A-filaments through the Z-line and independently of I-filaments.

The protein of G-filaments

While we were attempting to characterise the protein of G-filaments, candidates for this role were emerging elsewhere. It seemed most probable that the "connectin" of Maruyama et al. (1976) was the protein of the G-filaments (Maruyama et al., 1980; Locker and Daines, 1980; King and Kurth, 1980). A better characterised material emerged in titin (Wang et al., 1979; Wang, 1982a). Connectin and titin appear to be essentially identical (Maruyama et al., 1981a, b).

An interesting development with apparent potential for exploring the physiological role of connectin is its isolation in a "native" state and in high yield by a very mild extraction procedure (Kimura and Maruyama, 1983a). These authors have already demonstrated a weak interaction of this protein with both myosin and actin (Kimura and Maruyama, 1983b). However Wang (pers. comm.) considers this "native" form to be titin-2, a partly degraded titin, which has become salt-soluble in the process (Wang and Ramirez-Mitchell, 1983b).

The link between titin and G-filaments seems to have been finally established (La Salle et al., 1983; Robson and Huiatt, 1983; Robson et al., 1983). Antibody to bovine skeletal titin labelled the filaments in the gap of beef fibers, over-stretched after Locker and Leet (1975).

There is of course no reason why G-filaments should be composed of a single strand or of a single protein. King (1984) has suggested that they may be compound and we have raised the possibility that nebulin might be involved with titin on the basis of changes in PAGE patterns during ageing of meat (Locker and Wild, 1984a). The idea of the involvement of both proteins in G-filaments has been developed in detail by Wang (1983b) on the basis of his immunochemical studies (see later).

How much titin is there in muscle?

This is an important preliminary question in seeking a role for titin and G-filaments. Twenty years ago the balance sheet for structural proteins seemed more or less complete. It has proved remarkably elastic with the discovery of a host of new myofibrillar proteins. "Stroma" has been a convenient "throw-away" label for the largest untapped reservoir of new proteins. While this must contain connective tissue and membranes, there is clearly a great deal more. Indeed washed beef sternomandibularis myofibrils leave a residue of 16-20% of the protein nitrogen after extraction with M.KI, and all but 1-2% of this is soluble in 5M guanidine hydrochloride -DTT (Locker and Daines, 1980). This dissolved material, when made water-soluble by maleylation, gave a fraction unabsorbed by DEAE-cellulose (5-8% of the myofibril) which appeared to be connectin. Maruyama et al. (1976) estimated connectin in rabbit psoas at 5% of the myofibrillar protein, but the drastic treatments used in the preparation make this figure suspect. This criticism also applies to their estimate of 18% in cardiac muscle (Maruyama et al., 1977), indeed we found the content in heart and skeletal muscle to be much the same (Locker and Daines, 1980), an assertion borne out by later experience of four different cardiac muscles (Locker and Wild, unpublished results).

Wang et al. (1979) estimated titin-1, -2 and -3 in chicken breast at 10-15% of myofibrillar protein, based on the Lowry estimation of 20 eluates from agarose columns (stain intensity of gels gave only half these values). Titin-3, a distinct protein, has since been renamed "nebulin" (Wang and Williamson, 1980). Our own gel staining for beef (Locker and

Wild, 1984a) shows nebulin to be about half as abundant as titin (also the conclusion of Wang, 1982a). This means that the above column data, probably the best available, translate to 7-10% titin.

King (1984) has estimated titin in sheep semimembranous muscles in much the same way as Wang et al. (1979) and found it to be 12% of the myofibril (dry weight).

Our measurements of stain intensity in gels (Locker and Wild, 1984a) show the titin content of beef to be a third that of myosin, equivalent to 14% of myofibrillar protein, if 43% is taken as the best value for myosin (Yates and Greaser, 1983). This method must be suspect due to uncertainties about staining. Curiously the value is high, in contrast to Wang's low value from this method.

Finally, in our survey of the occurrence of titin across the animal kingdom (Locker and Wild, unpublished results), visual comparison of myosin and titin bands, suggest their relative amounts to be remarkably constant in skeletal and cardiac muscles, suggesting a consistent role for titin in a structure common to a wide range of vertebrate and invertebrate muscles.

Considering all the above data together it seems that the round figures of Wang (1982a) for titin and nebulin (10% and 5% respectively of the myofibril) are a very reasonable choice. The figure mentioned earlier of 9% for through-running S-filaments makes an interesting comparison. Titin is therefore a major component of the myofibril, lying between actin and tropomyosin in abundance. On these grounds alone it must be the leading candidate for the substance of an extra set of filaments. There would clearly be enough for the G-filaments of my model (Fig. 1).

It may also be noted that nebulin, about as abundant as tropomyosin, is also a major constituent. There seems to be more than would be required for an $N_2$-line (Wang and Williamson, 1980).

The G-filament as a possible core to the A-filament

The manner in which A-filaments are dragged one way or the other and are themselves stretched in over-stretched beef fibers, shows a strong tensile link between A- and G-filaments, but this does not prove that G-filaments continue inside the A-filament. However the various extraction studies described earlier, indicate that they do extend into the A-band, and there seems to be no place in this region, other than the A-core, in which to hide G-filaments, which are about as thick as I-filaments (Locker and Leet, 1975). Huxley (1957) found the H-band devoid of fine filaments and this has been the general conclusion since. But it should be noted that McNeill and Hoyle (1967) disagreed. Their Fig. 5b appears to show many "superthin filaments" between the thick filaments in the H-band.

Our micrographs clearly show continuity of A- and G-filaments in sarcomeres with A-bands either dislocated (Locker and Leet, 1975, Fig. 15) or undislocated (Locker and Leet, 1976a, Fig. 3). Moreover, when myosin is extracted from undislocated sarcomeres, the fine filaments left in the A-band still appear to emerge from the A-filament stubs, preserved within the M-line (ibid., Fig. 11). When dislocated sarcomeres are extracted the residual pattern of fine filaments mimics that of the original A-filaments, even to the surviving overlap of about 0.6 μm at the centre (ibid. Fig 8). This suggests a core running right through the A-filament.

There is room for a core

The accumulation of evidence for the structure of the vertebrate A-filament derived from X-rays, biochemical studies, quantitative mass measurements or direct morphology in the electron microscope, now lies heavily in favour of the 3-stranded model (see Kensler and Stewart, 1983; Ip and Heuser, 1983). This was the preferred model of Squire (1973), who discussed its implications for the core of the A-filament. He favoured a 3-stranded core for vertebrate muscle and a 6-stranded core for insect flight muscle, based on his detailed X-ray diffraction studies of frog sartorius and Lecotherus flight muscle. These models have internal spaces of clear diameter about 30A and 100A respectively. The vertebrate "hole" is actually triangular and would best accommodate a 3-stranded core of 20A filaments, while the insect "hole" would accommodate best a hexagonal array of 18 such filaments. The "hollowness" of A-filaments is visible in many electron micrographs. It is not uncommon in vertebrate muscle (e.g., Huxley, 1957) and is very striking in insect flight muscle (e.g. Ullrick et al., 1977). As pointed out by Squire it should not be regarded as emptiness, but as a central region of a lower staining capacity. His estimate of the diameter of this region, from direct observation of micrographs is 50A and 100A in the vertebrate and insect filaments respectively. While there is no evidence from his work for a core, he estimates that a vertebrate core, as above, should comprise not more than 4% of the myosin mass. This could not accommodate the titin estimated above at 10% of the myofibrillar protein, although nebulin at 5% comes close.

A-Filament cores do exist

There is precedent for A-filament cores in the case of molluscan adductors where paramyosin definitely fills the role (Szent-Gyorgyi et al., 1971). However these A-filaments differ markedly from the mammalian ones in having much larger and variable diameters of 300-1200A. Paramyosin may account for half the structural protein in some cases. This protein occurs in the musculature of various arthropod classes, annelids and nematodes, although its location is not known (Waterston et al., 1974). Paramyosin has proved abundant in some insect flight muscles (Bullard et al., 1973). Assuming a core location, that is an A-filament composed of myosin and paramyosin, the paramyosin content was equivalent to about 11% of the A-filament in Lethocerus (close to the predicted 12-13% of core permitted in the Squire 6-strand model), 18% in the rosechafer beetle, but only 2% in the blowfly. Since bumble bee flight muscle contains titin and nebulin (Locker and Wild, unpublished results) the possibilities for cores in insect flight muscles become complex.

Filament counts in the I-band

In the I-band, G-filaments cannot be hidden and the evidence is conflicting on whether they can be

counted with the actin filaments. Huxley (1960) found in serial cross sections of rabbit psoas, a mean ratio of 1.9 for I-filaments: A-filaments, close to the 2.0 predicted for the double hexagonal array. Again using serial sections of rabbit psoas (and taking pains to employ an impartial counter), dos Remedios (1969) obtained a ratio of 2.8, intermediate between the prediction of his model (3.0 for through-running residual filaments) and mine (2.5). Guba et al. (1968b) using the same material (but not serial sections), partly extracted for myosin, found a ratio of 3.1. Ullrick et al. (1977), using serial sections, found a ratio of 2.1 in frog sartorius and 2.2 for chameleon tongue. In short the present situation is unsatisfactory, but hardly excludes a model requiring a ratio of 2.5.

The classic electron micrographs of Huxley (1957) defined the hexagonal packing of the interdigitated thick and thin filaments in the A-band. His very special sections, cut at about 150A, were only one layer of filaments thick. Those lying exactly in the 1120 plane of the lattice, had in the A-band two thin filaments between each pair of thick filaments. But my counts of filaments in the I-bands, using his best plates, bear a ratio to thick filaments well in excess of 2.0, and closer to the 2.5 predicted by my model. This excess appears to be due to filaments emerging from thick filaments (see next section). Although counts on longitudinal sections are suspect, in these special sections they should be more reliable than usual.

Thin extensions to A-filaments

Numerous papers from 1962 onwards, leave no doubt that in insect flight muscle there are thin extensions of the A-filament to the Z-line (C-filaments). These are seen clearly in a break which opens up at the I-Z junction when Lecotherus flight muscle is stretched in rigor by 10% (White and Thorson, 1973, their Fig. 11). In transverse sections each thick filament gives way precisely to a thin filament in an equivalent hexagonal array (their Fig. 12). The ratio of filaments in the I-Z region to A-filaments was found by Ullrick et al. (1977) in this muscle to be 4.1, as to be expected with a set extra to the actin filaments (which in this muscle are three times as numerous as A-filaments). The C-filaments have been shown beautifully in honey bee flight muscle (Trombitas and Tigyi-Sebes, 1974, their Fig. 7).

The presence of C-filaments in these highly specialised muscles is no guarantee of their presence in vertebrate muscle. One of the best indications that equivalent structures may be present comes from the special one-layer sections of Huxley (1957) referred to above. I wish to point out that most thick filaments there have a thin extension at one end and some at both ends. These extensions appear indistinguishable from other I-filaments. In ordinary sections, about four times thicker, this could be dismissed as superposition of thick and thin filaments, but it is harder to dismiss so many occurrences in one-layer sections, especially as the thin filaments in the next layer up or down in the lattice do not overlie the thick filaments.

There is also evidence from isolated A-filaments. In a paper claiming to disprove the existence of an A-core, Morimoto and Harrington (1973) show micrographs of A-filaments and neglect to point out the stumps of thin filament protruding at one end of three out of four A-filaments, in their Plate V. These somewhat resemble the "end-filaments" of Trinick (1981) (see below).

It is interesting that Morimoto and Harrington could not isolate A-filaments at an early stage when dilute tris-buffer had already removed the M-line (but not the Z-line) from their myofibrils. After a week, when Z-lines had gone, A-filaments were released by gentle agitation. I believe they had remained moored by their G-filaments until released by slow proteolysis. This problem has not really been solved up to the latest methods for the preparation of A-filaments. Trinick (1982) claims only 5-10% of A-filaments are liberated on homogenisation of freshly glycerinated rabbit muscle, and that A-segments can be prepared in the same way, but best if the muscle is first aged for several days. Our own experience is that ageing is necessary to get significant yields of A-filaments from beef sternomandibularis muscle.

Morimoto and Harrington (1973) found by PAGE that myosin and C-protein were the only significant components of the A-filament. The apparent necessity to age A-filaments during preparation leaves a nagging doubt about such studies. I have previously argued strongly (Locker, 1982a, b) that an A-core would be fully protected from autolytic change, and indeed will show later that titin is resistant (although nebulin is not). While I still hold to this argument, I have to concede that an element of doubt exists. If the G-filament passes through the A-filament in any location other than as a central core, then the question of its vulnerability to proteolysis during A-filament preparation becomes a serious one.

Some recent disassembly of A-filaments reveals no sign of a core. Maw and Rowe (1980) found that rat (but seldom rabbit) A-filaments frayed into three strands when rinsed with water on a grid. These presumably correspond to the three myosin strands of the favoured A-filament model (Kensler and Stewart, 1983). Trinick (1981) found that rat or rabbit A-filaments frayed more reliably in 2 mM imidazole at pH 7.3. Again there was no sign of a core but he observed at each end a single "end-filament" (85 x 5 nm) with a period of 4.2 nm and sometimes a globular head. He considered these end-filaments were normally folded back inside since they were not visible in unfrayed A-filaments (although he quotes Craig as having seen them in unfrayed frog filaments). Pepe (1982, his Fig. 4) has observed a similar fraying in 10 mM tris-citrate buffer, pH 8.0.

The apparently core-less frayed A-filament suggests a possible alternative to a central core : that each of the three myosin strands might incorporate a strand of titin as a "mini-core", the three mini-cores uniting to form a triple-stranded G-filament. Such a concept would be difficult to reconcile with the withdrawal of a core under stretch or retraction during contraction (see below).

With regard to a multistrand G-filament we have on one occasion observed an unusually large number of filaments in the gap (Locker et al., 1977). This was on a sample of beef sternomandibularis, glycerinated at 100% stretch and then incubated 24 h

at 25°C with a crude muscle protease preparation and calcium at pH 7. Over most of the sample, A- and I-bands remained just in contact, but in a few places (Fig. 2) had separated leaving an abundance of beautifully defined filaments in the gap. Instead of the usual sparse array seen there (usually half the number of A-filaments) the filaments in the gap are here in excess (by about 1.2 times). This could be interpreted as the unravelling of a two- or three-stranded G-filament, possibly due to proteolytic removal of some cementing substance. On the other hand it could be taken as evidence for a G-filament emerging from every A-filament, on a different model (see later).

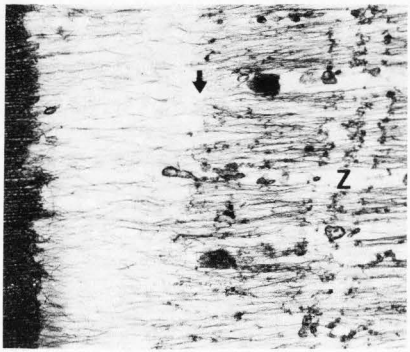


Figure 2. Beef muscle, glycerinated at twice excised length and incubated 24 h at 25°C in a crude muscle protease preparation (+Ca, pH 7). I-filaments touched the A-band except in a few places (as here) where a gap had opened up containing an unusually large number of sharply defined filaments. (Arrow shows edge of I-band.)

Shortening and stretching of G-filaments

If G-filaments exist in parallel array in the I-band, where do they go when the I-band shortens and disappears? There is no evidence for fine filaments coiling up around the Z-line. The thickening of the Z-line into "contraction bands" seems adequately accounted for by a pile-up of A-filament ends, bending or penetrating the Z-line (Marsh and Carse, 1974). I suggest that the G-filaments retract completely inside the A-filaments, reaching their minimum length of 1.5 μm. (Note that this length, as used here and below, is the length within one sarcomere. In my model, each G-filament extends into the next sarcomere, so that the total length is double that quoted.) G-filaments must then be withdrawn from the A-core on stretching muscle. In excised beef muscle the equilibrium sarcomere length is 2.1 μm (ibid.) which implies a G-filament length of 1.8 μm. Over-stretched sarcomeres may reach 12 μm (Locker and Leet, 1975) with filaments clearly surviving in the gap. Recently, observing over-stretched fibers in the light microscope, I have frequently seen 12 μm sarcomeres, 13 μm not uncommonly and once a record 14 μm. In the latter case the G-filament should be 7.3 μm long (allowing for residual overlap of 0.6 μm in the dislocated A-filaments). This means a stretch of five times minimum length, rather high for a biological filament. This alone should be enough to dismiss claims that G-filaments are wisps of myosin or actin.

If the G-filament merely joined the A-filament but did not penetrate, it would have a length in an equilibrium sarcomere (2.1 μm) of only 0.3 μm. In a 14 μm sarcomere the G-filament would then be stretched about thirteen times. Such a degree of stretch becomes hard to believe, and is a good argument either for a core or at least for continuation into the A-filament in a stretchable form. In short, there has to be something to stretch.

In pre-rigor muscle the G-filaments must have a high extensibility since a load of only 0.1 kg/cm² is necessary to extend the muscle by 80%. This, in terms of the model, means stretching G-filaments to about 1.5 times excised length. A high extensibility appears to be preserved into the rigor state, since when a muscle "yields" by fracturing of actin filaments (Locker and Wild, 1982a), the G-filaments survive in the I-band and stretch without further loading. It is also possible to over-stretch muscle that has gone into rigor at maximum stretch (100%), that is with no actin-myosin overlap (Locker and Leet, 1976a).

As discussed above, the Squire model has a central space in the A-filament with a clear diameter of 30A, but actually triangular and best suited a three stranded core (3 x 20A). Direct observation of micrographs suggested a core of about 50A diameter. These estimates appear to impose a maximum diameter of 30-50A to a G-filament core at the fully retracted length of 1.5 μm. This is in serious conflict with our observation that the filaments seen in the gap of overstretched muscle were of about the same diameter as actin filaments (60-70A). In these sarcomeres of 9-10 μm the G-filament length would be about 5 μm: i.e., stretched by 3.3 times. The maximum allowable diameter should then be 30-50A divided by $\sqrt{3.3}$, that is 16-27A. The gap filaments reported by Sjostrand (1962) were considered to be 30A in diameter, and being less stretched than ours are not too far from Squire's prediction. Guba et al. (1968a) found the filaments remaining after KI extraction to be 40A in diameter.

In the Squire model any core is likely to be a tight fit, and its extension might pose problems, accentuated by the predicted 3° helical twist. The central space resembles the bore of a rifle with three grooves of gentle pitch. The problem of fitting a core to a helically-grooved internal space is not affected by the bipolar arrangement of the myosin molecules. Given the same "handedness" of the twist, the internal grooves can pass uninterrupted through the whole A-filament. Extension of the core might at first be difficult and impose a twist, but as extension progressed the fit would rapidly become looser and twisting might cease.

The photo width for each of the three micrographs, Figs. 2, 4 and 5 is 3 micrometers.

The concept of retractable or stretchable A-core implies firstly that core and cortex are not firmly bound together, and secondly that the core must be composed of material which because of its nature or organisation is unusually elastic. It seems possible that the equilibrium length of the G-filament, whether core or not, could determine the equilibrium length of the resting sarcomere (2.1 μm). It is not the meeting of opposing I-filaments which determines this, since at 2.1 μm these are already overlapped by 0.3 μm (beef I-filaments are 1.2 μm long).

This idea is supported by the previously quoted observation of Huxley and Hanson (1954): "stretched myofibrils shorten spontaneously to a little less than rest length during extraction" of myosin. I have confirmed this for fibers using beef sternomandibularis, glycerinated at 2.0 x and 1.5 x excised length. The muscle was then chopped into fiber pieces or fiber bundles and extracted with Hasselbach-Schneider (H-S) solution. The 1.5 x sample reverted to a sarcomere length of 2.04 ± 0.29 μm (S.D.), while sarcomeres of the 2.0 x sample retracted less completely, to 2.77 ± 0.28 μm (104 fibers each). An attempt to achieve the same result on extracting long sarcomere beef myofibrils produced a lesser degree of retraction. However extraction of chicken myofibrils from muscle stretched only 22% during rigor, produced a rather precise retraction to excised sarcomere length.

The spring-back of fibers, cut after over-stretch and H-S extraction, has been recorded by Locker and Leet (1975, Figs. 23, 24, S=2.4 μm). I have repeated this experiment by over-stretching fibers directly into H-S solution. When cut after measurement and again measured under phrase contrast, the sarcomeres were found to have contracted from 11.8 ± 0.9 μm to 2.9 ± 0.4 μm (20 fibers), shorter in fact than fibers cut after stretching into isotonic neutral solution (3.6 ± 0.5 μm).

The symmetry of the G-filament connections

If G-filaments pass from the center-filled hexagonal array of the A-filaments to the tetragonal array at or near the Z-line, they are likely to do so in a systematic way.

If the G-filaments emerge from both ends of the A-filaments there is no problem. However to link half the A-filaments in each direction (as in my model), while preserving a hexagonal symmetry is not possible. This requires one third of the cores to emerge in one direction and two thirds in the other (Fig. 3a). However the same number of links in both directions can be achieved by selecting alternate A-filaments along two of the directions parallel to the sides of the hexagons, but the third direction then has all its connections the same way (Fig. 3b).

At the Z-line, the only feasible place for a G-filament to pass seems to be through the centres of the "Z-squares". If G-filaments emerged at both ends of A-filaments the ratio of I-filaments to G-filaments would be 2:1. This can be achieved symmetrically with the arrangement in Fig. 3c, using all centres on alternate diagonals. The 4:1 ratio required by the model can also be achieved by using alternate centres in alternate rows (Fig. 3d).

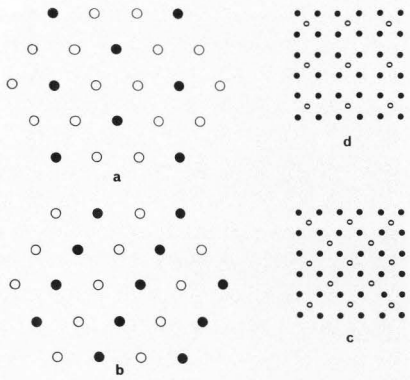


Figure 3. The possibilities for symmetrical connections of G-filaments within A-band and Z-line.

(a) The two kinds of circles represent A-filaments linked to a Z-line one way or the other by G-filaments. Only this arrangement preserves hexagonal symmetry, but it produces a 2:1 ratio of the directional links.

(b) This arrangement produces a 1:1 ratio, but is not fully symmetrical. It alternates directions along two sides of the hexagons but not along the third.

(c) A symmetrical arrangement of I-filaments (solid) and G-filaments (open centers) at the Z-line, which produces the 2:1 ratio required by a modified model with G-filaments emerging from both ends of an A-filament.

(d) A similar symmetrical arrangement which produces the 4:1 ratio required by the model of Fig. 1.

Extension of A-filaments during over-stretch

In the original paper on over-stretched beef-fibers (Locker and Leet, 1975) it was found that the A-filaments not only slid in one direction or the other, thus dislocating the A-band, but that the A-filaments themselves stretched. The dislocated A-band in a 10.6 μm sarcomere was 4.6 μm long (ibid., Fig. 12). Allowing for an 0.6 μm central overlap of A-filaments this means that each was 2.6 μm long (or stretched by 73%). It seems doubtful that an A-filament consisting entirely of packed myosin molecules would survive such stretch without

breaking, and I suggest that its survival was due to an intact elastic core. For the A-filament to have stretched, the core must have been attached initially to the cortex of myosin. It must have retained some attachment, with slippage, since the A-filaments continued extending up to the longest sarcomeres recorded in the cited figure, but to a lesser degree than the G-filaments.

Some extremely curious fibers were observed which, having been over-stretched with dislocation of the A-band, had then apparently lost tension and sprung back (Locker and Leet, 1976a). Except in the central overlap the A-filaments had totally lost their characteristic rigidity. They were thrown into waves and had apparently dissociated into strands (ibid., Figs. 20, 21). This effect suggests to me the violent recoil of a core.

A minority of over-stretched fibers did not dislocate (Locker and Leet, 1976a). Sarcomeres of over 7 μm were obtained with an A-band more or less intact. The M-line was sometimes intact, sometimes not (ibid., Figs. 2, 3). In these fibers the A-filaments were not stretched, so if there is a G-filament core, it must have been freely withdrawn. Again the G-filaments appear to be definitely continuous with the A-filaments.

Why the difference in behaviour of different fibers? Is it a question of fiber type in this "mixed" muscle? It appears not. Orcutt and Dutson (1984) have repeated the over-stretch technique with the same muscle and always obtained non-dislocated sarcomeres. The only difference between their procedure and ours appears to be that they stretched the muscle immediately post mortem, whereas ours was stretched generally several hours later (when the muscle is still however, far from rigor). Another difference is that A-filament:G-filament ratios are close to 2:1 in our fibers (either kind), but they found near 1:1. On the other hand La Salle et al. (1983) obtained both kinds of over-stretched beef fibers, closely resembling ours (personal communication).

This seems an appropriate point to comment on a peculiarity of my model: the notion that half the A-filaments in any A-band are connected by G-filaments through one Z-line and half through the other. This seemed necessary to account for the dislocation. The only reasonable alternative explanation ever offered to me was by Prof. K. Maruyama, who suggested that A-filaments are connected at both ends by G-filaments and that under stress there is random breakage of these, one side or the other, and the A-filaments slide accordingly. I take this suggestion seriously and admit that it would eliminate one of the oddest features of my model. Recent evidence that "ageing" of muscle begins at death (Marsh et al., 1981) may be relevant. If ageing is indeed a factor so early, and G-filaments are certainly weakened by ageing (Locker et al., 1977), Maruyama's explanation seems possible. It could be argued in the light of Orcutt and Dutson's experience that when over-stretch occurs very early, G-filaments remain intact at both ends of the A-filament, and no dislocation occurs. However even in our non-dislocated fibers, only half the A-filaments were connected in any one direction, so it would have to be concluded that random breakages had occurred there too.

A core in relation to the assembly of A-filaments

The in-built ability of the myosin molecule to self-assemble into thick filaments argues against the need for a template. Purified myosin solutions can be induced, merely by lowering the ionic strength, to produce "synthetic A-filaments" which closely resemble the real thing in all respects but length (for a review see Pepe, 1983). However Pepe found that a carefully programmed two-step reduction on ionic strength produced the correct length. This is, of course, not an option open to the cell.

Davis (1981a, b) found the rate of association to be independent of length, while the rate of dissociation increased rapidly with length. He therefore concluded that a kinetic equilibrium defined the length of the filament, and that this basic mechanism could be fine tuned by various factors in the cell. One of the factors he also suggested was co-polymerisation of other thick-filament proteins, an idea which others have also explored (see Pepe, 1983). A core filament with an equilibrium half-length of 1.8 μm could be a very suitable regulator, if its effective length was reduced to 1.5 μm by an N-line structure.

A length determining role is distinct from that of template for assembly. The former does not demand such a close association of myosin molecules with the core, and would be more compatible with its withdrawal or retraction.

Quite apart from either role it should be easier to assemble the A-filament around a core than to thread it in afterwards. The only relevant evidence on myogenesis published so far appears to be that of Gruen et al. (1982), who found that myosin heavy chains appear in fetal lambs some weeks earlier than connectin (which appears at about 7 weeks). They conclude, very reasonably, that this "argues against a possible role for connectin as a framework for the assembly of the sarcomere in vivo and that this result, together with the known susceptibility of connectin to hydrolysis, suggests "an exposed environment". It might however be asked to what extent the organisation and genetic expression of contractile proteins at this early stage resembles that in adult muscle. Histological data on embryonic lamb muscle are scarce, but at 45 days (about the time at which connectin appears) myotubes with just perceptible striations are present (Joubert, 1956, his Fig. 28).

I am aware of other work, as yet unpublished, which does not support the lag of titin behind contractile proteins during development and it therefore seems too early to make judgements in this area.

A role for G-filaments in contraction?

The constancy of the A-filament in length and structure is basic to the sliding filament theory. There have however been a number of reports of changes in the length of A-filaments or in their packing, most of which are suspect. Some of these have already been discussed (Locker and Leet, 1976a, Locker et al., 1976). Some unorthodox theories of contraction have been proposed, including cyclic changes in the backbone of the A-filament. For example, the "quantum" contraction theory of Davydov (1974) envisages excitation of the peptide groups that form three parallel chains of hydrogen bonds down the myosin helix. A wave of compression

travels down the molecule shifting the myosin heads relative to actin. The highly original theory of dos Remedios (1969), involves dissociation of I-filaments, changes in the packing of myosin molecules, the movement of A-filament cores and the re-making of I-filaments.

A much more plausible theory, also from Australia, came recently from Obendorf (1981). He suggests that the problems of matching the helically arranged myosin heads and binding sites on the I-filament during cross-bridge formation would be solved if the A-filament rotated during contraction. This novel idea retains the essentials of the conventional theory, and is not in conflict with its basic premises. So far it has drawn no fire.

This theory has interesting implications for G-filaments. If these are in fact the A-core, then the A-filament cortex might rotate on the core as on an axle. It might be argued that the tight M-line structure would prevent this, but it must be remembered that the center of the A-filament is the smoothest part, that isolated M-proteins do not show any binding affinity to myosin in vitro (Woodhead and Lowey, 1983) and that A-filaments under the stress of over-stretch can slide through M-lines (Locker and Leet, 1975).

The G-filaments seem much too elastic to be effective as transmitters of tension (as proposed by dos Remedios, 1969) unless they were transformed in some way by stimulation.

Localisation of titin in the myofibril

The original paper on titin used fluorescent antibody to locate the protein in chicken myofibrils (Wang et al., 1979). They conclude that "titin is present in M-lines, Z-lines, the junction of A and I bands, and perhaps throughout the entire A-band". This conclusion needs re-examination. The myofibrils (a-d) in their Fig. 4 appear to be artefacts of the kind we have described in some detail (Locker et al., 1976). They are the usual product of blending pre-rigor muscle, and are seen less commonly on blending rigor or glycerinated muscle. A-bands with their material apparently concentrated at the outer edges, as in (a-d) are typical. It seems significant that these are the ones staining most intensely across the A-band. Myofibrils (f-h) appear to be normal and stain strongly only in the H-zone and at the A-I junction. The relative intensity in these two regions varies. The Z-line is barely stained at all, and only heavily in short sarcomeres (j), where the line is in fact a contracture band, representing a pile-up of A- and I-band material against the Z-line. Apart from H-zone staining, these results seem not incompatible with a core role for titin: that is exposure in the outer I-band, and in the A-bands only when there has been damage.

The location of titin by the antibody-peroxidase method in the electron microscope is the subject of a preliminary communication by La Salle et al. (1983). The results were "complex and somewhat variable" for myofibrils (artefact problems?) but "always demonstrated labelling in the region of the A band-I band junction with some labelling extending into the A band." In fibers over-stretched after Locker and Leet (1975), the G-filaments were clearly labelled. This is the only direct link so far between titin and G-filaments. Often the outer A-band and the outer half of the I-band were also labelled, which seems to agree entirely with the light micrographs of Wang et al. (1979). Various models for the A-filament suggest a looser packing of myosin at the ends, and it is possible a core could be more accessible to antibody there. The failure of the inner I-band to label might be due to N-line structures there (see later).

Having criticised the early results of Wang et al. (1979) I must now acknowledge that the far more sophisticated approach which the group has since undertaken, using four distinct monoclonal antibodies to rabbit titin (Wang and Ramirez-Mitchell, 1984), bears out the earlier claims. These antibodies collectively indicate that titin passes from the M-line through the A-band and just into the I-band (Wang pers. comm.). The results suggest a degree of exposure not compatible with location in a central core. The results of Greaser's group (S.M. Wang et al., 1983) using a single monoclonal antibody to bovine cardiac titin are in agreement. Their staining occurred at the A-I junction, (but also at M-line and Z-line after extraction of major proteins).

Wang's third filament

When this review was almost complete, I received a paper I had not seen (Wang, 1982b) and another in press (Wang, 1983b). These offer a quite new alternative to G-filaments in terms of "an elastic filamentous matrix consisting of titin and nebulin as additional sarcomere constituents." The new concept, derived from his immunochemical studies, sees the filament as continuous from Z-line to Z-line. In the A-region it is composed of titin, wound spirally on the outside of the A-filament (and therefore able to react with antibody) and extending into the I-band on to an I-filament (or two I-filaments?). Here nebulin, in series, takes over, enveloping the I-filament in a manner unspecified. No extra filaments are needed, except of course where the titin thread bridges the A- and I-filaments. On contraction, the titin section in the A-band remains unchanged, but the nebulin section bunches up on the I-filament.

The model is speculative and has problems of its own, but brings an exciting new concept. It will be of great interest to see how it develops with new information.

The N-lines and the G-filaments

The N-line was first seen over a century ago and has been as much neglected as the G-filaments. It is now believed that there are two: the $N_1$- and $N_2$-lines. Curiously, studies on G-filaments and N-lines have converged recently in two laboratories. We claimed that $N_2$-lines are suspended on the G-filaments (Locker and Leet, 1976b), while in Austin, Texas, titin and nebulin have been studied together, and the latter located on the $N_2$-line (Wang and Williamson, 1980).

The disappearance of nebulin, but not titin, during tenderisation of beef by ageing (Locker and Wild, 1984a) led us to suggest that nebulin might be an essential part of G-filaments (which largely determine the myofibrillar contribution to meat tenderness). We queried the nebulin antibody work on the same grounds as the titin antibody experiments

(i.e., damaged myofibrils), and suggested that the nebulin antibody distribution was just as compatible with a location of nebulin in the G-filaments.

The elusive nature of the N-lines has defied systematic study. However we have just completed a survey of micrographs made here and elsewhere for other purposes (Locker and Wild, submitted for publication). My conclusion is that there are at least seven N-lines: an $N_1$-line always close to the Z-line; a group of four $N_2$-lines in the mid I-band, sometimes sharp, sometimes diffuse and often embedded in an "extra density"; and an $N_3$ doublet, seen near the edge of the I-band in fully stretched sarcomeres. It seems that both the $N_2$ and $N_3$ group may be suspended on the G-filaments. These groups were apparently confused in Locker and Leet (1976b), that is in one case it was the $N_2$-lines and in the other the $N_3$-lines which were found to be suspended on the G-filaments.

This new classification of N-lines into three distinct groups should provide a base for new research on these intriguing structures and on their relationship to the G-filaments. It may be noted that nebulin antibody labelled more than just a line in the I-band, and, if it is an N-line protein, could have attached to the whole $N_2$ and perhaps the $N_3$ groups. It is also possible that the failure of the inner part of the I-band to label with titin antibody (as noted in the previous section) could be due to the interference of N-structures.

G-filaments in moderately stretched muscle

While G-filaments can be reliably demonstrated in over-stretched fibers, we have never found oriented G-filaments after extraction of A- or I-filaments from muscle at excised length or stretched by up to 100%.

Extraction of beef sternomandibularis, glycerinated at 100% stretch, with Hasselbach-Scheider solution (1 h) left a drifting M-line (Fig. 4). At the ends of the residual A-filament stubs was a tangle of fine filamentous material with only a few slack filaments spanning across to the I-band. This is in contrast to the taut filaments emerging from such stubs in extracted over-stretched sarcomeres (Locker and Leet, 1976a, Figs. 9, 11). It is possible that some proteolysis may have occurred during glycerination (2-3 weeks at 2°C in EDTA, pH 7), but the same drifting M-lines were seen in thin strips of fresh muscle extracted at the same stretch for 24 h at 2°C in H-S solution.

When the same stretched glycerinated muscle was extracted for 1 h with a KI solvent (after Guba et al., 1968a) the sarcomere was reduced to a mass of disorganised fine filaments largely out of the plane of the section, but with some tending to longitudinal orientation (Fig. 5). The previously observed pile-up of material on the Z-line was evident.

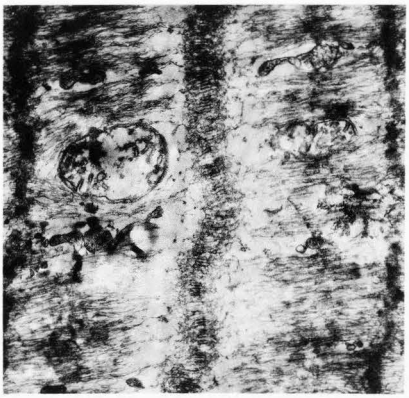

Figure 4. Beef muscle glycerinated at twice excised length and extracted 1 h in H-S solution. Continuity has been lost in the center of the sarcomere, but filamentous material is bunched up at the ends of the thick filament stubs preserved by the M-line.

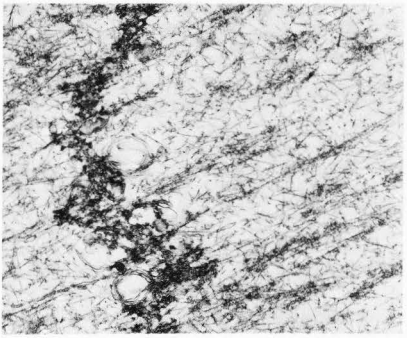

Figure 5. The same glycerinated muscle as in Figure 4, but extracted with strong KI solution (Guba et al., 1968a). Many fine filaments survive, but largely in random array. Only patches of longitudinal orientation remain. A pile-up of dense material on the Z-lines has occurred.

In short, there is no difficulty in demonstrating residual fine filaments (which I believe to be G-filaments) within the myofibril when muscle that has not been extended beyond its natural limit is extracted with H-S or KI solution. However

organisation is lost. Of course on the basis of my model, solution of myosin from the A-filament should leave both extremities of each G-filament free to retract and tangle. However the filament should be held taut between the M-line (where this survives) and the Z-line. This does not appear to be the case in Figure 4.

If it is a question of anchorages, why do these survive so well in over-stretched fibers, but not at lesser extensions? When A-filaments dislocate they never completely lose their overlap for reasons unknown. Why does this overlap of 0.6 μm survive, and why does it still provide anchorage for G-filaments after H-S extraction (Locker and Leet, 1976 a, Fig. 8)? These questions have intrigued me for years, but I still have no answers.

The cytoskeleton and the G-filaments

The concept of the cytoskeleton is now as fashionable in muscle as in other cells. It began in muscle with the observation of the Z-line "scaffold" of desmin by Grainger and Lazarides (1978) and has been further explored by Richardson et al. (1981). It has been reviewed by Stanley (1983), Robson and Huiatt (1983), Robson et al. (1983) and by Wang (1982b, 1983a, 1983b).

Wang and Ramirez-Mitchell (1983a) extracted myofibrils on a grid with KI and concluded that there is both a transverse and a longitudinal network of desmin, lying outside the myofibril but closely associated with it, so closely that it may survive partial disintegration of the fiber during homogenisation. Tokuyasu et al. (1983) have confirmed this external longitudinal network in intact tissue, using ferritin-labelled antibody to desmin.

Wang and Ramirez-Mitchell consider the longitudinal "residual filaments" of dos Remedios and Gilmour (1978) and others are not internal elements of the sarcomere at all, but merely these external intermediate filaments. This explanation could account for our failure to find organised filaments after extraction of moderately stretched muscle (previous section).

They note a heavy aggregation against the Z-line of material which they consider to be titin. This may well be so, since titin is the dominant electrophoretic component of their KI-residue, while desmin is present only as a trace. We have seen a similar accumulation on the Z-line after KI-extraction of both muscle at 100% stretch (Fig. 5) and over-stretched fibers (Locker and Leet, 1976a, Figs. 14-18). In both these cases enough filaments remain through the sarcomere to account for titin (in disorder in the first case and in parallel array in the second). I suspect that the Z-line aggregate may here be largely N-line substance or the associated "extra density".

The Ghosts Become More Ghostly

Coming back to the point at which this review began, we must now ask why, if no ordered, through-running filaments remain within sarcomeres after H-S or KI extraction, the ghosts of stretched myofibrils still shorten to near original rest length? If the G-filaments are no longer capable, is it a surviving longitudinal sheath of intermediate filaments that causes retraction? For fibers (which I used earlier to verify the effect) this is quite possible. But I suspect that isolated myofibrils are normally quite nude. I have noted earlier that my attempts to induce beef myofibrils of long sarcomere length to shorten by H-S extraction were less successful than those of Huxley and Hanson (1954) using rabbit myofibrils, and certainly less effective than experiments with beef fibers.

A new candidate for the ghostly spring has recently appeared in the "covalently cross-linked matrix" of Loewy et al. (1983). Exhaustive extraction of skeletal, cardiac or smooth muscle with 6M guanidine. HCl (+ thiol) leaves only a 1% residue (in good agreement with our earlier results; Locker and Daines, 1980). Treatment with collagenase reduces the residue to only 0.2% of original protein, but the shape of the fiber is perfectly preserved and the matrix extends throughout its volume. Although the protein is distinct in composition from titin, it cross reacts, in spite of the fact that the antibody was made from the residue of chicken gizzard, which has no titin. These authors did not observe filaments, but Ozaki and Maruyama (1980) found a residue in the slime mould Physarum polycephalum, after extraction with 6M guanidine. HCl (+ thiol) or 1% SDS, which contained filaments less than 5 nm thick. In the same slime mould Gassner et al. (1983) also found a residue resistant to 4% SDS + 7M urea, which contained filaments 2-3 nm in diameter. This recalls the "superthin" filaments of Hoyle (1983).

It now seems that we may need to contemplate within a fiber not only a third longitudinal filament, but a fourth of desmin (Wang, 1983b) and even a fifth of "covalently cross-linked matrix".

## MEAT TENDERNESS AND THE G-FILAMENTS

A new theory of tenderness

My interest in G-filaments arose from a chance observation of the ability of beef fibers to stretch to an extraordinary degree, far beyond previous reports for vertebrate muscle. This observation was pursued purely from an academic interest in this neglected avenue of muscle biology, which seemed to me potentially important. It was several years before I realised that G-filaments were also relevant to meat tenderness. The first convincing demonstration came from my colleagues Davey and Graafhuis (1976), who found in beef neck muscle, cooked at maximum stretch, that G-filaments survived well in the "gap", but not if the muscle had previously been aged. This and other observations led to the beginning of a theory (Locker et al., 1977). The crucial subsequent observation was that in cooked meat the A- and I-filaments disperse, leaving an actomyosin coagulum with a through-running array of fine filaments, which we identified as G-filaments (Locker and Wild, 1982b). Thus tensile continuity becomes dependent on G-filaments. As other evidence accumulated, a more comprehensive attempt at a tenderness theory was published (Locker 1982a, b). Some special attention was given to cold shortened meat (Locker, 1984). Now, several papers on, some foundations of the theory appear to have crumbled a little, and a re-assessment is due. The detailed argument behind the theory has been presented (Locker, 1982a). The following are its main points.

In the normal cooking of meat, only the G-filaments survive within the myofibril. As the temperature rises from 60° to 70°C, the A-filaments and I-filaments disintegrate, leaving G-filaments embedded in an actomyosin gel, while the collagen net denatures, becoming tensioned and elasticised. These two elements, heat modified myofibril and perimysium, stretch in unison under stress. They give cooked meat its essential character. The G-filaments denature slowly at 60°C, and more rapidly at higher temperatures. They survive with strength and elasticity for several hours at 100°C (whereas collagen does not).

Treatments which improve the myofibrillar component of tenderness, work by weakening the G-filaments. This is true of ageing and of pressure-heat treatment (Locker and Wild, 1984b), where the filaments are vulnerable to proteases and to pressure respectively.

The toughening due to cold shortening is attributed to an increasing incidence of sarcomeres without I-bands. The latter, having only half the number of G-filaments present in the A-band, constitute the weak link in cooked sarcomeres.

The failure of cold shortened meat to age is claimed to be due to retraction of G-filaments into the A-core, where they become completely protected against proteolytic attack.

Conflict between morphological and protein studies

The theory was based largely on morphological studies, which still appear valid. However study of changes in structural proteins during tenderising treatments and cooking has raised difficulties. Since the antibody work of La Salle et al. (1983) has shown that G-filaments, as expected, contain titin, its behaviour during such treatments is important to the theory.

In a series of papers, Australian workers had already shown that titin was rapidly degraded by the proteases in muscle at elevated temperatures, within its denaturation range (see King, 1984). Most recently King (1984) has shown that it is also degraded at 0° and at 15°C in beef and sheep muscles (using light scattering and PAGE). His result is contrary to our recent PAGE study of the fate of the large myofibrilar proteins during ageing, pressure-heat treatment and cooking (Locker and Wild, 1984a). We found that in beef sternomandibularis, titin appeared resistant to ageing at 15°C for 10 days and even 20 days whereas most of the tenderness improvement occurs in 2 days in the muscle. More in line with this time scale of tenderisation was the disappearance of nebulin and the appearance of a new band B midway between titin and nebulin. The rate of disappearance of nebulin varied somewhat between animals (as indeed does the rate of ageing), although generally it was largely gone at two days whereas band B appeared reliably in that interval. While the position of band B suggested titin as a source, densitometry showed insufficient loss of titin, but an adequate loss of material from the gel top. This top protein might be an aggregated form of titin. There was little evidence of titin doublets or conversion of titin-1 to titin-2. To sum up, there was no significant change in the titin content during the period of tenderisation, but a closely parallel appearance of faster band B, apparently derived from material unable to penetrate the gel. The disappearance of nebulin also followed tenderisation, more or less.

We have since found (unpublished results) that ageing of lamb longissimus followed the same pattern very consistently. On the other hand chicken leg muscle aged one day at 15°C retained titin and nebulin, but produced band B (the sharpest and densest we have seen) while chicken breast also retained titin but lost nebulin and did not generate band B. The patterns for the same muscles of a thrush were different again.

A variety of rabbit muscles was also aged one day at 15°C. In every case the titin band survived, and in all but gluteus minimus, the nebulin band. The disappearance of protein from the gel top was a consistent feature, while the appearance of band B was seen in multifidus dorsi, vastus intermedius, soleus and gluteus medius, but not in psoas, gluteus minimus or longissimus. However when a sample of the longissimus was aged 3 days at 15°C, nebulin disappeared and B appeared. These particular muscles represent a range of essentially "white", "pure red" and "mixed" muscles (Locker and Hagyard, 1968) but their ageing behaviour cannot be so divided. It is clear that the pattern of ageing varies widely between muscles.

At the same time, and quite independently, the Iowa group has been involved in a similar study of ageing in beef longissimus (Lusby et al., 1983) using three storage temperatures 2°, 25° and 37°C for 1, 3 and 7 days. In the intact muscle they claim a graded conversion of titin-1 to titin-2 with increased time and temperature, although the photographs of their gels are not convincing, showing no resolution of these bands. Titin remained up to 7 days at 2° and 25°C but vanished within 3 days at 37°C.

They also found that nebulin disappeared, apparently faster (1 day at 2°C) than in our muscle, and they record breakdown products just below the original band (as we did). They also report a breakdown product apparently corresponding to our band B, but only after severe ageing (7 days at 25°C, or 3 days at 37°C).

Considering only ageing conditions of practical significance, it seems that our results and theirs are in substantial agreement: conservation of titin, and rapid loss of nebulin. Their ageing differs in the claimed conversion of titin-1 to titin-2, and in the rather sluggish appearance of band B. It is possible that our gels did not resolve titin-1 and titin-2, and we propose to investigate this futher.

If titin is not substantially degraded, but G-filaments are during ageing, some other component, essential to their integrity, may be suffering. We have suggested that nebulin may have such a role (Locker and Wild, 1984a). The new model of Wang (1983b), which has nebulin in series with titin in the G-filament, fits well with this idea. Attack on nebulin would inevitably weaken the filament.

It may be noted that Maruyama et al. (1981a) found that when chicken myofibrils were prepared in the presence of EGTA, both titin and nebulin were conserved. In the absence of EGTA there was some loss of titin and nebulin had disappeared. The equivalent of band B was present. If mM Ca was added, titin was much depleted and nebulin absent.

These workers also subjected myofibrils, prepared with EGTA, to purified calcium activated neutral protease (CANP) and serine protease (both at pH 8.0). In the case of CANP, nebulin disappeared rapidly, but the titin doublet remained (although becoming distinctly more mobile on the gel with time). Serine protease destroyed both proteins at the same rate.

Heart muscle

We have subjected heart muscle from lamb, rabbit, chicken and thrush to PAGE and find in all cases a level of titin comparable to that in skeletal muscle, but no nebulin at all. The material at the gel top is also missing. Ageing in every case had little apparent effect, and did not generate band B.

If, as suggested, nebulin is part of the G-filament we must conclude that either heart has no G-filaments, or they differ in a major respect. We have in fact cooked fully stretched papillary muscle and find filaments in the "gap". Recently I have also managed to over-stretch papillary muscle within a few minutes of death, but the few samples obtained so far have not reached the electron microscope due to mishap. However I conclude that G-filaments are an essential feature of cardiac as well as skeletal muscle.

Cold shortening and ageing

King (1984) reported that cold shortening did not influence the rate of ageing in terms of survival of titin. Our study has also shown that the gel patterns are the same whether the muscle has been cold shortened, set at excised length or stretched by two times. The only differences seen were at longer times in the fully stretched muscle, when myosin heads appeared to have become vulnerable due to loss of interdigitation allowing better access of proteases (Locker and Wild, 1984a).

Of course if titin is not attacked at all, it is not possible to demonstrate its protection by contraction. But there has definitely been no protection of nebulin nor suppression of band B. It can only be concluded that the present results offer no support for the attractively simple idea of protection of G-filaments by retreat into the A-core.

Survival of the G-filament proteins on cooking

The Australian group has repeatedly claimed that titin degrades in cooking at 80°C, and cannot therefore be significant to meat tenderness. This is in contrast to the undoubted survival of the G-filaments themselves (Locker et al., 1977, Locker and Wild, 1982b). We have pointed out in discussing King's work, that although little of the original titin survives, most of the "smeared" product represents material with a molecular weight of over half a million, and could therefore still be capable of contributing to structural strength, particularly with the "healing" possibilities of cross-linking during heat denaturation (Locker and Wild, 1984a). At 100°C the degradation becomes more serious, although after an hour, half the material is still larger than $10^5$ daltons. We have not investigated the degradation after another 3½ hours at 100°C when the shear force falls by only a quarter (Davey and Niederer, 1977). It may be noted that most of the myosin heavy chain has also disappeared after an hour at 100°C, so that any theory of residual strength based on this protein is likewise in difficulty.

Whether meat is tenderised by ageing (where the titin survives), or by pressure-heat treatment at 60°C (where it is largely degraded), the gels derived from the cooked material (80°C, 40 min) differ remarkably little from each other or from untreated cooked controls. However the remnant of the titin band seen in the cooked controls is absent in the cooked treated samples.

With respect to the possibility that nebulin may be part of the G-filaments, it may be noted that this protein generally (but not always) survives cooking at 80°C better than titin. The variation may be due to variable degrees of ageing during cooking. However the band disappears entirely on cooking at 100°C.

Denaturation point of G-filaments

A feature of the theory was that G-filaments denature slowly at 60°C, with a sharp decrease in their elasticity. However they remain strong and extensible. Numerous pieces of circumstantial evidence were listed in support of denaturation (Locker, 1982a). More direct evidence has now been obtained from beef fibers, over-stretched in 0.15 MKCl + 5 mM K phosphate, pH 7.0, and then heated for 1 hour at 60°C in that medium. On cutting, the fibers (n = 13) shortened from 11.3 ± 0.5 μm to 9.5 ± 0.5 μm (S.D.), that is to 84% of stretched length. When this is compared with shortening from 11.4 ± 0.8 μm to 3.6 ± 0.5 μm (31% of stretched length) in unheated samples it is clear that denaturation of G-filaments must have occurred within an hour at 60°C.

More basic information on G-filaments is needed

The present dilemma arises from the failure of what appears to be the major constituent of G-filaments to suffer during ageing periods which damage G-filaments, and the failure of this protein to survive severe cooking, which does not destroy G-filaments, nor greatly decrease the tensile strength of meat. The relationship between G-filaments and tenderness now seems much less tidy in the light of PAGE studies than it did when the theory was put together. The revision of the theory must wait for more basic knowledge about the nature of G-filaments, for firm answers on their components and assembly. If indeed they prove to be compound in structure, some of the difficulties may be resolved. The question of the strength of the anchorages of G-filaments, and how this survives tenderising treatments and heat, must not be overlooked and may be as important as the strength of the filaments themselves.

If my model (Fig. 1) were revised on Maruyama's suggestion of G-filaments emergent at both ends of the A-filament, the G-filament count would be the same in both A-band and I-band and the concept of the I-band as the weak link in cooked meat is itself weakened. However the reinforcement of the A-band with actomyosin gel should still provide a significant margin of strength.

Whatever the nature and properties of G-filaments, it is hardly possible to deny the accumulated evidence that they are there and that they are survivors in cooked meat.

Previously I have emphasised that my theory is over-simple in considering only longitudinal strength in meat, and that lateral strength must also be important. This is emphasised by the recent review paper of Stanley (1983) on the cytoskeleton. His own results confirm for beef earlier observations that emptying of the sarcomere becomes possible only after an ageing period. This indicates that decay of the cytoskeleton could be an important part of tenderisation.

## CONCLUSIONS

This review has of necessity been speculative, raising more questions than answers. However it represents an attempt to gather many scattered pieces of information and to weigh honestly the evidence, inadequate as it may be, for and against the concept of G-filaments as A-cores. This may prove to be effort wasted on an erroneous idea, but I still feel it is one which should not be discarded without adequate consideration. A heavy dependence on this laboratory for work on G-filaments is regretted, but over most of the last decade little else has come forward. The existence of G-filaments has been challenged only once in the literature (Ullrick et al., 1977) but otherwise has been ignored, until quite recently. The situation reminds me of that in the sixties when I was busy characterising the light chains of myosin, which then tended to be dismissed as impurities. It is pleasing that a number of highly capable groups are now busily studying the relationship between the myofilaments and the new large proteins. Papers have begun to appear in rapid succession, bringing important new evidence and stimulating ideas. It seems that a new chapter in muscle biology will soon be written. It will be concerned with the "extra" filaments, their composition and disposition in relation to A- and I-filaments; a more detailed picture of the architecture of the I-band, and the relationship within it of longitudinal filaments, transverse structures such as the N-lines and the "extra density". Some important and basic evidence on G-filaments has come from meat-oriented research. Those who work in meat research institutions tend to do experiments which scientists in university biology departments would not dream of. The use of these unorthodox materials and treatments sometimes pays dividends, and opens new vistas.

My model for G-filament connections, and tenderness theory based upon them, already have some dents and more are in store. Both are bound to be remodelled before many years have passed, as indeed are the bold and welcome ideas of Wang (1983b). I have given some attention in various places to Maruyama's suggestion that the G-filaments may emerge at both ends of the A-filament and run right across the sarcomere. I consider this a real possibility, which would bring my ideas somewhat closer to those of Wang.

The role of G-filaments in muscle function must remain for the moment an entirely open question, but in meat seems already better defined. That G-filaments are significant in both fields appears well enough established. They are not likely to go away. I was going to say, like S-filaments, but the G-filaments are in my view, the rightful successors to that extinct species.

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## Discussion with Reviewers

D.W. Stanley: Considering the concept of self-assembly and your hypothesis about the location and length of G-filaments, how do you conceive of the formation of these elements in vivo?
Author: I am aware of the work going on in embryological development (still unpublished) and it is too early to speculate in this direction.

D.W. Stanley: A recent paper by Koohmaraie et al., 1984 (Koohmarai M., Kennick W.H., Anglemier A.F., Elgasim, E.A. and Jones T.K. Effect of Post Mortem Storage on Cold-shortened Bovine Muscle: Analysis by SDS-Polyacrylamide Gel Electrophoresis, J. Food Science, 49, 290-291) also explores cold-shortened muscle.
Author: Thank you.