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THE EFFECT OF STRETCH ON THE ULTRASTRUCTURE OF A RIGOR SHORTENED MUSCLE

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Introduction: During the first few hours post-mortem skeletal muscle undergoes an extensive series of biochemical changes culminating in rigor mortis (Bate-Smith & Bendall 1949), and the ultimate quality of the meat is determined by the rate and extent of these changes. The physical and chemical changes accompanying rigor mortis have been reviewed by Bendall (1960) and include shortening of the muscle, loss of ATP and creatine phosphate, decrease in pH and loss of extensibility (Bate-Smith & Bendall 1947, Bendall 1951).

The shortening accompanying rigor mortis coincides in time with the disappearance of ATP (Bendall 1951) and causes changes in the banding patterns of the fibres similar to those indicated by the sliding filament model for in vivo contracting muscle (Stromer & Goll 1967a). According to the sliding filament theory (Huxley & Niedergerke 1954, Huxley & Hanson 1954) muscle contraction depends structurally on a sliding of interdigitating thick and thin filaments - the latter being attached at one end to the Z line with the other end located between adjacent thick filaments. Changes in banding pattern are then due to changes in the overlap or relative positions of the thick and thin filaments and this is also reflected in the sarcomere length.

Interest in the effect of the degree of muscle contraction on tenderness of meat was first stimulated by Locker (1960), who stated that relaxed muscles were more tender than partly contracted muscles; beef, poultry and fish muscles excised pre-rigor have all been shown to be less tender post-rigor than control muscles allowed to pass through rigor restrained by normal skeletal attachments (Ramsbottom & Strandine 1949, deFremery & Pool 1960). Also, the degree of contraction as measured by sarcomere length when altered in different portions of the same muscle by treatment or when varying naturally in different muscles has been shown to be associated with tenderness (Herring, Cassens & Briskey 1965). Sarcomere length as an indicator of the degree of contraction is an important criterion of muscle tenderness and this is especially true for muscles where the effect of connective tissue is small (Ramsbotton & Strandine 1949).

All the evidence to-date suggests that muscle in rigor is inextensible. This is due to the formation of tight cross-linkages between actin and myosin thus preventing sliding of the filaments. The evidence has come mostly from investigations of the physical changes occurring during rigor mortis (Bendall, 1960). The investigations included the measurement of extensibility (Bate-Smith & Bendall 1949), hardness (Marsh 1952), elasticity (Marsh 1954, Bahler & Fales 1966), contractility and excitability (Forrest & Briskey 1966), and changes in molecular configuration from X-ray diffraction studies (Huxley & Brown 1967). These are reviewed by Forrest (1968). More recently sarcomere length was shown not to change when muscle was subjected to an applied load 90 hours post-mortem (Davey & Dickson 1970). This observation further supports the inextensibility theory.

Goll (1968) distinguishes between two types of inextensibility occurring in muscle as rigor develops - (1) macroscopic inextensibility or loss of the ability of the muscle fibre to stretch under the influence of a given weight or force and (2) molecular inextensibility or inability of the actin and myosin filaments to slide past each other. Goll concludes that a partial reversal of the molecular inextensibility phase of rigor occurs in bovine muscle after 3-4 days post-mortem

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(Gothard et al. 1966, Stromer & Goll 1967b).

This suggested an investigation as to whether a reversal of the molecular inextensibility phase occurs in muscle in rigor but before any autolytic changes can take place. It had already been found (Hegarty & Herlihy, in preparation) that mouse muscles allowed to go into rigor for 4 hours and then stretched post-rigor showed an increase in sarcomere length when this was measured in isolated fibres from the stretched muscle. It became of interest to examine these muscles electron microscopically to confirm that a sarcomere length change occurred in muscle as a whole under the same conditions and to investigate possible ultrastructural changes associated with the stretching.

Methods: Mice weighing 28-30 grams from Falconers Q strain were killed by ether anasthesia. In the first series of experiments they were immediately laid on their backs on a cork board with both their forelimbs fixed with tape in a folded-over position. After 4 hours post-mortem in this position the bone to which the biceps brachii muscle is attached was placed in fixative while the companion limb was pulled out straight to 180° and pinned for an hour in that position before fixation in the same manner.

In a second series of experiments one of the limbs was extended to 180° pre-rigor and pinned in that position for 4 hours before fixation while the companion limb was folded maximally pre-rigor for 4 hours before stretching out for an hour and final fixation.

Fixation was carried out either for 3 hours or overnight in 2.5% glutaraldehyde made up in (1) 0.05M cacodylate buffer pH 7.4 (Gordon et al 1963) (2) Ringer-Locke buffered with 0.05M Tris-HCl or 0.05M phosphate pH 7.4. The initial fixation was always carried out with the muscle biceps brachii attached to the bone. The muscle was then dissected from the bone, further fixed for a time in flutaraldehyde and then washed for one hour in 10% sucrose solution made up in buffer. The muscle was then cut into 1mm cubes in 1% OsO_4 at 0-4° for 1 hour (Palade 1952). Samples cut for fixation were taken from the area of maximum fibre diameter of the biceps brachii in each limb (Goldspink 1962). Dehydration in graded ethanol was followed by infiltration and embedding in Araldite (Glauert, Rogers & Glauert 1956). Thin sections were cut on glass knives using an LKB Ultramicrotome. Sections were stained for 15 minutes in 2% uranyl acetate in methanol (Stempak & Ward 1964). After thorough drying lead citrate was applied for 15 minutes using the method of Reynolds (1963). Sections were examined in a Hitachi Electron Microscope Type HS-7. Sarcomere lengths were measured at X4,000 using latex particles as a suitable standard at the same magnification.

Results: It was found that the incorporation of Ringer Locke into the glutaraldehyde fixative resulted in better preservation of the muscle structure with less swelling of the mitochondria and less spacing between the myofibrils. Whatever the method of fixation employed a significant (20%) difference in sarcomere length ($P < 0.01$) was found between a folded muscle in rigor and its companion muscle stretched post-rigor after folding. These measurements are summarised in Table 1. An increase in sarcomere length due to stretching was found in the case of every muscle examined in each experiment. A slight through significant increase in A band length was also noted due to stretching post-rigor ($P < 0.01$). A length of 1.30 nm was obtained for the A band of normal fresh muscle. This value lay between the two A band lengths obtained fo a pre-rigor folded muscle (1.25 nm) and a muscle stretched post-rigor after folding pre-rigor (1.40 nm). This suggested that both slight shrinkage and stretching of the A band occurred during maximum contraction and post-rigor stretching respectively, accounting for the difference in A band length.

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Table 1 - Comparison of sarcomere lengths of mouse biceps brachii muscle in rigor and after stretching in rigor

Experiment No.		Fixation	Folded Pre-Rigor		Stretched Post-Rigor from Folded Position	
			Sarcomere Length (nm)	A Band Length (nm)	Sarcomere Length (nm)	A Band Length (nm)
I	3 mice	Glut. in cacodylate 3 hours	1.78 ± 0.06	1.40 ± 0.08	2.20 ± 0.20	1.50 ± 0.
II	3 mice	Glut. in cacodylate overnight	1.80 ± 0.02	1.30 ± 0.06	2.05 ± 0.09	1.40 ± 0.
III	3 mice	Glut. in Ringer Tris 3 hours	1.50 ± 0.07	1.20 ± 0.06	1.80 ± 0.06	1.34 ± 0.
IV	3 mice	Glut. in Ringer phosphate 3 hours	1.70 ± 0.08	1.20 ± 0.04	2.10 ± 0.20	1.40 ± 0.
V	4 mice	Glut. in Ringer phosphate overnight	1.73 ± 0.06	1.20 ± 0.02	2.00 ± 0.09	1.34 ± 0.
	Mean (16 mice)		1.70 ± 0.03*	1.25 ± 0.03	2.03 ± 0.06	1.40 ± 0.

* standard error of mean

Further evidence for the increase in sarcomere length due to stretching of a muscle folded pre-rigor can be seen by comparison of the micrographs of Fig. I and Fig. II. Lengthening or relaxation of the stretched muscle appears to have occurred by a sliding of the interdigitating filaments past one another. It is interesting to note that despite the application of stretch, the sarcomeres of a muscle stretched post-rigor after pre-rigor folding (Fig. II) are not as long as those seen in a muscle allowed to go into rigor in a stretched state (Fig. III). This indicates that post-rigor stretching can not effect complete reversal of inextensibility in a muscle folded pre-rigor. The sarcomere measurements from these muscles are summarised in Table 2.

Table 2 - Comparison of the sarcomere lengths of mouse biceps brachii stretched pre-rigor and stretched post-rigor

Experiment		Fixation	Stretched from folded position		Stretched Pre-Rigor	
			Sarcomere Length (nm)	A Band Length (nm)	Sarcomere Length (nm)	A Band Length (nm)
I	3 mice	Glut. in cacodylate 3 hours	2.20 ± 0.20	1.50 ± 0.04	2.46 ± 0.10	1.30 ± 0.
II	3 mice	Glut. in cacodylate overnight	2.05 ± 0.09	1.40 ± 0.04	2.40 ± 0.04	1.30 ± 0.
III	3 mice	Glut. in Ringer-Tris 3 hours	1.83 ± 0.06	1.30 ± 0.05	2.58 ± 0.09	1.35 ± 0.
	Mean (9 mice)		2.02 ± 0.08*	1.40 ± 0.04	2.50 ± 0.05	1.32 ± 0.

* standard error of mean

Some of the ultrastructural damage seen in a muscle stretched post-rigor from the pre-rigor folded position could be attributed to the observation that inextensibility is only partially reversible. It could also be put down to overstretching of the limbs, a it occurred only in some of the fibres. It was not possible to quantify the amount of tension exerted by stretching the muscle post-rigor from the fully contracted state to 180°.

The ultrastructural damage seen was of two types and occurred in small areas of some of the fibres. It must be emphasised that the remaining sarcomeres were well preserved and had an increased sarcomere length relative to those seen in a folded muscle in rigor.

The first type of damage seen was a breakdown in the orderly arrangement of filaments and a change in the normal fibrillar ultrastructure. This fibrillar damage was especially evident in the centre of the I band. The Z line in such sarcomeres varied from a wavy appearance to complete absence.

The second type of damage which occurred less frequently was characterised by a definite breakage in the sarcomere. This occurred between the Z line and the actin filaments and less often at the A band-I band junction. These findings tend to confirm the hypothesis that the weakest part of the muscle fibre is the junction between the actin filaments and the Z line. The Z line had a wavy appearance in some of these sarcomeres and in some cases it was broken in part, thus facilitating the breakage between the Z line and the actin filaments. A similar type of breakage was seen less frequently in the control pre-rigor folded muscle which casts some doubt as to the breakage being due to the stretching of the pre-rigor folded muscle.

Discussion: The increase in sarcomere length associated with stretching of a rigor shortened muscle suggests that the cross-linkages formed between actin and myosin during rigor are not as permanent and tight as had been predicted on the basis of extensibility measurements only. These results confirm earlier measurements of sarcomere length with the light microscope (Hegarty and Herlihy, in preparation), though in all cases a larger value for sarcomere length was obtained. This discrepancy between absolute values for sarcomere length could be due to differences in the preparation of the muscles. Huxley (1969) reported a 20% decrease in cross-bridge length due to processing of muscle for electron microscopy. Glutaraldehyde, in particular, is known to cause some shrinkage of tissues during fixation (Torack 1966).

Evidence for the full development of rigor in these muscles before the application of stretch was obtained from ATP and pH measurements - the ATP concentration decreased from 5.25 μ moles/gm immediately post-mortem to 0.74 μmoles/gm at 4 hours post-mortem and pH decreased from 7.3 to a constant 6.6 in the same time period.

The occurrence of ultrastructural damage after stretching in some of the fibres in rigor shortened muscle is consistent with the theory that muscle cannot be stretched in rigor mortis but will tear rather than elongate. However, the presence in these fibres of well preserved myofibrils with stretched sarcomeres compared to those of muscles folded in rigor suggests that sliding of the filaments also takes place. The breakages in the Z line found here were also reported to result from stretching of an aged muscle post-mortem (Dickson, 1968). This supports the conclusion that the Z line is the location of minimum resistance in the fibre. According to Bendall (1969) any forcible attempt to stretch a muscle after it has passed into rigor and become rigid is accompanied by fracture, usually in the I band. It was surprising that such breakages were not found more often. The finding of similar breakages in a folded muscle in rigor casts some doubt as to their being caused by stretching of the muscle. It has been reported (Voyle 1969, Bendall, 1960) that some fibres can undergo distortion and sometimes even rupturing during development of rigor mortis due to shortening of more actively contracting fibres.

The question now arises as to the nature of the changes in the actin-myosin interaction in the myofibril that resulted in the slippage of the filaments under the influence of tension. Goll (1968) considers that lengthening of contracted sarcomeres

in a muscle 2-3 days post-mortem originates primarily from a weakening or partial dissociation of the actin-myosin interaction and also in part from a degradation of the sarcomere at the level of the Z line due to proteolytic digestion. It is unlikely that proteolytic breakdown would have occurred in a muscle 4 hours post-mortem.

According to the sliding filament hypothesis of muscular contraction the capacity of the contractile system to produce tension is proportional to the number of interacting sites and hence the amount of overlap between the two sets of interdigitating filaments. In a study of the effect of sarcomere length on the tension exerted by a muscle Edman (1966) showed that active tension is virtually absent when the A and I filaments are at end to end positions relative to each other; it increases to a maximum when the degree of overlap provides the maximum number of interacting sites for propulsion of the thin filaments and then decreases again due to shortening of the sarcomere and tighter packing of the filaments. The maximum degree of contraction seen in a muscle folded pre-rigor could be responsible for the slippage of the filaments during post-rigor stretching. It is possible that the high degree of shortening of the sarcomeres permitted only a small number of sites on actin and myosin to interact resulting in minimum tension development between the two sets of filaments.

The most important result from this study may be that the immediate post-rigor stretching of muscle effects a significant increase in sarcomere length. In view of Locker's (1960) finding of a correlation between meat tenderness and sarcomere length this work may have some application in meat tenderization.

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