THIXOTROPIC AND TIME DEPENDENT MECHANICAL PROPERTIES OF RELAXED SKELETAL MUSCLES OF THE FROG (*RANA TEMPORARIA*).

A Thesis

Submitted to the University of St. Andrews for the degree of Doctor of Philosophy.

by

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CONTENTS

ABSTRACT

Isolated frog sartorius, iliofibularis muscles and single twitch fibres all show thixotropic tendencies. The stiffness of the muscle is variable, depending on the prior history of movement, and it is also highly dependent on both the force of the stretch, and on the duration that the force is applied for.

Stiffness recovery after a series of large perturbations is highly non-linear with a large proportion recovered within the first few seconds after the movement ceases. This is followed by a slower return which occupies the remaining time of the trial.

Temperature (range 3 to 17^{o}C) of the surrounding medium had no effect on either the size of the thixotropic effect, or on the rate of stiffness recovery in any of the muscle preparations.

Maximal isometric tetanic stimulation of frog sartorius muscle reduces the muscle's subsequent stiffness to a level equivalent to that produced by stirring. Stiffness is regained with a similar time course to that shown after a series of passive perturbations. The extent of the stiffness decline after stimulation varies with the time of year. In February the muscles are actually stiffer than the initial level for up to 1 s after the stimulation, before the muscle becomes less stiff than the initial values.

The thixotropic response is drastically reduced, or even abolished in muscles and fibres that have developed rigor, and also in fibres where the contractile material has been disrupted. The underlying mechanism for the appearance of thixotropy is thought to be related to a few attached

cross-bridges, higher stiffness values indicating a larger proportion of attached cross-bridges.

Frog sartorius muscle has a seasonal variation in its resting level of stiffness, being stiffer in early spring and least stiff in the autumn (a doubling in the level of stiffness from December to May). Possible explanations for these changes are: the expression of the myosin heavy chain; the configuration of the acetylcholine receptor; or the electrical properties of the muscle membrane.

Frog sartorius and mouse soleus muscles show the same time dependent response to stretches of varying durations, yielding more to stretches of longer durations, a discontinuity in yielding appearing consistently at about 3 s. The temperature had no effect on the time of the appearance of this discontinuity.

Adrenaline causes a reduction in the resting level of stiffness to a level equivalent to the post stirring stiffness values found in the untreated muscle and also a speeding up of the thixotropic recovery. The fast phase of the recovery is complete between 1 - 2 s, halving that seen with the untreated muscle, but only at unphysiological doses. Adrenaline may work by a speeding up of the cross-bridge cycling rate.

DECLARATION

I Lesley Robson hereby certify that this thesis has been composed by myself, that it is a record of my own work, and that it has not been accepted in part or complete form for any other degree or professional qualification

Signed Date 20.8.90

I was admitted to the Faculty of Science of the University of St Andrews under Ordinance General Number 12 in October 1986, and as a candidate for the degree of PhD in October 1986.

Signed Date

Resolution of the University court No. 1 1967.
I hereby certify that the candidate has fulfilled the conditions of the resolution and regulations appropriate to the degree of PhD.

Signature of Supervisor Date

20.8.53

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Lastly but by no means least I must thank my family for their support, without them this thesis would probably not have been written.

The work described here was supported by a research scholarship from the Medical Research Council.

I dedicate this thesis to the memory of my father and to my mother who died 5 days after my viva.

SUMMARY

1 Some of the mechanical properties of relaxed skeletal muscle of the frog *R.temporaria* and the mouse were studied.

2 The stiffness of frog sartorius muscle was measured by imposing small forces, measuring the resulting displacements, and calculating the Elastic Modulus (E).

3 There was no fixed level of stiffness in muscle: it was entirely dependent on the prior history of movement. Stiffness was reduced after movement and returned if then left at rest. This behaviour characterizes a thixotropic system.

4 Isolated frog muscle displayed clear thixotropic behaviour when imposed forces produced displacements within the range of the SREC (0.2 % of the muscle length). Forces that produced displacements greater than the SREC gave negligible thixotropic effects.

5 The time course of stiffness recovery after a series of large stretches (stirring) was highly non-linear, with most of the recovery within the first few seconds. This was followed by a slower prolonged return.

6 Prior isometric tetanic stimulation was found to cause a reduction in the stiffness soon after stimulation, but only at certain times of the year. The stiffness recovery was similar to that seen following stirring.

7 Thixotropy was also observed in the whole iliofibularis muscle (frog), and in single twitch fibres of this muscle. In the whole iliofibularis muscle the time

course of stiffness recovery is slightly slower than that obtained from the sartorius muscle, possibly because of its mixed fibre population.

8 The natural or artificial production of rigor causes a concomitant reduction in the thixotropic response and an increase in the stiffness. Increased stiffness is probably linked to the development of permanently attached cross-bridges.

9 Muscle plasticity was studied. The duration of the stretch greatly influenced the muscle stiffness. Long duration stretches reduced muscle stiffness to a greater extent than short duration stretches, with a discontinuity in the response appearing around the 3 - 5 s mark. The same plastic behaviour was seen with both frog and mouse muscle.

10 Temperature had no effect on the size of the thixotropic response, the rate of stiffness recovery after stirring, or on muscle plasticity.

11 Frog muscle undergoes seasonal changes in resting stiffness, probably related to the hormonal activity. A number of possible explanations are proposed.

12 Adrenaline produced a marked reduction in the general level of resting stiffness and speeded up the recovery of stiffness after stirring in frog iliofibularis bundles.

CHAPTER ONE

THIXOTROPY: THE EFFECT OF STRETCH SIZE

INTRODUCTION

The mechanism by which muscles contract, and the control of movement, have been extensively studied, while the properties of relaxed muscle have largely been ignored. This omission has been in part due to the assumption that relaxed muscle reaches a constant state once it has come to rest, and that its properties change only when the muscle is stimulated to contract and generate force. This simple supposition has recently been challenged and shown to be incorrect. Relaxed muscle is not such a static material as at first appears, and certain of its properties are constantly changing. One property which is in a constant state of flux in relaxed muscle is its stiffness.

THIXOTROPY

"Thixotropy" derived from the Greek; *thixis* - touch, and *tropos* - state.

Thixotropy is a characteristic of solutions with large colloidal or polymeric molecules; when the solution is agitated there is a disruption of the physico-chemical bonds formed between the molecules. This loosening then persists until the bonds can reform. Observations of substances with this property have shown that a thixotropic gel thins out to the sol state when it is stirred, and will return to the gel state when left at rest.

An important characteristic of a thixotropic system is that the loosening is reversible, the thixotropic recovery time being anything from a microsecond to a few hours, depending on the material, and the method of measurement (Dintenfass 1965). Thixotropy is a property of some everyday

materials such as tomato ketchup, non drip paints, clay and quick-sand. It has also been observed in biological substances such as, blood (Dintenfass 1965) and synovial fluid (Dintenfass 1965 and Davies 1965).

THIXOTROPY IN HUMAN MUSCLES AND JOINTS

Thixotropy has been shown in a variety of human muscle and joint systems (Lakie, Walsh and Wright 1979, 1983 and 1984; Lakie and Robson 1988c: Hagbarth, Hagglund, Nordin and Wallin 1985, Walsh and Wright 1987, 1988 and Hufschmidt and Schwaller 1987). Lakie *et al* (1984), subjected the wrist to small sinusoidal torques and demonstrated that the amplitude of the steady state displacement response was increased several fold by a transient large perturbation. The original state could be restored if the wrist had several seconds of inactivity. The resonant frequency of the wrist was also higher when small as opposed to large rhythmic torques were used.

Similar results have now been reported in other human muscles and joints such as the knee, wrist, ankle, hip and the metacarpophalangeal joint of the finger, but the underlying mechanism is still a matter of speculation. In humans the thixotropic effect may have its origin in the muscle, tendon or joints.

Synovial fluid, a complex solution found between the joints of humans and animals is known to be a thixotropic substance (Davies 1965, Dintenfass 1965, and Nuki and Ferguson 1971). The chemical composition of synovial fluid in simple terms is a dialysate of plasma, to which has been added the long chain, glycosaminoglycan hyaluronic acid. The

role of synovial fluid in joint stiffness is thought to be the result of weeping from the pores in the cartilage, allowing the joints to bear large loads with the minimum of friction, because the pressurized fluid is able to bear much of the load, but at the cost of a temporary increase in friction when movement ceases. Evidence that the synovial fluid does not play a significant role in the muscle/joint stiffness come from rheumatoid arthritis patients (Helliwell, Howe, and Wright 1987, 1988). Rheumatoid arthritis sufferers have similar, or even less stiffness than normals in the metacarpophalangeal joint, and the "stiffness" they experienced may be the result of confused terminology. Stiffness may be mistakenly associated with pain or restriction in the range of movement (Helliwell *et al* 1987). Stiffness is more prominent in arthritis patients after a long period of inactivity ("morning stiffness"), which may well reflect the synovial fluid weeping and the articular cartilage rubbing together.

The rheological characteristics of synovial fluid alter radically in inflammatory joint diseases, such as rheumatoid arthritis, becoming less viscous and more like a Newtonian substance, with the thixotropic properties disappearing. It is the hyaluronic acid/protein complex of the synovial fluid that is affected, being reduced, or degraded, causing both the viscosity and hyaluronic acid/protein complex concentrations to be lowered in those with inflammatory joint diseases (Nuki and Ferguson 1971). A degradation of synovial fluid decreases its lubricating properties, preventing it from supporting a constant load at any speed of movement,

allowing the fluid to be squeezed out from the gap between the surfaces, causing wear and tear of the cartilage (Dintenfass 1965).

As the stiffness in arthritis is not significantly different from that of normal subjects it can be reasonably assumed that synovial pluid does not play a major role in the stiffness of the joint.

THIXOTROPY IN ANIMAL MUSCLES AND JOINTS

The use of animal tissue in thixotropy studies allows the investigation of the individual parts of the system. Wiegner (1987) concluded that in the rat ankle joint 40 to 65 % of the stiffness that he measured could be accounted for by the joint, suggesting that joints are capable of contributing substantially to the thixotropic effects measured at relaxed joints. However this value can not be extrapolated to other joint systems, or species because of anatomical differences. Thixotropy was demonstrated in the isolated rat soleus muscle preparation, and its contribution to the whole system was comparable to the contribution made by the joint (Wiegner 1987). In the cat's paw, the muscles and joint each contribute approximately 40 % of the total stiffness (Johns and Wright 1962), confirming the values given by Wiegner (1987). Wiegner attributed the thixotropic behaviour at relaxed joints to both joint structures and to the short range stiffness of the muscles.

Buchthal and Kaiser (1951) demonstrated thixotropic properties in isolated bundles of frog semitendinosus muscle fibres. In frog muscle the thixotropic changes lasted for minutes rather than the seconds seen in Wiegner's mammalian muscle and the short time observed in early human studies by

Lakie *et al* (1984). Buchthal and Kaiser (1951), found their best effects were produced by movements of only 0.2 - 0.3 % of the muscle length. Larger stretches than this produced only a small degree of thixotropy.

THIXOTROPY IN MUSCLES - UNDERLYING MECHANISM

Assumptions as to the underlying mechanism for the appearance of thixotropy can be made from the nature of the effect itself. It is likely to involve the long term re-arrangment of bonds between the muscle's two filament populations (actin and myosin), and is therefore likely to be related to the Short Range Elastic Component (SREC) described by Hill (1968), today sometimes referred to as Short Range Stiffness (SRS) (Rack and Westbury 1974 and Wiegner 1987).

SREC

The first studies investigating in any detail this elastic behaviour of relaxed muscles were conducted by D. K. Hill (1968). By using hypertonic solutions Hill noticed that the muscle displayed a reluctance to yield at the beginning of a stretch. He was not, however the first to notice that muscle is stiffer for small movements than for larger ones. Denny-Brown (1929) when stretching cat muscle, found what he called a "stationary" or "preliminary rigidity" in the muscle. This elastic property provided an almost spring like resistance at the start of any length change, even for movements made at very slow velocities. Denny-Brown reported that preliminary rigidity was observable over a very small range of movement before the muscle yielded to give a frictional resistance thereafter. Hill renamed this

reluctance by the muscle to yield at the start of a stretch, the "Short Range Elastic Component" (SREC).

Hill (1968) reported that the SREC had a range of movement which accounted for only 0.2 % of the muscle's length, and concluded that the SREC was the product of the mechanical stiffness of a small number of cross-bridges attached even in the resting muscle. When the muscle was stretched slowly the strain on the cross-bridges increased and the tension rose. If the velocity of the length change is not too low the cross-bridges do not break spontaneously and instead slip along the actin filament. The tension developed in these early stages is almost linearly related to the amount of stretch and hence the term of "elasticity" is a just one as the muscle is obeying Hooke's law. Later when the length change approaches the elastic limit of 0.2 % the rate of slip increases. If the length change continues at the same velocity the tension levels off to give a constant frictional resistance. It is frictional rather than viscous because of the fact that the force developed is only slightly dependent on the velocity of the length change.

CROSS-BRIDGES IN THE RELAXED MUSCLE

Evidence now exists for cross-bridges being attached in relaxed muscle, which are known as "stuck" or "locked on" cross-bridges (Huxley 1960). Brenner, Schoenberg, Chalovich, Greene and Eisenberg (1982) and Schoenberg, Brenner, Chalovich, Greene and Eisenberg (1984), showed that there are cross-bridges in relaxed skinned rabbit psoas fibres at low ionic strength, which are sensitive to the concentration of free Ca^{2+}. Moss, Sollins and Julian (1976) gave further

evidence for attached cross-bridges in relaxed skinned frog muscle fibres, as well as in glycerinated rabbit papillary muscles. They demonstrated that these attachments are sensitive to the concentration of Ca^{2+} in the cytoplasm. This work suggested that it was these attachments in the resting muscle that underlie the presence of the SREC, as removing the external Ca^{2+} in these fibres where the myofibrils were directly exposed to the bathing medium abolished the SREC.

OTHER POSSIBLE FORMS OF ATTACHMENT

If it is the attached cross-bridges which are the underlying mechanism for the SREC, as well as the observed thixotropic effects, then the SREC should be decreased with extensions of the muscle beyond the optimum sarcomere length of around 2.25 μm, and should eventually vanish at sarcomere lengths of around 3.6 μm (where there is no overlap between the actin and myosin filaments). Haugen and Sten-Knudsen (1981a) using single twitch fibres of the frog semitendinous muscle were able to show the SREC increased by a factor of 2.9 as the sarcomere length was increased from 2.3 to 3.0 μm, and then declined to a very low level at 3.6 to 3.7 μm. This paradoxical result suggests that other factors besides filamentary overlap, such as filamentary spacing, may play a part in the generation of the SREC.

There may be other forms of interaction between the actin and myosin filaments besides cross-bridges, such as molecular forces between the two filament populations. Certainly when hypertonic solutions have been employed the SREC is increased with no extra filamentary overlap (Hill 1968; Lannergren 1971). Studies on the mechanism underlying the hypertonic

effect have shown that the solution acts on the contractile mechanism through a change in the intracellular milieu. As muscle fibres shrink in hypertonic solutions, the resulting increase in internal ionic strength, may inhibit myofibril adenosine triphosphatase (ATPase) activity, but also increases the percentage of free calcium surrounding the muscle's contractile material, leading to an increased number of interactions between the filaments (Podolsky and Sugi 1967).

Berman and Maughan (1982) reduced the fibre width by the addition of a non-penetrating long chained polymer "Dextran T500", which causes a decrease in the filamentary spacing without penetrating the fibre unlike hypertonic solutions. When Dextran T500 has been added to single fibre preparations, the stiffness of the fibre is increased. The increased stiffness may reflect an increased interaction between the cross-bridges as the actin and myosin are compressed together, so creating an environment where more cross-bridges could be formed, consequentially increasing the SREC. But it could equally be a reflection of increased molecular bonding between the filaments.

Although thixotropy in humans, rats and frogs, and the SREC have been investigated in isolated muscles and fibres, there has been no systematic investigation of the role of the SREC in thixotropy, and the part that thixotropy plays in the behaviour of the muscular system. It is the aim of this study to determine if thixotropy can be observed in isolated whole frog muscle and if the SREC can be linked to this property, and to consider any possible physiological consequences of the effects.

METHOD

In this section general details of the animals, muscles, dissection, mounting, apparatus and the notation for stretches, will be described. Where modifications to the methology have been made these will be described in the appropriate chapters.

ANIMALS

Common frogs *Rana Temporaria* were used. They were imported from Ireland, and kept in tanks without light and food, maintained at approximately $4^{o}C$. Before a series of experiments the frogs were kept for several days to equilibrate to the conditions of the tanks. Males and females were used randomly in all studies. Even without any obvious external stimuli the frogs followed a normal mating pattern, the males displaying amplexus in February onwards and the females spawning in mid Spring.

DISSECTION AND ANATOMY

Frogs were stunned, decapitated and pithed and the sartorius muscle dissected out. This is a sheet muscle, lying on the ventral surface of the thigh, thin enough to be aerated by diffusion, and made up of long parallel fibres. The tibial tendon was cut and tied with a small piece of stainless steel wire (0.05 mm diameter Goodfellow Fe/Cr18/Ni8 FE205115/1). The sartorius was then separated from any further connective tissue up to the pelvic bone insertion. The muscle was removed with a piece of the pelvic bone still attached. The bone was trimmed from around the muscle until a small piece was left (approximately 5 mm square).

MOUNTING (APPARATUS A)

FIGURE 1a shows a diagrammatic representation of the apparatus used for these experiments. The muscle was mounted by sliding the piece of pelvic bone under a stainless steel hook at the bottom of the muscle chamber. The tibial end, with the stainless steel wire attached to the tendon was connected to a slot in a light 4 cm aluminium lever. The wire was retained in the slot by a small tapered wedge. With the muscle held vertically in the apparatus the sides of the chamber were raised (not shown in FIGURE 1a), and the bath filled with 10 ml of isotonic Ringer solution to cover completely the muscle (for Ringer composition see TABLE 1a). The chamber was constructed of perspex and double jacketed so that water could be circulated around the chamber. To facilitate the removal of the Ringer solution from the chamber there was a drainage tube at the bottom of the chamber, so fresh solutions could be added at any time.

The aluminium lever was mounted on the shaft of a miniature electric motor (ESCAP 15M61). This motor was used in conjunction with a torque servo system, allowing precisely controlled forces to be applied to the muscle. There was a small amount of mechanical dither in the motor and this was used to reduce the friction of the apparatus to a negligible amount. This meant that there was very little resistance to movement and very small forces could be utilized. The muscle length was altered by moving the whole lever and motor arm up or down, by using a micrometer adjustment screw, so the length of the muscle in the apparatus corresponded to that of the resting muscle measured in the body with the leg at 90^{o} (L_{o}). A backstop was moved into position, once the muscle had

FIGURE 1a: A schematic representation of apparatus A.

M Motor generates small torques

L Lever arm

S Muscle attached to lever by fine stainless steel wire

H The pelvic bone is held in place by a stainless steel hook

T Eddy current transducer looks at a metallic target (TA)

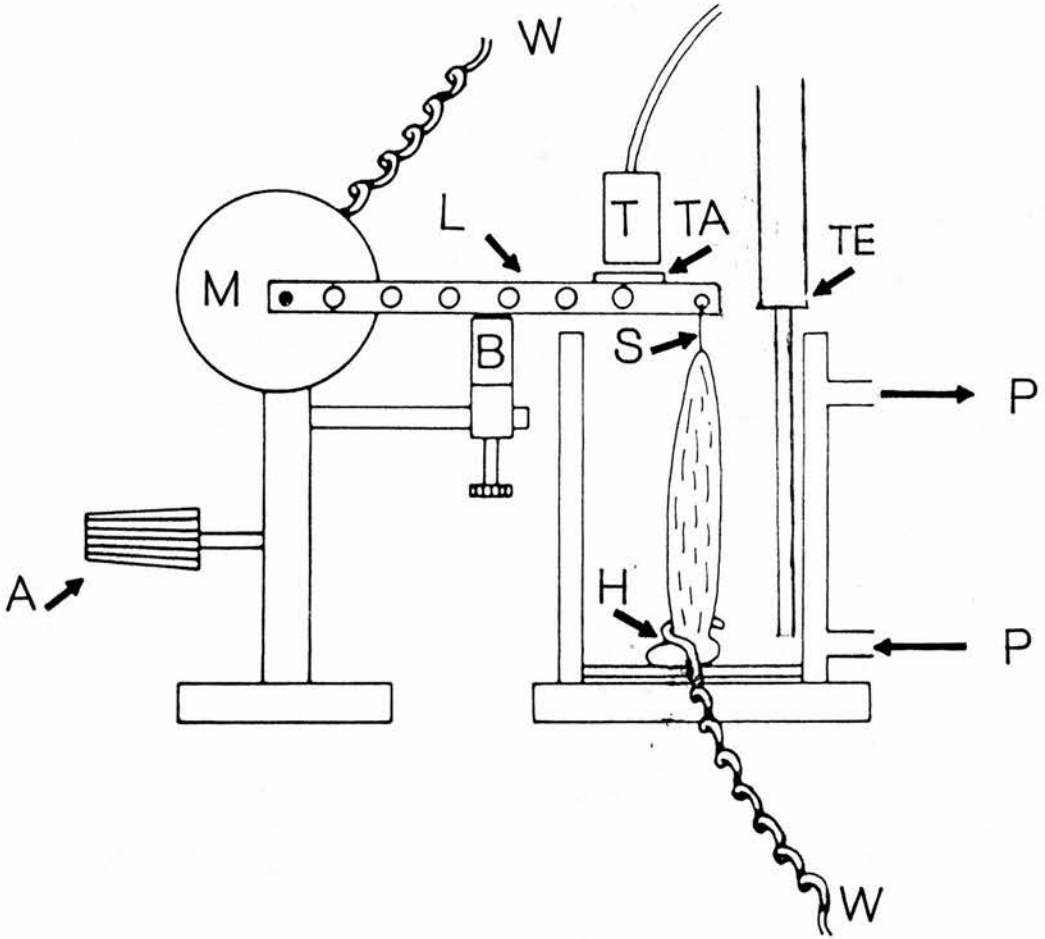
A Adjuster to set muscle to L_o

B Backstop prevents shortening upon stimulation

W Wires for stimulation

P Water is circulated via ports to maintain set temperature

TE Temperature probe monitors temperature of the muscle and surrounding Ringers, connected to thermostat (not shown)


W
L
T
TA
TE
M
B
S
P
A
H
P
W

been set at the correct length, with the lever just balanced to be in contact with the backstop. The force needed to do this represented the torque required to balance the weight of the muscle and the lever. Once this balancing force had been determined it was not altered at any time during the experiment. This backstop prevented the muscle from shortening during the experiment, and provided a base line length to which the muscle returned after a series of stretches.

TEMPERATURE CONTROL

The temperature of the bathing Ringer was monitored by a temperature probe situated in the muscle chamber next to the muscle. This temperature probe (platinum resistance probe (PT100) class A) was connected to a thermostat (platinum temperature controller), controlling the temperature of the chamber to within +/- 1^{o}C of the set temperature, by switching on and off a water pump which circulated iced water around the chamber. The temperatures used were 3, 10 and 17^{o}C.

STRETCHES

For all the experiments reported here the muscles were studied under isotonic conditions with measurements of length changes being made to imposed forces. These applied forces will be referred to as "stretches".

The following notation will be used throughout this thesis for the stretches used in the experimental protocols:

S_1; (First stretch) Used as an indication of the baseline stiffness for later comparison with the S_2 and S_3 stretches. In all chapters (except this one) the S_1

stretch is applied at exactly 3 min intervals after the stirring or stimulation had ceased. The experiments therefore have a fixed cycle length, continuously repeating. In this chapter the cycle length was set to 10 mins.

S_2; (Second stretch) This stretch is applied to the muscle at a fixed time after a period of stirring or stimulation. It therefore provides a measure of the effect of the stirring or stimulation.

S_3; (Third stretch) This stretch is applied to the muscle at a chosen time after S_2 and before the end of the cycle. It therefore indicates the degree of stiffness recovery.

TREATMENT OF THE RESULTS

-DEFINITIONS-

Stiffness; (Young's Modulus, E value): A measure of the resistance to the imposed stretches by the muscle at any given moment in time. It was calculated from the displacement that each stretch produced and from the muscle dimensions. The units used are g/cm^2.

Thixotropy: The changes in the muscle's stiffness that occurred as a function of time, and as result of the muscle's previous history of movement.

-CALCULATION OF YOUNG'S MODULUS-

Following each experiment the muscle was removed, gently patted dry, and weighed. Data was collected from a number of sartorius muscles, of varying dimensions, so that the results had to be normalized. This was completed by converting the data into a value of elastic modulus; ("Young's modulus"

(E)), using the following formula.

$$E=(F/A)/(\Delta L/L_o)$$

F = Force of the stretch applied to the muscle

A = Area of the muscle

ΔL = The change in length as a result of the stretch

L_o = The original length of the muscle

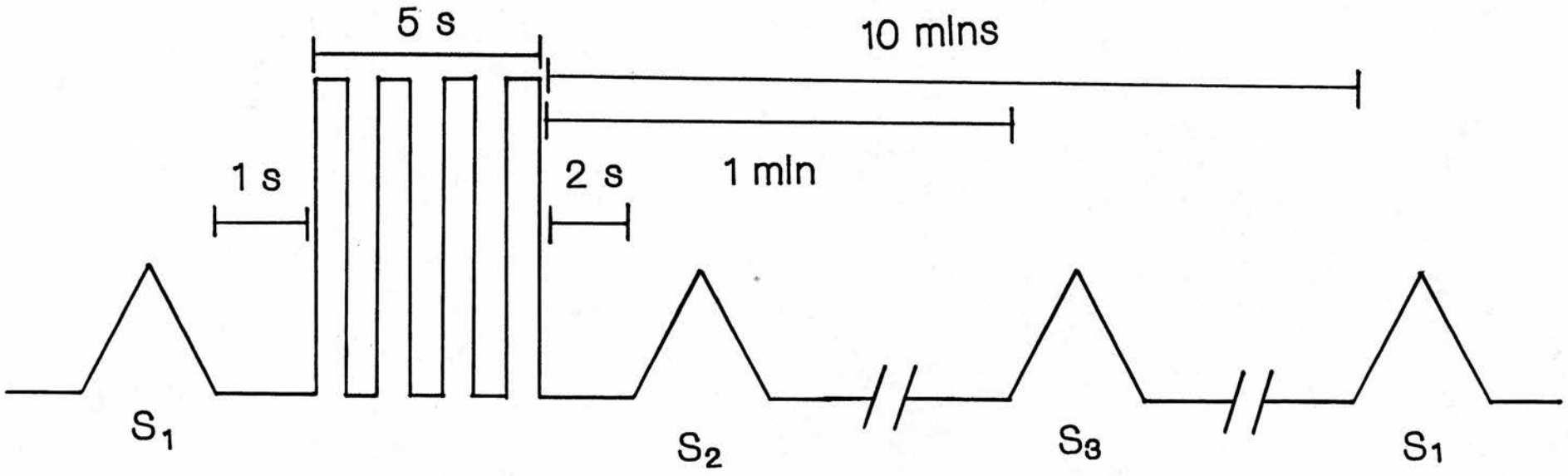
Young's modulus will remain constant for any material which obeys Hooke's law and is independent of the size of the specimen. Hooke's law states that the extension ΔL is proportional to the force, so that the material would give the same stiffness values no matter what force was selected, within appropriate limits. However muscle does not follow Hooke's law, and is highly non-linear. The elastic modulus was assessed at the point where each of the three stretches was applied. The higher the E value the stiffer the muscle was at that particular moment.

PROTOCOL

The experiments were carried out in November and December.

FIGURE 1b shows a schematic diagram for the timings of the stretches and stirring used in this protocol. The muscle was subjected to a series of three single triangular stretches in each trial, controlled by a Digitimer D4030 (accurate to 1 μs). S_1 was generated on a 10 min continuous cycle following the last period of stirring the muscle had received. One second after S_1 the muscle's received 5 s of 'stirring', comprising a series of square wave oscillations (3 Hz, maximum movement of 2.5 mm). The stirring waveform was so arranged that it always ended on a downward stroke. This was to ensure that the muscle was always resting

FIGURE 1b: Schematic diagram (not to scale) of the protocol used in Chapter 1, showing timings and relative order of the test stretches and stirring.


5 s
10 mins
1 s
2 s
1 min
S1
S2
S3
S1

against the backstop at the commencement of S_2. Stirring was followed 2 s later by S_2, identical in force to S_1. The third and last stretch (S_3) in each test run was 1 min after the end of stirring, again with the same force as the previous two stretches.

The movement that each stretch produced was recorded by a contactless eddy current transducer (EMIC 502F, resolution 1 μm). The signal was displayed on a Nicolet 3091 digital oscilloscope which allowed accurate measurements to be made at the time of the stretch; or the data could be sent to an IBM AT microcomputer, which was interfaced to the oscilloscope, using the PC/31 software package, for data storage and later analysis of the results. All the triangular stretches in any particular trial were of the same magnitude, but the force was varied randomly between the trials. The peak forces of the triangular stretches used in this study were 0.2, 0.4, 0.8 and 1.2 g. The duration of the triangular stretches was 300 ms for the complete stretch.

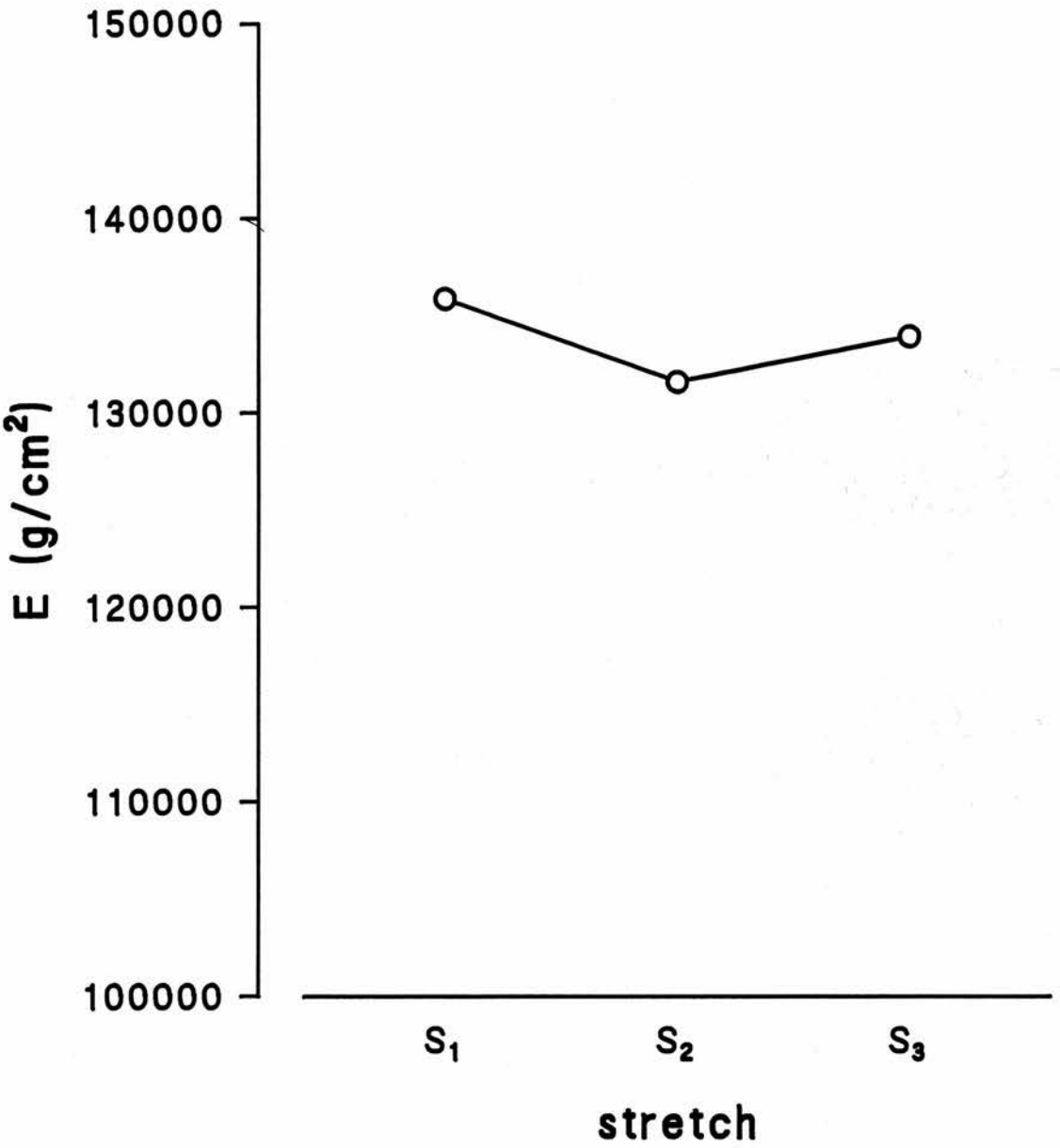
CONTROLS

A number of controls were also undertaken to show that the observed changes could not be attributed to artifacts or originate in the apparatus itself.

-CONTROLS FOR THE APPARATUS-

The muscle was replaced with an elastic band, of approximately the same dimensions as the muscle. Attached to the apparatus by stainless steel wire at both ends and tied into place, then surrounded with isotonic Ringer solution. All four forces, and three temperatures were tested with the elastic band and the results indicate that stiffness was much

FIGURE 1c: The effect of replacing the muscle with an elastic band on thixotropy (mean, SD not shown), with approximately the same mean dimensions as the sartorius muscle's (length 25 mm, width 0.5 mm n=3, 3°C, only 0.2 g peak force shown other forces gave values not significantly different from these).


150000
140000
130000
120000
110000
100000
E (g/cm²)
S1
S2
S3
stretch

higher than for the muscle, and that stirring did not produce any decrease in the stiffness values (FIGURE 1c). The four forces produced small movements proportional to the force used. Temperature had no effect on the stiffness values for the elastic band.

-EXPERIMENTAL CONTROL-

A muscle was set up in the standard manner as for the test experiments, but the 5 s of stirring was omitted, with the muscle only receiving the three test stretches. All force levels were tested in this way.

TABLE 1a: The compositions of the Isotonic Ringer solution used to bath the frog muscles. The various drugs tested in the later Chapters were made up in this basic solution.

Salt	mM
NaCl	111.0
NaH_2PO_4	0.04
$NaHCO_3$	4.7
KCl	1.8
$CaCl_2$	1.08
Glucose	11.1

This salt solution was unaerated.

RESULTS

EFFECT OF STIRRING

The results presented in FIGURE 1d show the mean and standard deviations (SD) for the 0.2 g peak force and 3°C trials only. The S_2 stretches applied 2 s after 5 s of stirring have lower stiffness values than the S_1 stretches. The decrease from S_1 to S_2 was approximately 50 %. The third stretch (S_3), occurring 1 min following the cessation of stirring, yields stiffness values which are intermediate between those of the S_1 and S_2 stretches. The S_3 values of stiffness can be used to give an indication of the amount of stiffness the muscle has regained in that period of time. The muscles regain approximately 90 % of the original starting S_1 stiffness value, after 1 min at rest.

It must be emphasized that the S_1 stretch is a purely arbitrary base line indication of the muscle's stiffness with which the relative increases and decreases in the muscle stiffness can be compared. Any change in the stiffness of the muscle must represent the effects of the previous history of movement of the muscle.

EFFECT OF STRETCH SIZE

FIGURE 1e shows the effect of altering the peak force of the stretches on muscle stiffness. The E values for the 0.2 g peak force trial stretches are larger than those recorded from the 1.2 g peak force stretches, indicating that the muscle is less stiff for the larger force. The 0.4 g trials do show a small decline after stirring, and a recovery of stiffness after 1 min at rest, but the relative amount of change is reduced compared to the values obtained for the

FIGURE 1d: The mean stiffness values and SD for the 0.2 g peak force, 3°C trials only (n=8). S_1, Control stretch; S_2, 2 s after stirring and S_3, 1 min after stirring.

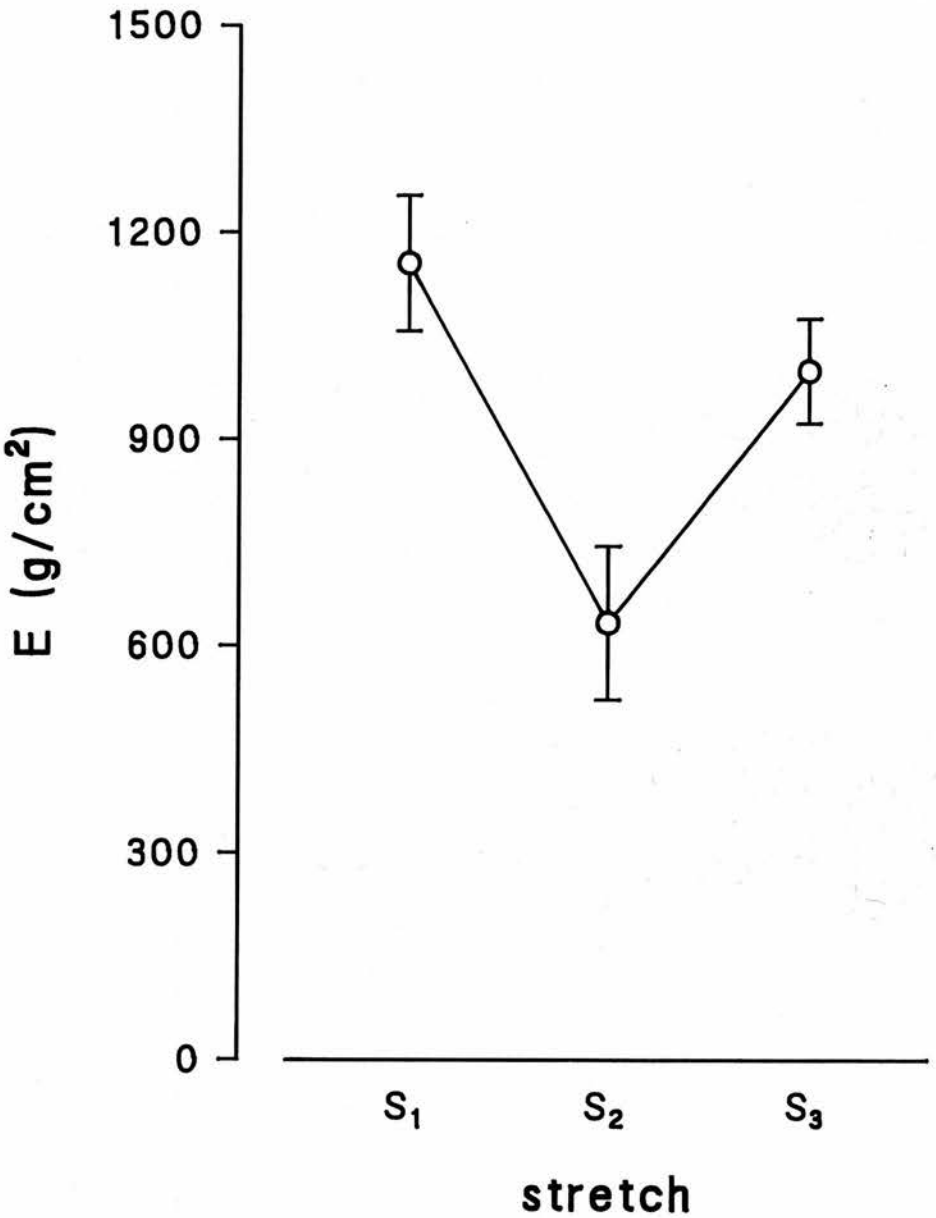

1500
1200
900
600
300
0
E (g/cm²)
S1
S2
S3
stretch

FIGURE 1f: The effect on the mean (plus SD) stiffness values of raising the temperature from 3 to 17°C, 0.2 g peak force only plotted, n=8.

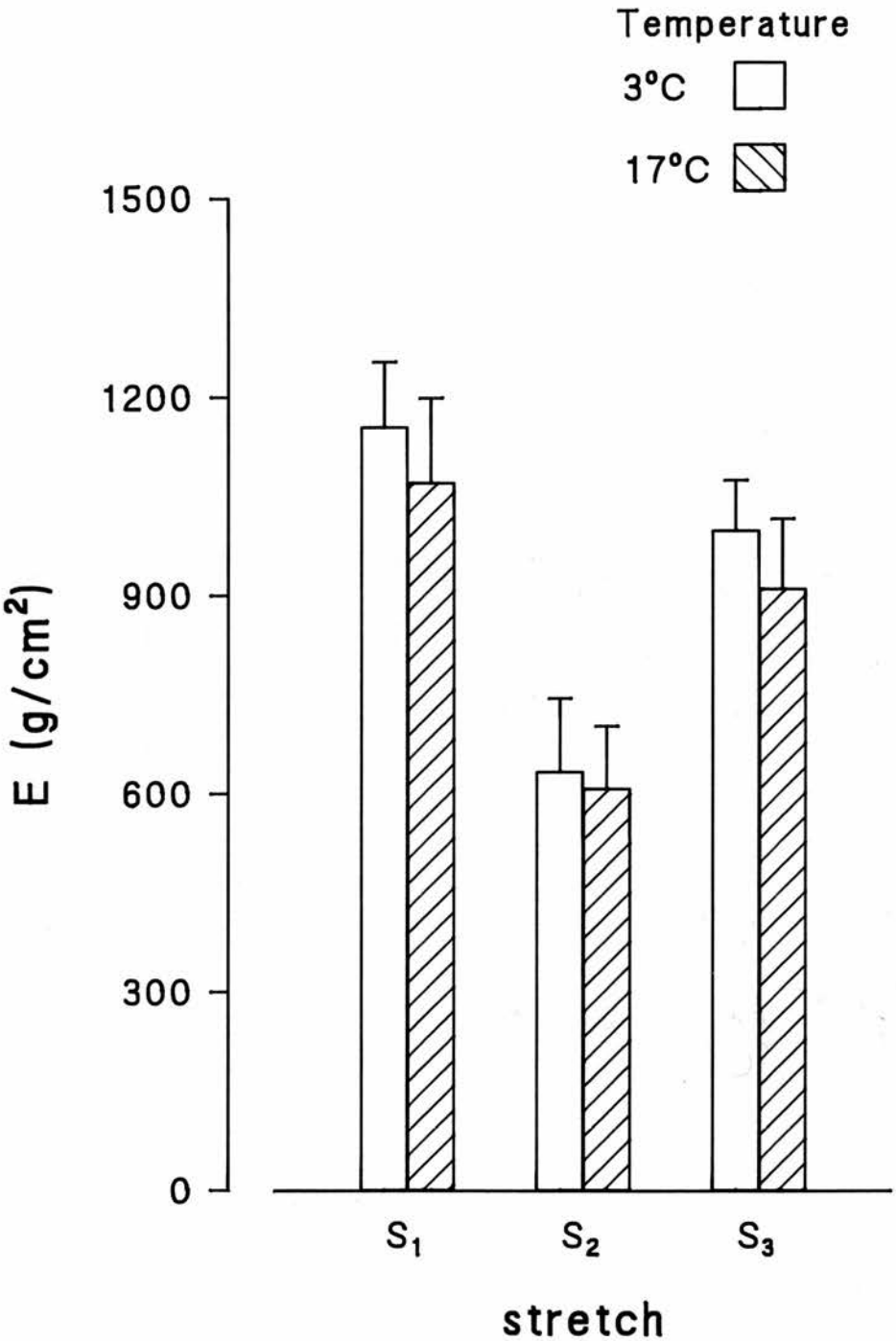

Temperature
3°C
17°C
1500
1200
900
600
300
0
E (g/cm²)
S1
S2
S3
stretch

FIGURE 1g: The effect on the stiffness values of removing the stirring waveform (3°C only, n=8, SD's are not plotted for clarity)

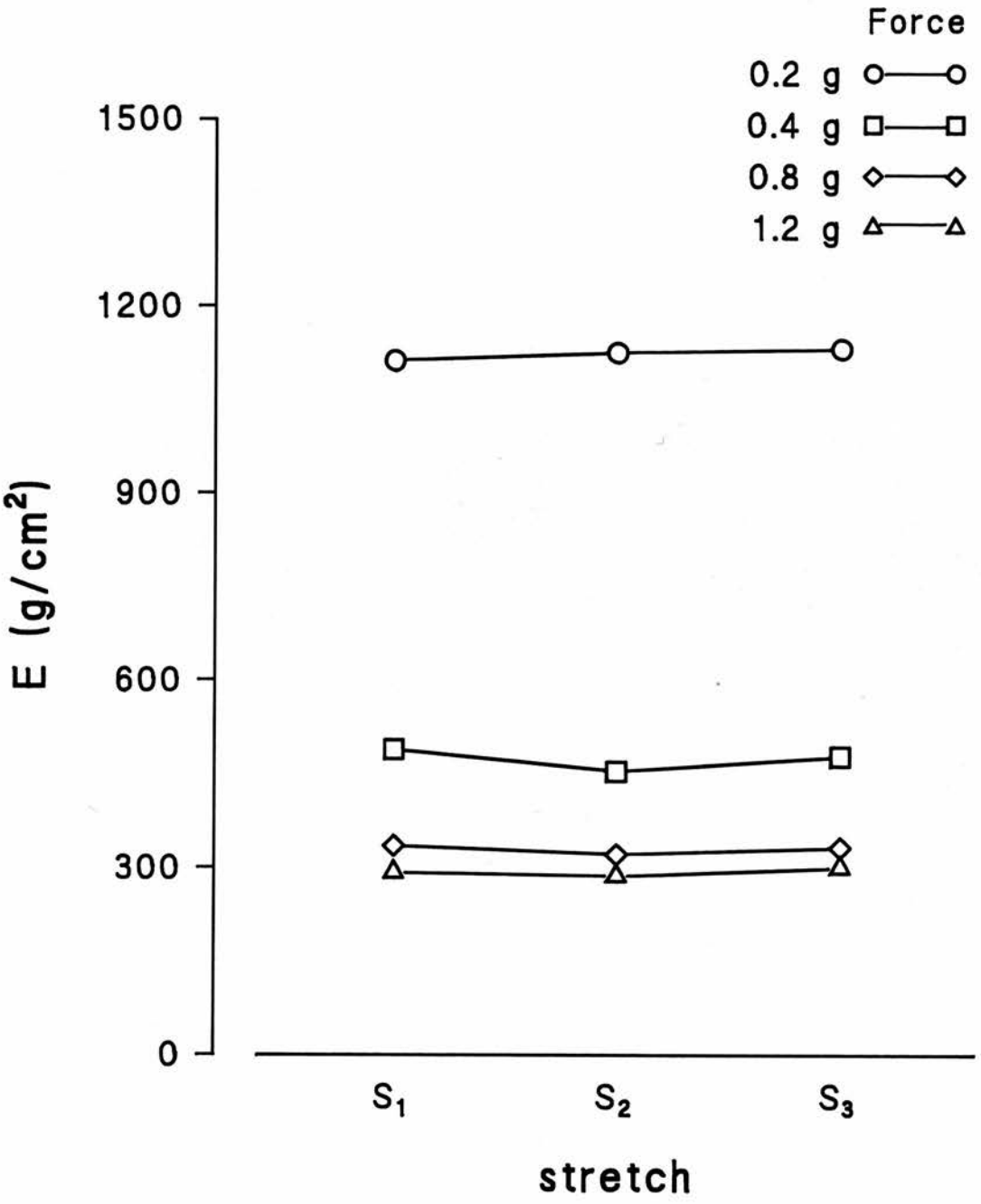
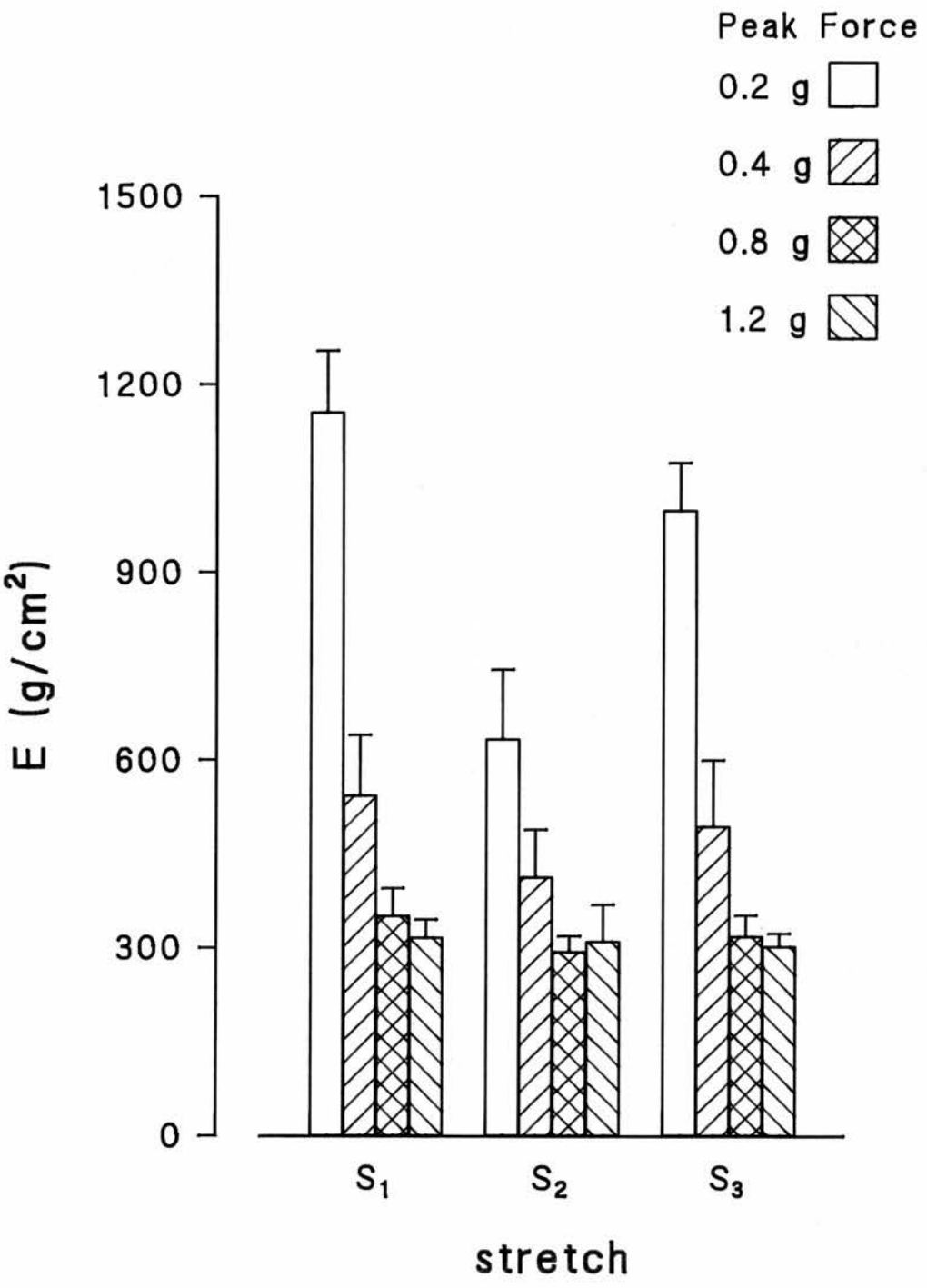

Force
0.2 g
0.4 g
0.8 g
1.2 g
1500
1200
900
600
300
0
E (g/cm²)
S1
S2
S3
stretch

FIGURE 1e: The effect of increasing the peak force of the stretches on the stiffness values (mean and SD are shown) at 3°C only (n=8).


Peak Force
0.2 g
0.4 g
0.8 g
1.2 g
1500
1200
900
600
300
0
E (g/cm²)
S1
S2
S3
stretch

0.2 g trials. The stiffness decrease following stirring for the two larger forces are even less, and there is little difference in the stiffness over the three stretches.

When an analysis of variance (ANOVA) was conducted on the results it was found that for the 1.2 g trials the difference between the stretches is significant at only the $p < 0.05$ level, compared to the very highly significantly difference ($p < 0.001$) that was found with the 0.2 g trial.

FIGURE 1e also shows the muscle's non-linear stiffness characteristics to increases in the peak force of the stretches. For the S_1 stretches a doubling of the peak force from 0.2 to 0.4 g approximately halved the stiffness values but a doubling of 0.4 to 0.8 g only caused a reduction in the stiffness values of about a third.

TEMPERATURE

FIGURE 1f shows the effect of temperature on stiffness, (from 3 to 17^{o}C). Although there is a slight indication that the muscles are less stiff at the higher temperature, this difference is not significant. There was no significant difference in the responses obtained at the higher temperatures with the other three peak forces either.

CONTROLS

When the muscle received no stirring, there was no drop in the stiffness values of S_2. All three stretches had similar stiffness values (FIGURE 1g). The stiffness values obtained for the S_1's in the test trials are not significantly different from the values plotted here.

DISCUSSION

It has been recognized for some time that resting muscle is stiffer for small movements than for larger movements (Denny-Brown 1929). Cat muscle, when stretched passively, shows at least two distinct phases in its tension response. The first of these phases which he called the "preliminary" or "stationary" rigidity, was found to be a characteristic of both the extensors and the flexors, whenever the muscle had been allowed to rest for any period of time longer than 1/10 s. Denny-Brown therefore concluded that this stationary rigidity was a peculiarity of muscle and in particular the muscle fibre. He also noted that the extent of this rigidity was reduced by any previous stretches applied to the muscle shortly before the test, and was restored when the muscle rested at a stationary length.

D. K. Hill (1968) re-investigated this "stationary rigidity" using pairs of frog sartorii in hypertonic solutions, giving them stretches in the form of length changes at constant velocity and recording the tension from these resting muscles. He noted the same reluctance of the muscle to yield at the beginning of any stretch, and re-named the phenomenon the SREC. The SREC constitutes approximately 0.2 % of the muscle's length before it abruptly disappears and the muscle yields and shows a frictional resistance thereafter. It was concluded that the resting muscle possesses a few attached cross-bridges, which are very stable and long lived, and confer upon the muscle a permanent tension by being so orientated as to provide elastic resistance. The whole muscle thus becomes an efficient system for holding a small tension indefinitely. This early

work of Denny-Brown and Hill forms the basis for an explanation of the results obtained here, and a working hypothesis to explain thixotropy.

In the study the lowest force (0.2 g) produced both the highest levels of resting stiffness, and the greatest amount of thixotropic change. At this small force the S_1 stretch averaged displacements of 27 μm, where L_o averaged 25 mm. This means the displacements for S_1 were 0.11 % of L_o, less than 0.2 % of the muscles resting length, and are therefore within the range of the SREC. S_1 stretches produced by the 0.4 g peak force yielded displacements averaging 130 μm, around 0.5 % of the muscles length. Thus the 0.4 g stretches produced movements just beyond the muscle's 0.2 % elastic yield point for the SREC. The two remaining forces, 0.8 and 1.2 g, produced movements well outside this 0.2 % range (1.4 and 2.4 % respectively). The response of the muscle to the increasing peak force is highly non-linear, a doubling of the force, 0.2 to 0.4 g and 0.4 to 0.8 g, does not result in a halving of the stiffness in each case. The fact that the 0.8 and 1.2 g peak force stretches gave E values which are approximately of a similar order of magnitude, and that there was little difference between the S_1, S_2 and the S_3 stretches, tends to suggest some limit had been reached which these larger forces had exceeded. The 0.4 g stretches are producing displacements that represent a borderline situation. A small but significant thixotropic change is still elicited, but is greatly reduced from the changes seen with the 0.2 g peak force stretches, whereas with the 0.8 g peak force trials, thixotropic changes are virtually absent.

The effect of the 5 s of stirring on the 0.2 g peak force

stretches was to cause a dramatic transient reduction in the level of stiffness, possibly by destroying any attached cross-bridges that existed in the resting muscle. For the larger forces (0.8 and 1.2 g peak force) the S_1 stretch was sufficient in its self to rupture the bonds forming the SREC and stirring had little, or no, further effect on the already reduced level of stiffness. In the case of the 0.2 g peak force stretches the reduction after stirring was to 50 % of the resting value. With the 1.2 g peak force trials the reduction after stirring is only about 10 % at the most.

The lost stiffness after stirring can be recovered if the muscle is allowed time to rest without any movement. The recovery does not follow a linear time course, as by the end of 1 min the muscle has regained about 90 % of the control stiffness value for the 0.2 g stretches, with the remaining 10 % regained in the following minutes after S_3 and the start of the next trial. This time scale for the recovery of the muscle's stiffness can be compared to that reported by Lannergren (1971) who found in single iliofibularis fibres of the frog that stiffness recovery after movement took up to 3 mins. Buchthal and Kaiser (1951), using isolated bundles of frog semitendinosus muscle, found if the bundles were given repetitions of stretches of such a magnitude as to just exceed the 0.2 - 0.3 % limit of the "plastic" yield point of the muscle's resting length, there was a loosening of the muscle over successive stretches. However, if the muscle was allowed to rest without movement the stiffness was recovered in "a few minutes". Buchthal and Kaiser named this property "Thixotropy".

Wiegner (1987) studied the thixotropic response in the

rat soleus muscle, and found the recovery time for mammalian muscle was in the order of only 4 - 10 s, faster than seen in the frog, but similar to that seen in human studies (Lakie *et al* 1979, 1983 and 1984). In humans only a few seconds was necessary for the recovery of the stiffness. However, recent studies by Lakie and Robson (1988c) have followed stiffness recovery in the human interphangeal joint of the finger for up to 30 mins and have found that the system continues to stiffen. This is a good indication that any level of stiffness is a purely arbitrary value as the initial stiffness depends on the time the muscle has been at rest before the measurements are taken. Even the very act of taking a reading will by definition have a distortion factor as the muscle has been disturbed.

Temperature did not seem to affect the thixotropic properties of the muscle. This confirms the results of Wiegner (1987) who showed in the rat that thixotropy was not affected by the temperature. He concluded that temperature affected some muscle structures other than those responsible for the SREC, as the SREC appeared to be unchanged by changes in the temperature, whereas the temperature sensitive viscoelastic response for larger perturbations were affected. The results presented here show that the initial stiffness values at $3^{o}C$ were generally slightly higher than those at $17^{o}C$. For the 0.2 g force stretches the effect of temperature was not significant as the SREC is dominant for these small stretches. Temperature may have had a small effect with the larger forces where the SREC is no longer an important factor, and the more temperature dependent viscoelastic system is in operation.

The SREC is thought to be caused by a small number of attached cross-bridges present in the relaxed muscle. Cross-bridges have been identified in the resting muscle of rabbit psoas muscle at low ionic strength and in the frog (Brenner, *et al* 1982, Schoenberg *et al* 1984 and Moss *et al* 1976). In all cases these cross-bridges were linked to the presence of the SREC and the external concentration of Ca^{2+}; removal of the external Ca^{2+} abolished the SREC in skinned fibres. These cross-bridges must be connected long enough for the muscle to develop a measurable tension, and to cause the increase in the stiffness. They only break when the force applied to the muscle reaches a critical limit. The frequency of breakage of any unstressed or slightly stressed cross-bridge must be low, indicated by the higher stiffness values for the small force stretches. Eventually a limit must be reached where the maximum number of possible connections has been made and an equilibrium develops between the formation and destruction of the cross-bridges.

The attached cross-bridges confer on the muscle a stability in the face of imposed small movements. It has been shown that frog sartorius is thixotropic and the greatest thixotropic change is obtained with displacements in the range of the SREC, and so it can be supposed thixotropy is related to the number of attached cross-bridges in the resting muscle at any particular time. Thixotropy is a potentially useful muscle mechanism: it means that the animal is less susceptible to displacements as a result of random movements arising both from within and from outside the body.

CHAPTER TWO

THIXOTROPY: STIFFNESS RECOVERY RATE

THIXOTROPY IN FROG MUSCLE

Frog muscle has been shown to be thixotropic, and is disproportionately stiffer for small movements than for larger movements (Hill 1968). This stiffness can be reduced when the muscle has been stretched to a length that takes it beyond the range of the short range stiffness (SRS/SREC), calculated to be around 0.2 % of the muscle's resting length. The reduction in the muscle's resting level of stiffness is transient. The muscle will, on being allowed to rest, regain its initial stiffness level.

RECOVERY OF STIFFNESS

In Chapter 1 stiffness recovery was measured 1 min after a movement and also at the end of the trial period. It appeared that the recovery of stiffness was a fairly fast process, 90 % of the original stiffness being recovered within the first minute after stirring had ceased. The remaining 10 % of the stiffness was presumably regained in the remainder of the trial period, as by the end of the 10 min period the initial stiffness value had been restored.

FURTHER DETAILS ABOUT THE ORIGIN OF THE SREC

The origin of the SRS/SREC is still somewhat in doubt, although the hypothesis of Hill (1968), that it is the result of a small number of cross-bridges attached to the actin filaments even in the resting state of the muscle, would seem to be the most favoured explanation. Even in the absence of activation a muscle exerts a significant level of resting tension. A. V. Hill (1952) wrote about tension in the resting muscle as follows:

"The tension of a resting muscle diminishes continually with decreasing length; there is no sharp end point and it is impossible to define a length at which the tension becomes zero."

Various structures within the muscle have been implicated in this tension production; attached cross-bridges, sarcolemma and other fibrillar material, are all possible contenders. Helber (1980), supports the notion that folds form along the actin filaments themselves, so that they fold back on themselves. The properties of the SREC can thus be interpreted completely in terms of a critical elongation of the elastic actin-filaments. Helber (1980) calculated this to be around 5 - 6 nm per sarcomere at low frequencies but extending to 100 nm per sarcomere at high frequencies. Helber's explanation for the origin of the SREC could also support the thixotropic results, as stretching of the fibre would straighten the actin filament and produce a looser muscle, and as the folds re-formed stiffness would increase. The SREC can be abolished in a Ca^{2+} free medium and this would seem to support the cross-bridge theory, rather than any other theory, which rely on other molecular bonds between the filaments. However Gillis and O'Brien (1975) and O'Brien, Gillis and Couch (1975), reported that when actin filament paracrystals (reconstituted muscle thin filaments) have Ca^{2+} added to the solution the actin helix will unwind slightly. The unwinding is reversible by changing the Ca^{2+} concentration over the range 10^{-6} - 10^{-5} M, and the extent of the unwinding was estimated to be equivalent in size to a full rotation of the myosin heads. Such Ca^{2+} dependent

unwinding would be a feature of stimulation rather than passive stretches, but is direct evidence that the folding of the actin filament may play a significant role in the production of the SREC.

The cross-bridge theory, the "meander" model of folds in the actin filament and the interaction of other bonding between and within the filamentary structures of the muscle could all explain the formation of the SREC. One muscle structure that can probably be discounted as a factor in producing the SREC is the sarcolemma. Rapoport (1972) found the sarcolemma played no part in the resting tension of muscle fibres when the sarcomere lengths were below 3 μm, and contributed only 10 % of the intact fibre's tension when the sarcomeres were between 3 and 3.75 μm, and linearly greater amounts at even greater sarcomere lengths. It would seem therefore that an important contributor to the resting tension are the bonds between actin and myosin and it is these that probably underlie both the SREC and thixotropy (Hill 1968, Halpern and Moss 1976, Morgan 1977, Wiegner 1987 and Lakie and Robson 1988a).

It is unlikely that the question of the origin of the SREC can be so simply explained, by one theory. The aim of this series of experiments was to look at the recovery of the stiffness after stirring, over 3 mins. The effect of changes in temperature, and the force of the test stretches were also investigated.

METHOD

ANIMALS

These experiments took place November and December. The frogs were kept several weeks before being used, at 4^{o}C and in the dark.

MODIFICATIONS

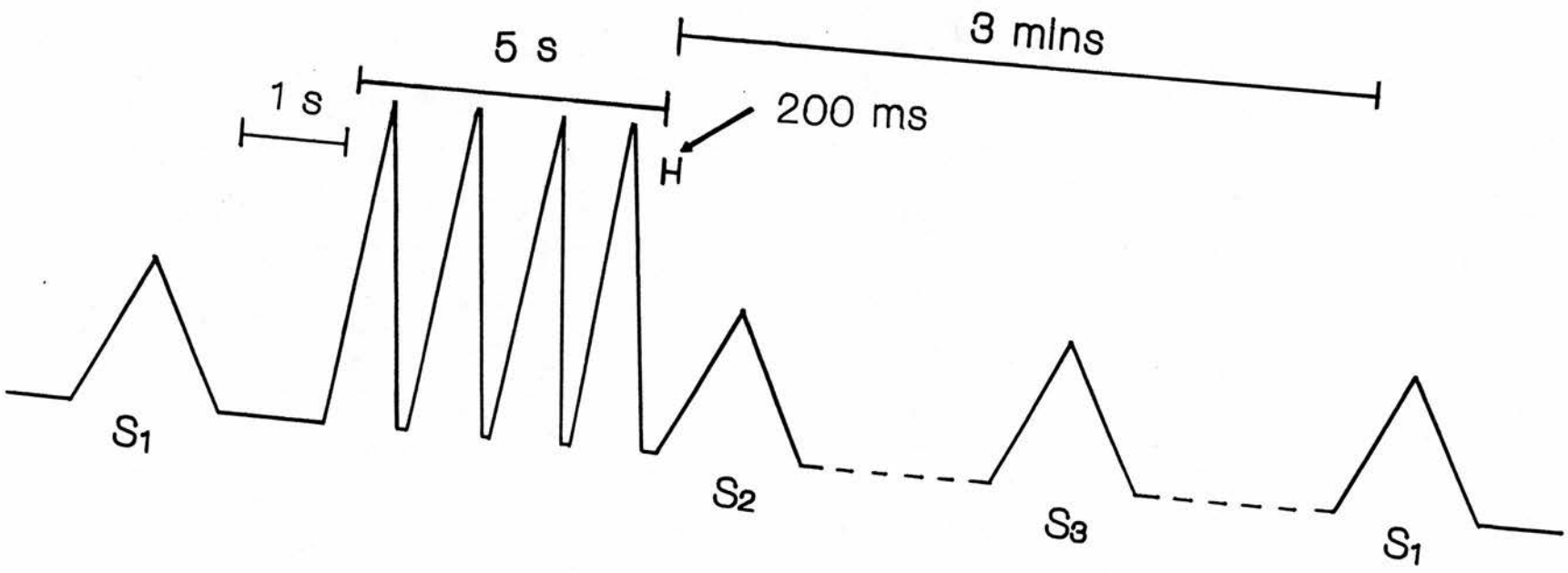
Two major modifications were made to the protocol that had been used in Chapter 1.

First, the force of the stretches used was set so as to produce the optimum thixotropic change. This meant the force of the stretches was arranged so that the displacements produced were less than 0.2 % of the muscle's length. After calculation it was found that a force of 0.3 g produced the desired effect. A larger force of 0.6 g was also tested which produced displacements outside the 0.2 % yield point.

Second, symmetrical triangular stretches for the stirring waveform were used instead of the rectangular stretches used in Chapter 1. The change in force was therefore at constant velocity rather than an abrupt jump. This had no effect on the size of the thixotropic effect elicited from the muscle. The three triangular test stretches had a duration of 400 ms for the full stretch.

The preliminary results described in Chapter 1 indicated that the resting stiffness level of the muscle varied with the time that the muscle rested. As the majority of the muscle's stiffness appeared to return by the end of the first minute, the trial cycle length was set to 3 mins rather than the 10 mins used in Chapter 1. This shorter time cycle would still allow significant alteration in the stiffness values of

FIGURE 2a: A schematic diagram (not to scale) of the protocol used in Chapter 2 for the recovery of stiffness, showing timings and relative order of the test stretches and stirring.


5 s
3 mins
1 s
200 ms
H
S1
S2
S3
S1

the muscle, and the period where the greatest stiffness changes occurred could be examined in detail.

PROTOCOL

FIGURE 2a is a schematic diagram of the protocol that was used in these experiments. S_1 was followed 1 s later by 5 s of stirring (3 Hz and a maximum movement of 2 mm). The stirring was followed by the S_2 stretch which was generated 200 ms after the stirring had finished. The S_3 stretch, occurred at a variable time generated between 1 s and 180 s after the stirring had ended. There was only one S_3 stretch per trial, and the time at which it was generated varied randomly from trial to trial.

Since the force within each trial for the test stretches was identical, the stiffness measured will be inversely proportional to the peak height of the resulting displacement.

TEMPERATURE

In these experiments 3 and 17^{o}C were used.

RESULTS

RAW DATA

FIGURE 2b shows a typical example of the raw data. It clearly shows that the S_2 displacements are nearly 100 % larger than those of the S_1 displacements. The stirring occurring between these two stretches had the effect of approximately halving the stiffness of the muscle. The size of the S_3 displacement was dictated by the time elapsing between it and the end of the stirring waveform. If the S_3 stretch was applied close to the cessation of the stirring then the displacements produced were larger, and if the time between the stirring and the generation of S_3 was lengthened then the size decreased.

This decrease in the response of the muscle to S_3 stretches in the trial was found to be very non-linear in character. The greatest drop in the size of the stretch was recorded soon after stirring, with a more progressive decline for the stretches occurring later in the trial.

FIGURE 2b also shows that the S_1 displacements for each trial were of the same magnitude as each other. This result was entirely dependent on using the same cycle length for each trial. If the trial length was increased for one of the cycles, then the S_1 stretch would have been smaller (a higher E value). Conversely if the trial length was shortened, the S_1 stretch would then have produced a larger displacement. The size of the S_2 displacement was not dependent on the trial length, but on being generated at precisely 200 ms after the stirring waveform, in every trial.

The displacements produced as a result of the stirring waveform were large in comparison to the three test

FIGURE 2b: In descending order, four trials (trial length 3 mins from the end of stirring and S_1), the S3's are 1, 2, 4 and 6 s after stirring. In the three lower traces the displacement record for the stirring has been truncated. Force 0.3 g 3°C.

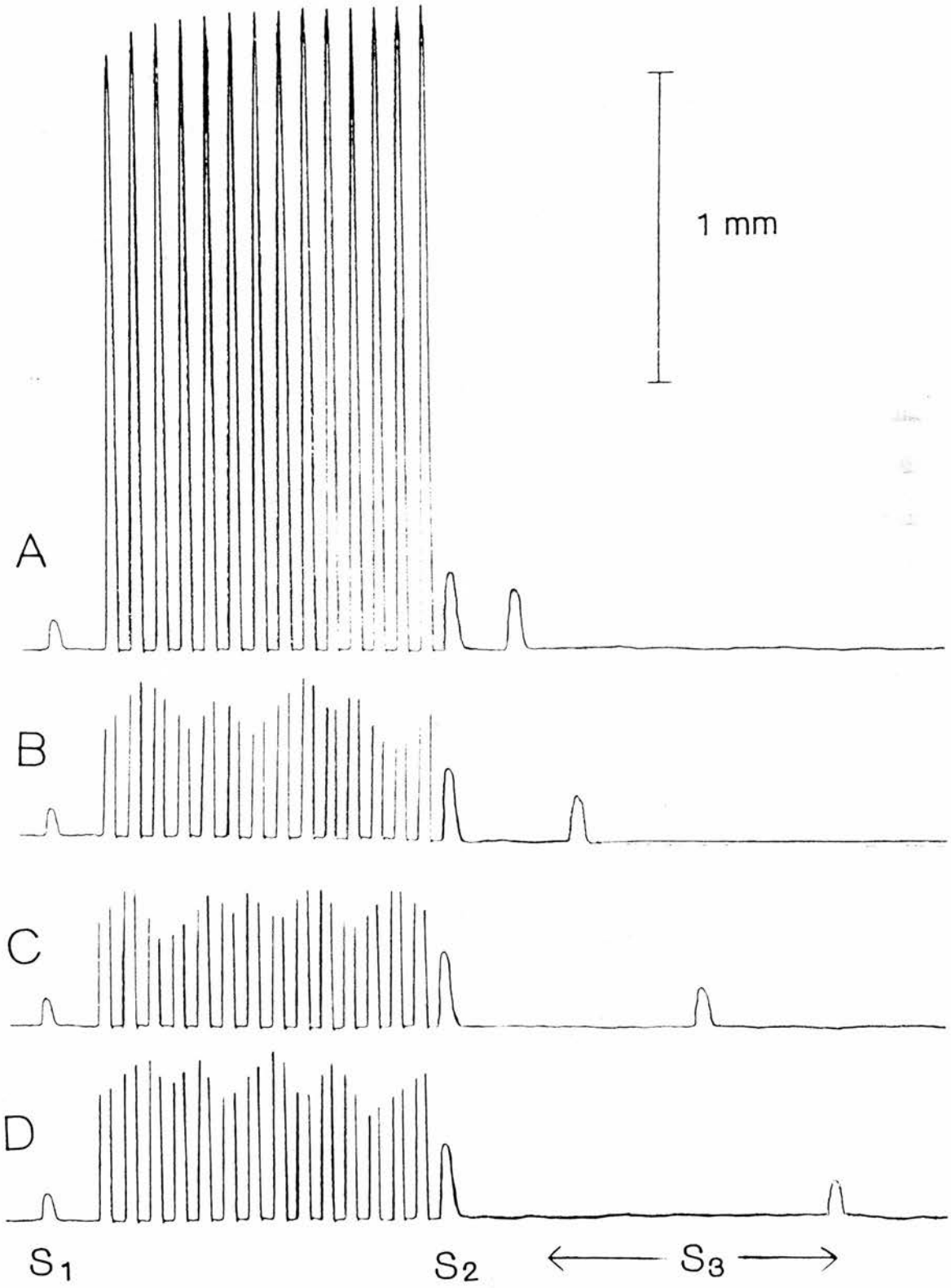

1 mm
A
B
C
D
S1
S2
S3

FIGURE 2c.A & .B: The mean values and SD of E for the S3 stretches at various times after stirring, for 3 and 17°C. Force 0.3 g, n=8.

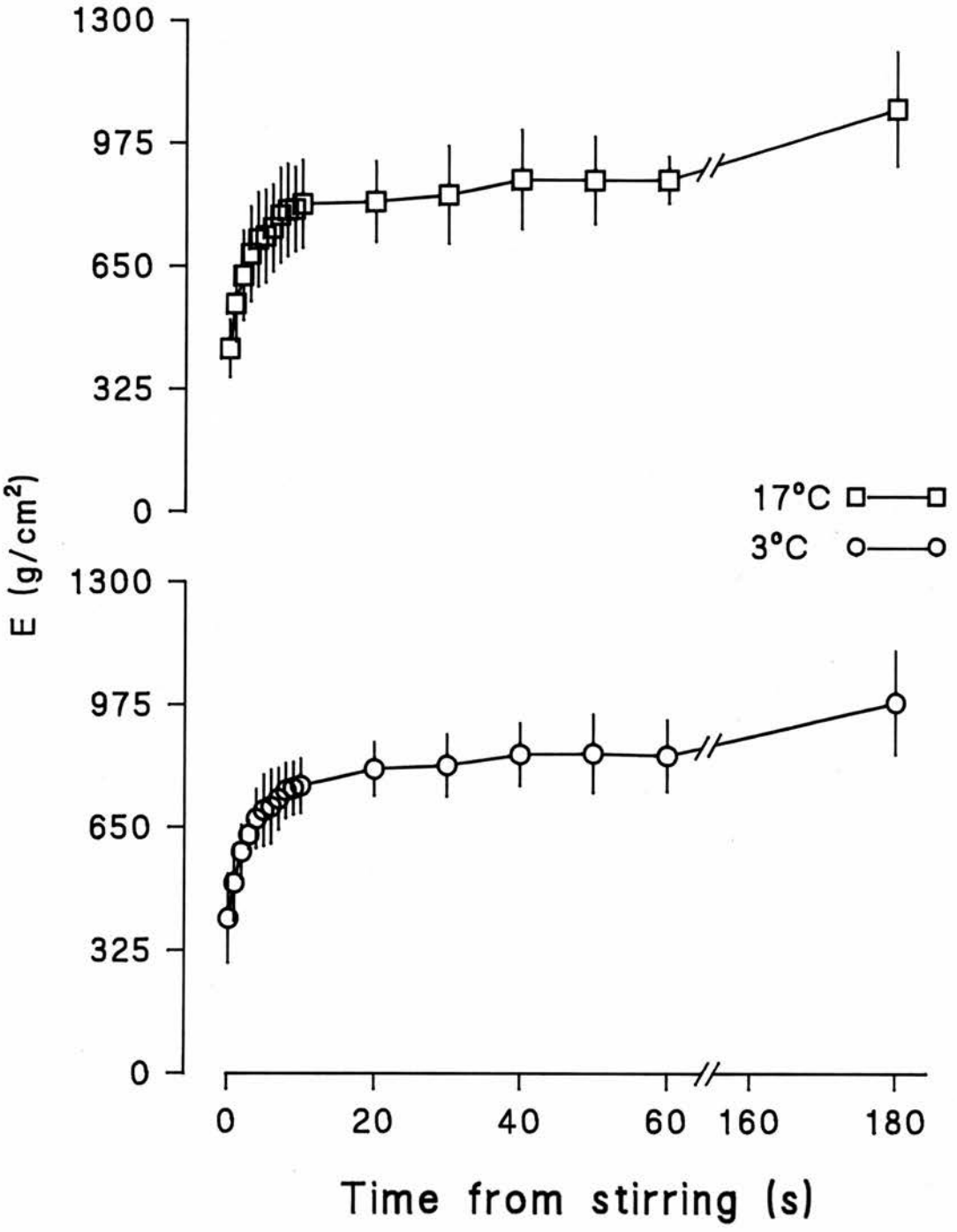
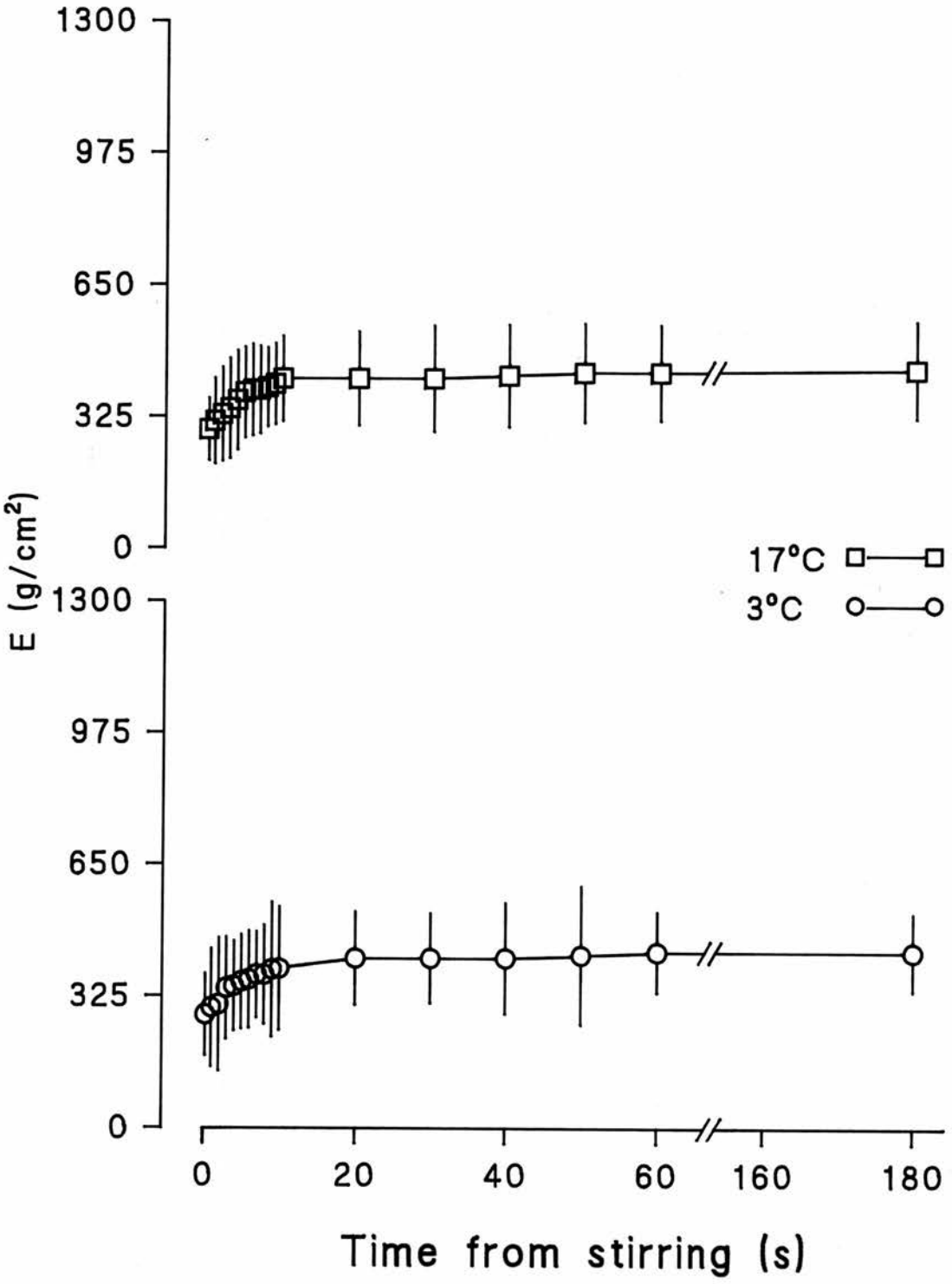

1300
975
650
325
0
E (g/cm²)
17°C
3°C
0
20
40
60
160
180
Time from stirring (s)

FIGURE 2d.A & .B: The mean and SD for the values of E for 3 and 17°C. Force 0.6 g, n=4.


1300
975
650
325
0
E (g/cm²)
17°C
3°C
0
20
40
60
160
180
Time from stirring (s)

stretches. The 2 mm movements caused by the stirring are within the physiological range of movements the muscle would be expected to make in the body. Even here, some degree of thixotropy can be seen. The first stretch did not produce the largest displacement, not until around the 4th or 5th stretch was the maximum displacement recorded.

STIFFNESS RECOVERY

FIGURES 2c.A & .B are graphs of the mean values for Young's modulus (E): for the recovery of stiffness following stirring. Recovery is almost complete within the first minute, with the return of stiffness most noticeable in the first few seconds after stirring. Recovery from 200 ms to 1 s after stirring is around 10 % of the initial stiffness (S_1). By the end of the first 10 s following the stirring 50 % of the stiffness has been recovered. By the end of the first minute 90 % has been recovered by the muscles. Therefore the recovery of stiffness by the muscle after a period of movement is highly non-linear.

Raising the temperature from $3^{o}C$ to $17^{o}C$ had no effect on the recovery rate or the size of the stiffness values.

SIZE OF STRETCH

FIGURE 2d.A & B shows the data for the 0.6 g trials, again at both temperatures. The same time scale of events for the stiffness recovery can be seen as with the smaller force, an initial fast recovery lasting up to 10 s after stirring, followed by a slower prolonged recovery over the remainder of the 3 mins. Recovery is over 90 % complete after 1 min. However the thixotropic loosening after stirring is reduced with the higher force; from S_1 to S_2 the stiffness

has dropped by a factor of only 1.5 compared to a drop of 2.4 with the 0.3 g peak force results (at $3^{o}C$). Temperature also had no effect on the results for this force; both curves are not significantly different.

S_1 displacements for the 0.3 g peak force, were of the order of 52 μm (S.D. +/- 12 μm), with mean L_o, 24 mm (S.D. +/- 3); this is around 0.2 % of the mean muscle length. Therefore these movements would be within the range of the SREC. The larger force (0.6 g) produced movements outside this 0.2 % range, displacements of 0.27 mm (S.D. +/- 15 μm), mean muscle length 24 mm (S. D. +/- 2.5). Consequently, the thixotropic change was much reduced in these larger force trials, but the time scale and the shape of the stiffness recovery was the same as for the smaller forces.

DISCUSSION

Isolated frog sartorius muscle displays a definite thixotropic effect most clearly observable when a small force is applied. After a series of larger movements the initial stiffness falls, and is then regained over the trial period. In this investigation a detailed examination of the rate at which the muscle regained its stiffness was carried out. Stiffness recovery by the muscle is not a linear process, but follows a seemingly exponential path. In the first 10 s after stirring 50 % of the stiffness had been regained, and around 90 % had returned by the end of the first minute. The remaining 10 % is recovered in the following 2 minutes of the trial period before the next S_1, as these stretches produced displacements that were not significantly different.

The stiffness of the muscle does not stop increasing after the end of the 3 mins trial. Consequently, for the S_1 stretches to produce repeatable sized displacements the same trial cycle length is required. It occasionally happened that the cycle was lengthened to half an hour or more, this caused a dramatic increase in the initial stiffness of the muscle. The effect on stiffness of increasing the trial length from 3 to 10 mins is small, because after 3 mins, stiffness recovers only very slowly. Lakie and Robson (1988c) have shown that human muscle continues to stiffen even 30 mins after the last movement. Thus the memory time of the muscle for previous movements may fade more slowly than was at first thought, and the history of movement of the muscle may have a significant effect on both the starting stiffness value, and also on the subsequent time for a full recovery.

Muscle stiffness can rise only to a finite level, determined by the maximum number of cross-bridges that can form between the actin and myosin filaments in the relaxed state. This will be only a small proportion of the total number of the possible cross-bridges that can be formed in an active muscle, but is a significant proportion of the population, for the forces employed in this study.

The stiffness of active muscle has been determined by a number of workers; their results are shown in TABLE 2a. These values can be compared with those obtained from resting muscle presented in TABLE 2b.

The proportion of the resting to active stiffness ranges from between 1 - 3 %. Haugen (1982), stated that tetanic stiffness was 99 % greater than the resting level. It can therefore be expected that at least 1 % of the cross-bridges attached in the tetanic muscle are also attached in the resting state of the muscle.

If stirring breaks the cross-bridges that are formed by the muscle when at rest, then the recovery must reflect the re-formation of these bonds. As the most significant recovery is found within the first few seconds after the movement has stopped, there must be more bonds formed at this time, than at any other time during the trial period. This seems plausible because there will be a higher proportion of actin binding sites unoccupied immediately after stirring. Certainly formation must outway any bond decay in these early stages following stirring. Morgan (1977) concluded that the variation in the SRS could be predicted by the cross-bridge theory. This is based on the assumption that the muscle's stiffness is generated by an array of parallel independent

identical bridges, which have a finite compliance and variations in the stiffness are the result of variations in the number of attached cross-bridges.

Muscle is probably undergoing continuous formation and decay of the cross-bridges, each occurring at a characteristic rate. If external forces are applied to the muscle, a larger proportion of cross-bridges will be broken than are formed and the total number of cross-bridges attached will fall to a new lower equilibrium state. If the movement is stopped the original situation is restored by cross-bridges being formed in preference to being broken, until the available sites on the actin filament are filled. If the muscle is given another large movement its stiffness will be further reduced or if the muscle is allowed to rest for a longer time than it had received previously, its starting stiffness would be greater.

Changing the temperature of the bath had no effect on the rate of recovery. The muscle regains its stiffness at the same rate at 3 or 17^{o}C. It can therefore be postulated that bond formation and break down rates are affected to the same extent by an increase in the temperature and a "balance between two opposing processes" (Hill 1968) has been effected. Temperature has been found to have little or no effect on the compliance of the SREC, and so it is not surprising that these stretches falling within the range of the SREC are relatively unaffected by a change in the temperature (Wiegner 1987 and Lakie and Robson 1988a). However, the recovery with the 0.6 g peak force stretches was also unaffected by changes in temperature, and this force produced displacements outside the range of the SREC.

Bressler (1981) found that the time to peak twitch tension and the half relaxation time are decreased markedly with an increase in temperature in both frog and toad sartorii. At 10^{o}C the half relaxation time was 25 % and the time to peak twitch tension was 31 % of their respective values at 0^{o}C. Bressler (1981) also found that there were changes in the twitch tension produced, in that for up to 0 - 4^{o}C the muscle remains within 10 % of the value at 0^{o}C, for 4 - 12^{o}C there is a small potentiation and from 12^{o}C there is a steady decline in the isometric tension. These temperature dependences might lead to the expectation that the muscle's stiffness would also be altered in some way, but there was no difference in the muscle's instantaneous stiffness. This was also seen in the results presented here. Changing the temperature made no difference to the stiffness values that were obtained.

Ford, Huxley and Simmons (1977) also demonstrated a lack of correspondence between the isometric tension and stiffness in single frog fibres at selected temperatures of -0.1, 3.8 and 8.1^{o}C. In both cases the stiffness values measured may have been influenced by the increase in the early recovery of tension following an applied change in length.

Kuhn, Guth, Drexler, Berberich and Rllegg (1979) have reported an increase in the isometric tetanic tension in single glycerinated insect flight muscle fibres at temperatures between 5 - 35^{o}C, that was not accompanied by an increase in the stiffness of a single cross-bridge, or in the number of attached myosin heads.

The rate of recovery mentioned here is very similar to

that reported by Lannergren (1971), for the recovery of tension after movement using frog single twitch fibres from the iliofibularis muscle.

> "When the fibre had been perturbed mechanically it was found that the tension response did not return to its original amplitude until the fibre had been allowed to rest for about 3 mins"

The results emphasize the danger of assigning a particular value of stiffness to relaxed muscle, since it is so dependent on what has gone before. The longer the time period between stretches the greater the stiffness, and vice versa.

TABLE 2a: The active stiffness of various muscle prepartions reported by other workers.

Muscle	Stiffness (Kg/cm^2)	Workers
Frog, sartorius	85.3	Halpern and Moss (1976)
Toad, sartorius	140.0	Bressler and Clinch (1974)
Frog, semitendinosus	135.0	Civan and Podolsky (1966)
Frog, semitendinosus	166.0	Huxley and Simmons (1971)

TABLE 2b: The resting stiffness values for a variety of muscles reported by other workers, as well as recorded in this thesis.

Muscle	Stiffness (Kg/cm^2)	Workers
Frog, sartorius	2.8	Halpern and Moss (1976)
Frog, sartorius	2.0	Hill (1968)
Frog, iliofibularis (fibres)	2.28	Lannergren (1971)
Frog, iliofibularis (fibres)	1.9	Lakie and Robson (1990)
Frog, Iliofibularis	2.4	Lakie and Robson (1990)
Frog, sartorius	1.2/ 2.6	Chapters 1 - 8
Frog, semitendinosus	1.96	Halpern and Moss (1976)

CHAPTER THREE

THIXOTROPY: THE EFFECT OF PRIOR ISOMETRIC TETANIC STIMULATION

INTRODUCTION

A muscle's stiffness (resistance to stretch) is greatly influenced by its previous history of movement; any movement prior to measurement results in a reduced level of stiffness. The stiffness returns if the muscle is allowed to remain at rest. The basis behind this thixotropic property of muscles is thought to lie in the interaction of the two filaments of the contractile material, specifically in the destruction and re-formation of bonds between the actin and myosin filaments.

STIMULATION

Muscle contraction is caused by the adenosine triphoshate (ATP) fueled cross-bridges, operating between the two interdigitating filaments. On neural stimulation, Ca^{2+} ions are released from the sarcoplasmic reticulum (SR) and interact with the contractile apparatus. Cohen (1975), in a review of the literature, described a Ca-activated protein "switch" residing on the actin filament allowing the myosin head to form a connection with the actin filament. On cessation of stimulation the Ca^{2+} ions are taken back up into the SR and the muscle returns to the resting state.

In the living resting muscle the cross-bridges are arranged in a regular pattern in the vicinity of the thick filament. When the muscle is activated, they move promptly towards the thin filaments to participate in tension generation. After activation, the myosin heads return to their resting position.

LATENCY RELAXATION

The first mechanical response detected after the onset of stimulation of a muscle held at constant length is a minute

drop in the tension. This immediately precedes the development of the contractile force and is known as the "latency relaxation". Hill (1968), suggested that the latency relaxation was due to the reduction of the filamentary resting tension (FRT), and therefore a result of a reduction in the number of attached cross-bridges that are responsible for both the SREC and FRT.

Other views as to the origin of this latency relaxation have been proposed. Mulieri (1972) claimed that the latency relaxation arose from a change in the compliance of the SR. It has been suggested that it is the result of an elongation by the actin filament, as a consequence of a conformational change, when the troponin molecules bind with the Ca^{2+} ions (Haugen and Sten-Knudsen 1976, 1981b, Bartels, Skydsgaard, and Sten-Knudsen 1979).

RELAXATION AFTER PRIOR STIMULATION

Jewell and Wilkie (1958), reported that the series elastic component of a muscle seemed to become slightly more compliant during relaxation after a stimulus. Podolsky, St Onge, Yu, and Lymn (1976), who followed the events after stimulation, found, from observations of the intensities of the equatorial x-ray reflections, that the myosin cross-bridge return is almost complete 100 ms after the fall of contractile tension. The time course plotted by Yagi, Ito, Nakajima, Izumi and Matsubara (1977) seemed to indicate that the return after a burst of tetanus occurred in two stages. The initial rapid recovery was the result of the return of the majority of the cross-bridges. By the end of 1 s when the tension had fallen to a resting level there was a second phase, made up of the slower return of the remaining 20 % of

the cross-bridges still attached, or certainly in the vicinity of, the thin filaments. Even 5 s after the end of stimulation about 10 % of the cross-bridges still have to return to the myosin backbone. This time scale for the return of the cross-bridges after stimulation was confirmed by the work of H. E. Huxley (1972), who concluded from axial X-ray reflections, that the return of the myosin heads to their resting positions takes at least several seconds.

There was found to be a discrepancy between the fall of tension and the return of the myosin heads. This discrepancy is due to the slow return of the myosin heads, but no mechanical experiments had been conducted at this time to try and analyse whether these cross-bridges are attached to, or merely in the vicinity of, the thin filaments.

Haugen (1982), looking at the SREC after tetanic stimulation, followed the relaxation period, dividing it into two phases. These were: a slow almost linear decline in tension, lasting up to 300 - 400 ms after stimulation, followed by an almost exponential decline, which he followed for 4 s. The transitional period between these two phases gave a "shoulder" in the tension recording. The slow return of stiffness was not paralleled by the response in the tension, with tension relaxation almost complete within the first second. The stiffness recovery was followed in detail up to the end of 4 s by which time the stiffness values had still not regained their resting values and at least a further 2 mins was required for it to be re-established. Haugen reported that the two phases of the relaxation period following a tetanus differed qualitatively in two ways.

Firstly, during the linear phase the relative drop in

tension is greater than the relative decline in stiffness, while in the exponential decline the two variables are proportional.

Secondly, a stretch at a constant velocity applied during the plateau of tetanic tension, or during the phase of linear decline, produced a rising phase of the short range elastic component (SREC), with a sigmoid shape. If the stretch was applied later, during the exponential decline, then the response had the same appearance as in a resting muscle. This sigmoid shape was concluded to be the result of the cross-bridges having non-linear elastic properties. The later response suggests that the cross-bridges also possess a linear elasticity (Ford *et al* 1977). The change from non-linear to linear elasticity may arise from a variation in the state of attachment of the cross-bridges. The sigmoid response might be associated with attachments to the actin sites controlled by troponin molecules with Ca^{2+} bound to them, and so able to perform force generation. The linear response on the other hand might result from cross-bridges attached to actin sites controlled by troponin molecules having the Ca^{2+}-specific site vacant and only capable of carrying force elastically. It should be pointed out that Haugen (1982) did not see a reduction in stiffness compared to the resting level in the relaxation period.

CROSS-BRIDGES AND STIMULATION

Following stimulation it seems that the complete return of the cross-bridges is delayed for at least 5 s. This is likely to have profound implications for the mechanical properties of a muscle after a period of stimulation. If

these cross-bridges were attached they might well be expected to contribute to the muscle's resting stiffness.

There has been much interest in modelling cross-bridge activity. From these models it has been suggested that cross-bridges may exist in several states while they are attached to the actin filament (Tregear and Marston 1979 and Eisenberg and Greene 1980). It has been proposed that these states could be associated with differing levels of stiffness. Immediately after tetanus the cross-bridges are in a configuration of high stiffness which declines in two phases. The decline may be a result of a change in orientation or, by a return of cross-bridges to the myosin backbone over time.

It has long been believed that the presence of Ca^{2+} ions are essential for cross-bridges to form. Mendelson and Cheung (1976), reported that for the cross-bridges to move towards the actin filament, Ca^{2+} is not needed, and as the distal tip of the myosin head is not more than 40 Å from the actin, small amounts of radial Brownian motion may be all that is needed. Even if there was no complete bond formation, the myosin head may well interact with the actin filament, making the muscle's stiffness a dynamic property.

The effect of prior isometric stimulation on the passive mechanical properties of the muscle was investigated over a three month period in this series of experiments.

STRETCHES

The test stretches were rectangular forces. These produced better defined timing of the stretches, so that accurate measurement of stiffness recovery phase could be made.

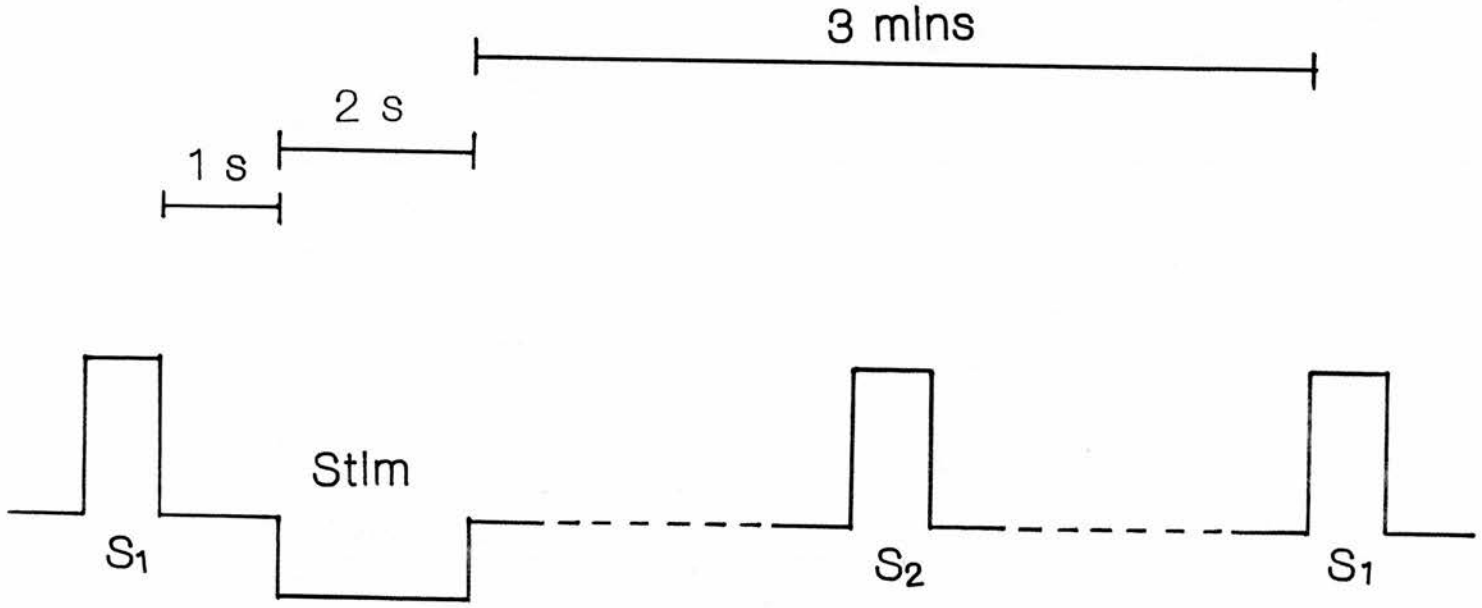
The stretches consisted of an increment in the force that rose abruptly from that of the steady biasing force (used to balance the weight of the muscle and the lever arm). The maximum force level was maintained for a duration of 500 ms, after which the force abruptly returned to the level of the steady biasing force used to balance the muscle. The increment in force was small, arranged to give a good thixotropic effect. This was found to be 0.25 g for an average sized muscle. The force needed to produce displacements that were within the SREC was smaller with rectangular stretches than with the triangular stretches as the peak force is acting for a greater time on the muscle.

STIMULATION

The muscle was stimulated by means of bipolar electrical impulses, administered to the muscle longitudinally; they consisted of a train of pulses at 30 Hz, 8 V and 1 ms in duration. During this maximal tetanic stimulation the muscle did not shorten appreciably as it contracted against the rigid backstop, which was also used to set the muscle to its resting length.

However the arrangement was not entirely isometric. A significant tetanic tension was generated by the muscle during the period of the stimulation, which caused a slight

FIGURE 3a: A schematic representation (not to scale) of the protocol used in Chapter 3, showing the relationship of the stimulation to the test stretches.


3 mins
2 s
1 s
Stim
S1
S2
S1

bending of the lever arm. Even under isometric conditions there will be some shortening of the sarcomeres, sufficient to cause a small degree of contraction. The bending of the lever amounted to only about 5 μm (which was measurable by the transducer). This movement by the muscle represents a shortening of the sarcomeres of roughly 5 nm. The time for the muscle to regain its initial length was called for the purposes of this study the "relaxation time".

PROTOCOL

-EFFECT OF STIMULATION-

FIGURE 3a is a schematic representation of the protocol used in this study, indicating the relationship between the stimulation and the stretches.

The S_1 stretch gave an indication of the resting muscle's stiffness before the start of each trial. It was followed 1 s later by the burst of tetanic stimulation lasting 2 s. The S_2 stretch occurred at one of the following times 0.5, 1, 2, 5, 10 and 30 s after the end of the stimulation with only one S_2 per trial. The time at which the S_2 stretch appeared was randomized from trial to trial. S_1 recurred at precisely 3 minute intervals after the end of the stimulation.

Experiments were conducted over a three month period, from December 1987, to February 1988. The temperature of the Ringers solution was maintained at $3^{o}C$ throughout the experiment.

A second set of trials was run at the same time as the stimulation trials where the stimulation was replaced by 2 s of rectangular stirring stretches (3 Hz and a maximum movement of 2 mm). The test stretches and conditions were the same as in the stimulation trials.

RESULTS

THE EFFECT OF PRIOR TETANIC STIMULATION

The effects of prior stimulation on the muscle's subsequent stiffness were followed over a three month period, and not until analysed did it become clear that the frog muscle had undergone a dramatic transformation in stiffness values.

DECEMBER

In December (FIGURE 3b lower) there is a clear decrease in the muscle's stiffness values immediately following stimulation. From S_1/S_2 (500 ms after stimulation) the E values were reduced by a factor of 2.87. The muscle's stiffness values return towards those of the S_1 values as the time from stimulation lengthens. The recovery of stiffness after stimulation is very similar in time scale to that seen after the muscle has had a period of stirring. In FIGURE 3c the effect of mechanical stirring for 2 s are shown; the results may be compared with the effects of stimulation.

JANUARY

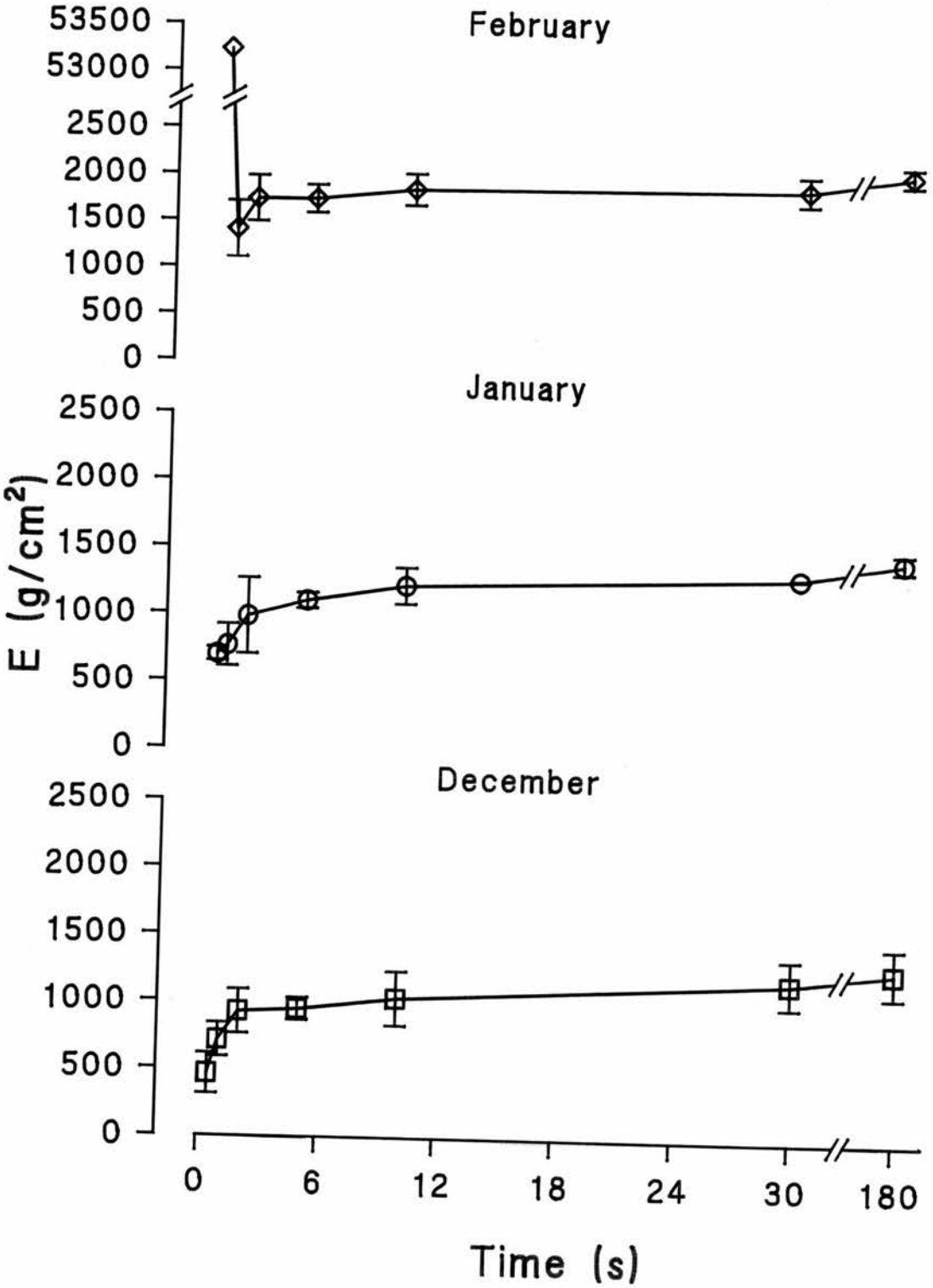
In January (FIGURE 3b middle), there is a similar pattern to that seen in December. The overall stiffness values are higher than they are for December, with the curve being displaced upwards. The thixotropic loosening seen soon after stimulation was reduced, the S_2 stretch 500 ms after stimulation was only reduced by a factor of 2 from the S_1 stretches, which is almost a third less than that recorded in December. For the remainder of the recovery period stiffness values are remarkably similar for the two months.

FIGURE 3b: The effect of 2 s prior tetanic stimulation on the mean (SD) stiffness values for a three month period. Force 0.25 g, 3°C.

LOWER; December n=10

MIDDLE; January n=6

TOP; February n=14 (reading at 500 ms is the mean of 4 muscles only)


February
January
December
53500
53000
2500
2000
1500
1000
500
0
E (g/cm²)
0
6
12
18
24
30
180
Time (s)

FEBRUARY

FIGURE 3b top, shows the dramatic alteration in the way the muscle responds to stimulation, the stretches 500 ms after stimulation are now very much stiffer; whereas for the previous two months all the stretches produced a decease in the stiffness following stimulation. In February of the fourteen muscle's studied, only four gave a measurable response to this 500 ms S_2 stretch, the other ten gave negligible displacements, and hence the extraordinary high stiffness values for this particular stretch is the mean of only the four muscles where a reading was taken. The remainder of the other S_2 stretches had stiffness values which were smaller than the S_1's, and followed a similar pattern of recovery already observed for the previous two months. The muscles are generally stiffer than in December and January.

The muscles have a tendency to become stiffer over the three months, but the amount of stiffness recovered within 30 s is almost the same for all three months, between 90 - 93 %. The increase in the S_1 values from December to January is 14 %, and from December to February, 63 %. Therefore the most dramatic changes in the muscle's stiffness are taking place at the end of January, beginning of February.

As such a dramatic change in the stiffness values was observed over the three months the study was extended and the stiffness throughout the year was monitored, these results are presented in Chapter 7.

RELAXATION

The relaxation times are given in TABLE 3a. In February

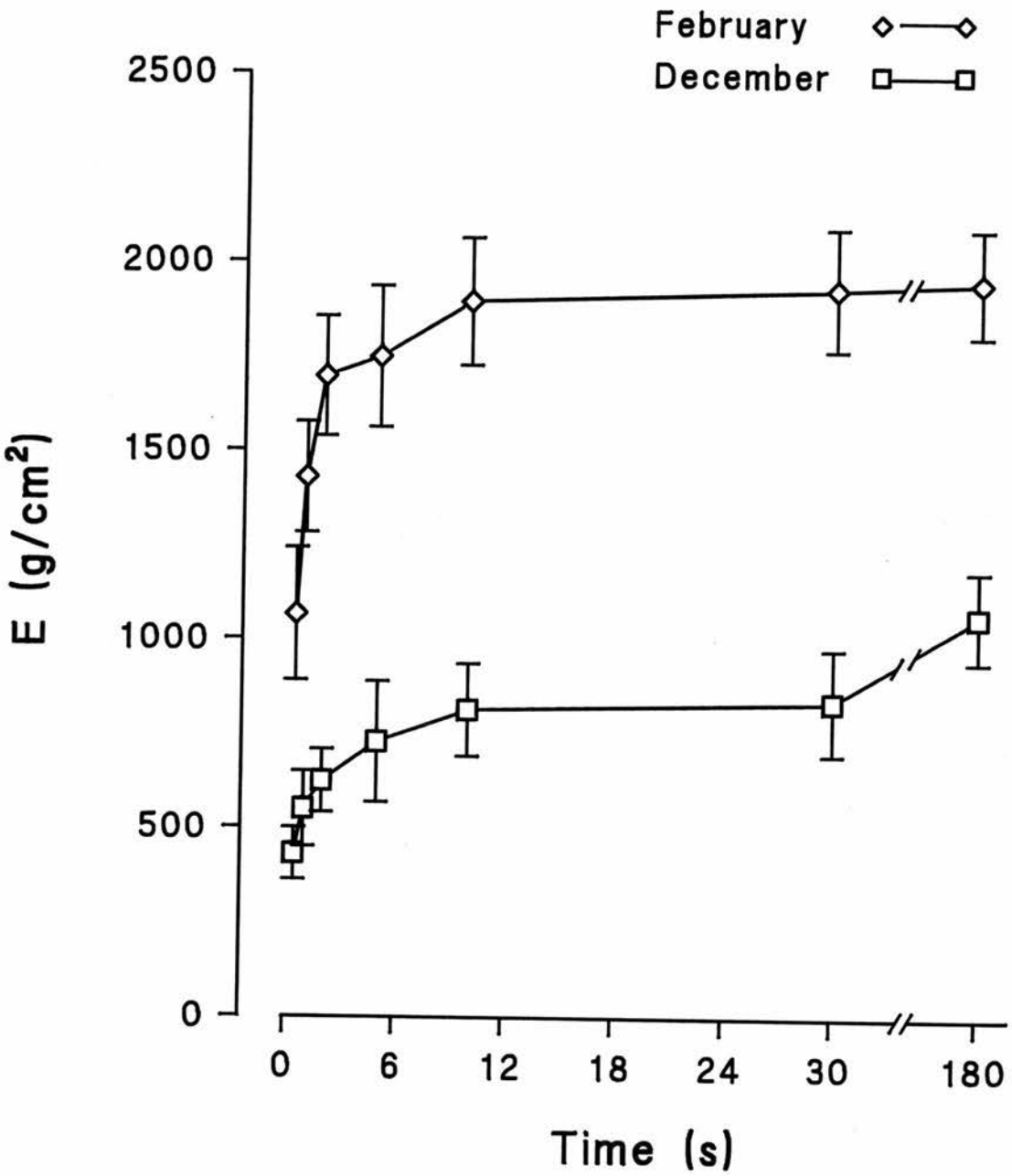
TABLE 3a: The crude relaxation times for December to February plus SD, measurements taken by recording the time from when the stimulation stopped to the muscle regaining its original length.

MONTH	n=	RELAXATION TIME (s)	+/- SD
December	10	0.764	0.017
January	6	0.842	0.02
February	14	1.46	0.17

FIGURE 3c: The effect of 2 s prior stirring on the mean (SD) stiffness values for December and February. Force 0.25 g, 3°C, as measured from the end of the stirring.

December n=10

February n=14


February
December
2500
2000
1500
1000
500
0
E (g/cm²)
0
6
12
18
24
30
180
Time (s)

the muscles spend twice as long in "relaxing" as they do in December, with the muscles in February showing a prolonged recovery following stimulation.

The results indicate that there is little difference between December and January. However February's results are very highly significantly different from both those of December and January (ANOVA, both $p < 0.01$).

STIRRING v STIMULATION

If the shape of the recovery curves obtained from the stimulation experiments is compared to the recovery reported following stirring, then it can be seen they bear a remarkable similarity to each other. FIGURE 3c shows the stiffness recovery curve from December after a period of stirring. An initial fast recovery lasting for up to 4 - 5 s is followed by a slower recovery for the remainder of the time. The values of stiffness are also remarkably similar for the two curves, as are the percentage changes seen. For February's recovery following stirring, although the stiffness values are larger, the recovery mirrors the changes seen in December rather than the results from this months stimulation stiffness recovery. The trials with stirring do not show the same increase in the stiffness for the 500 ms S_2 stretches, and so this would seem to be a feature of the stimulation.

DISCUSSION

Passive stretches have been shown to cause a temporary reduction in the resting level of stiffness of frog muscle. In this series of experiments maximal isometric tetanic stimulation also caused a considerable subsequent decrease in the level of stiffness, except in February where there was an initial increase.

The extent of the stiffness decline is similar to that seen with the stirring, as is the time scale for the stiffness recovery. In the light of the evidence presented in the introduction it might have been expected that stimulation would cause an increase in the level of stiffness, rather than the decease reported here.

The effects of the prior tetanic stimulation on the resistance to the passive stretches are notably different for the three months studied. The results obtained for December and January are similar to results obtained using stirring, the only significant difference being that the muscles in January are generally slightly stiffer than in December. In February however there is a complete reversal of the results seen for the previous two months. The muscles have a greater level of resting stiffness and a very high stiffness value for the 500 ms stretch post stimulation rather than the lowered stiffness values seen in December and January, and for the remainder of the post stimulation results. This change would seem to be a seasonal variation in some property of the muscle. There are a number of possible explanations. Chapter 7 will cover these in more detail.

FACTORS GIVING INCREASED STIFFNESS AFTER STIMULATION

Sugi (1972) stretched active fibres and found that the

cross-bridges become distorted from their initial positions of attachment on the actin filament causing an increase in the force exerted, producing an initial abrupt rise in the tension. Finally they break if the force reaches a critical limit. During isometric stimulation the cross-bridges can become distorted as they form attachments along the actin filament but are unable to move to their full extent, and so become strained. After stimulation stops these cross-bridges would still be in a distorted arrangement and might well cause a higher level of stiffness than the cross-bridges in a normal attachment state (Sugi 1972).

If the muscle remains active for longer in the spring months then there would be a greater proportion of cross-bridges attached at any one time, and as the muscle's stiffness is proportional to the number of cross-bridges that are attached then the muscle would appear to be stiffer. The drop in stiffness seen after 1 s in February may represent the time limit of the cross-bridges persistence.

Haugen (1982) found that for a stretch, 1 s after the end of stimulation, the stiffness of the fibre was reduced by about 99 % from when tetanically stimulated. During the interval 1 - 1.5 s to 4 s following stimulation the SREC was 21 % stiffer than when the fibre was at rest. He did not follow the exact dependence of the short range elastic stiffness beyond 4 s but established that the resting level of stiffness had returned after a period of 2 min. This slow return of the stiffness to the resting level he explained, by assuming some of the myosin heads remained attached to the actin filaments, slowly returning after a tetanus.

Haugen (1982) and Sugi (1972) do not mention the time of

year that their experiments were conducted at, and as the present results show this may have a bearing on the stiffness recorded by them and their subsequent explanations.

FACTORS GIVING REDUCED STIFFNESS AFTER STIMULATION

-CROSS-BRIDGE FORMATION AND THE ACTIVE STATE-

Stimulation does cause cross-bridges to form, but because the muscle is held under isometric conditions they are unable to form a normal attachment. These may spontaneously break down once the stimulation has ceased, or stretches occurring soon after stimulation could cause the destruction of these strained bonds, both possibilities lowering the muscle's stiffness. A stiffer muscle may be achieved, as mentioned earlier, if the muscle remains active for longer after the stimulation has stopped, due to some mechanical change.

-RETURN OF CROSS-BRIDGES AFTER STIMULATION-

Yagi *et al* (1977), reported a rapid return of about 80 % of the myosin heads in the first second after stimulation in frog sartorius muscle at 4^{o}C. This was followed by a much slower return of the remaining 20 %, with even 5 s after approximately 10 % of the myosin heads still apparently attached to the thin filaments. It should be mentioned that there is no direct evidence that these myosin heads are attached to the thin filaments at all, and they may just lie in the vicinity of the thin filaments. This rapid return of the myosin heads is mirrored by the drop in stiffness immediately after stimulation and the remaining 5 % of the heads forming the basis for the return of stiffness back to the resting level (resting stiffness has been calculated to be between 1 - 3 % of the myosin heads Chapter 2).

-CROSS-BRIDGE ATTACHMENT STATES-

Eisenberg and Greene (1980) and Tregear and Marston (1979) both suggested that cross-bridges may be able to exist in several states while attached to the actin filament. Stretching of any attached cross-bridge may either promote the transition to a form of attachment with a greater potential stiffness, or, to cause its detachment which lessens the muscle's stiffness. Increased stiffness could be brought about by some mechanism that re-orientates the heads into a stronger alignment with the active sites on the actin filament. It is possible that for a few seconds after stimulation has finished the cross-bridges are in a weaker attached state (lowered stiffness). As time passes the myosin heads can assume a new alignment causing the muscle to become stiffer. A number of cross-bridge configurations have now been identified in invertebrate muscle (Wray, Vibert and Cohen 1975).

Cross-bridges remaining in the vicinity of the thin filaments may well not be attached in the same manner as they are in the active muscle, but could well be attached by weaker molecular forces such as hydrogen bonding between the actin and the myosin heads. Passive stretches that moved these heads to a position above, or closer to an actin binding site might cause the attachment to become stronger (Keane, Trayer, Levine, Zeugner and Ruegg 1990). Haselgrove (1972), identified by X-ray diffraction the presence of some firmly attached cross-bridges in the relaxed muscle, although the tropomyosin had not moved before attachment. It would seem therefore that the tropomyosin does not cover the active site completely, but inhibits the myosin/actin interaction by

preventing attachment of myosin loaded with ATP, but allows the attachment of unloaded myosin (Lymn and Taylor 1971 Keane *et al* 1990).

-ONE OR TWO ACTIN FILAMENT BONDING-

The myosin cross-bridge is composed of two globular subunits making up the heads, attached at one end of a fibrous tail. During contraction the heads bind to the actin filaments and produce tension by tilting. The way in which this is shared by the two heads is not known (Huxley 1969). It has been supposed that the two heads of one myosin molecule interact with neighbouring subunits on the same actin filament. Offer and Elliott (1978) claimed however that in the highly ordered filament lattice found in striated muscles, tighter binding could occur if the two heads were to bind to different actin filaments. Muscle in rigor can be regarded as having reached the termination of the drive-stroke of the cross-bridge cycle, and shows a mixture of both single filament and two filament interactions. The two and single filament configurations produce differing tensions. Re-stiffening after stimulation may reflect a change from single to two filament binding.

-CALCIUM-

Johnson, Charlton and Potter (1978), plotted the half time for the removal of Ca^{2+} from the Ca^{2+} specific sites of isolated troponin C molecules. It is only about 3 ms. During the early phase of the muscle's relaxation the actin filament is brought out of its "on state" as the concentration of Ca^{2+} declines, suggesting that stiffness will drop immediately as Ca^{2+} is removed. However cross-bridges can reform without the need for Ca^{2+}, and

certainly Ca^{2+} may not in fact be needed for the first phases of attachment of the cross-bridges, with weak attachments attained by the myosin head pushing away the regulatory mechanisms on the actin (Mendelson and Cheung 1976).

-ACTIN FOLDING-

There is evidence that folding of the actin filament may play some role in the stiffness of the muscle. Ca^{2+} release produced by stimulation may cause an unwinding of the actin helix equivalent to a full rotation of the myosin heads (Gillis and O'Brien 1975 and O'Brien *et al* 1975). This unwinding of the actin filaments would make the muscle appear to be less stiff immediately after stimulation, with the muscle re-stiffening as the actin folds re-form, taking place in conjunction with the removal of the Ca^{2+}.

Re-folding of the actin helix can be considered with the re-formation of the cross-bridges as contributing to the return of the muscle's resting stiffness, as well as any other forms of bonding both inter and intra filamentary.

CONCLUSION

Stimulation prior to a passive stretch has pronounced effects on the muscle's stiffness recorded here. It may have been expected that stimulation would make the muscle subsequently stiffer and more resistant to external forces, but this was observed only at certain times of the year. Higher stiffness developed in the early Spring, the time of the year when frogs emerge from hibernation and begin mating. This period of extended stiffness after the muscle's have been active lasts for approximately a second, with the muscle system then becoming less stiff, before re-stiffening to the control level.

A period where the muscle is less stiff immediately after activation would allow the animal to be able to move freely and without restrictions for several seconds after the last period of activity, before the system reached its resting level of stiffness, making the system economic in expenditure of energy. This lack of restrictions on the muscle moving can be compared to what happens in human muscles after a period of activity. Tremor increases, the thixotropic behaviour and stiffness are reduced, returning as the period at rest increases (Lakie and Robson 1988c).

CHAPTER FOUR

THIXOTROPY: IN SINGLE ILIOFIBULARIS TWITCH FIBRES

INTRODUCTION

In the whole isolated muscles used in the earlier studies, there was connective tissue still associated with the muscle, including the sarcolemma surrounding the muscle fibres and the tendons in series. The role that these components play in the thixotropic effect is uncertain. Single fibres obviate some of these difficulties and were chosen for this series of experiments.

SINGLE FIBRES

Single fibres have been used extensively, yielding valuable data on the contractile mechanism of muscle (Hellam and Podolsky 1969 and Julian 1971). Single fibres can be dissected out with the sarcolemma intact, which leaves the fibre responsive to stimulation. Alternatively chemical or mechanical skinning of the sarcolemma leaves the fibre unresponsive to stimulation. The mechanical properties of single fibres are similar to whole muscle if the sarcolemma is left intact.

The muscles used for single fibre work in the frog are mainly the iliofibularis and semitendinosus, as both have less connective tissue surrounding the fibre, rather than the sartorius muscle with its close connective matrix.

SARCOLEMMA

The sarcolemma is an elastic material composed of a number of layers (Ramsey and Street 1940, Reed, Houston and Todd 1966 and Fields 1970). Street and Ramsey (1965) identified four distinct layers. The inner layer is made up of the plasma membrane, destroyed when the fibre is damaged and the contractile material clots. The next layer is a

matrix forming the basement membrane and beyond is a layer of collagen. The collagen layer presumably imparts the tensile strength to the sarcolemma tube. The outer layer is made up of unidentified fine filaments. These last three layers' structure indicates that they are merged (Reed *et al* 1966), and will separate *in vitro* from the inner plasma layer (Birks, Katz and Miledi 1959).

When an isolated single muscle fibre is gently squeezed, or the sarcolemma is touched, the cell contents appear to clot on either side of the injury, caused by a flood of Ca^{2+} into the fibre producing massive local contractions of the myofibrillar material (Fields 1970 and Fields and Faber 1970). Often denaturation continues, with the clots growing, retracting from the site of injury.

Following division of the coagulated sarcoplasm adjacent to, or between the clots, an anticlastic cylindrical zone of sarcolemma filled with fluid is left behind. Casella (1951) described the sarcolemma tube that forms after damage as having a curved longitudinal contour with the smallest diameter in the central region, with larger diameters nearer the ends (anticlastic surface). The diameter of such "reaction zones" is always less than the diameter of the intact fibre, whereas reaction zone length is always greater than the length of the same membrane in the intact fibre (Fields 1970).

The mechanical properties of the retraction zone membrane are considered to be representative of the sarcolemma of normal muscle fibres, as the outer connective tissue investments are believed to be responsible for the principal rigidity of the membrane and are retained following

retraction zone formation (Street and Ramsey 1965). If the assumptions regarding the retraction zone structure are correct then the morphology of the tubular segment of the membrane can be described in terms of a thin elastic shell of cylindrical geometry subjected to certain external deforming forces.

ATTACHMENTS OF THE SARCOLEMMA TO THE FIBRE MATERIAL

Areas of the sarcolemma can form attachments to the myofibrillar material underneath. Franzini-Amstrong and Porter (1964) reported that the sarcolemma was attached to the delicate transverse network of tubules at the level of the Z membrane. Reed, *et al* (1966), reported a larger area of attachment for the sarcolemma that stretched from the H zone to the Z line. The attachment was dynamic and not always present.

RIGOR

The development of rigor is associated with depletion of ATP (Bate-Smith and Bendall 1947, 1949). Cooke and Franks (1980), looking at rabbit psoas muscle fibres in rigor, in which there was complete overlap found that the values for the K-ATPase activities indicated that 94 +/- 4 % of the myosin heads were bound to actin. In frog muscle the comparable percentage has been found to be 95 %, while in sarcophaga muscles (insect flight muscle) it is 70 % (Lovell, Knight and Harrington 1981). A more detailed report of the development of rigor will be given in Chapter 5.

IAA RIGOR

The metabolic poison iodoacetic acid (IAA) produces rigor by the inhibition of muscle glycolysis by

direct suppression of the triose dehydrogenase system activity, as well as indirectly by irreversible dephosphorylation of ATP. Once the muscle's ATP stores have fallen the normal activity of the cross-bridge cycle is disrupted and the cross-bridges become permanently attached (Sandow and Schneyer, 1955, and Mulvany, 1975). IAA rigor develops most strikingly when the muscle has been active, although the essential features of rigor do appear in unstimulated muscles (Sandow and Schneyer, 1955).

Whole iliofibularis muscles were investigated for their thixotropic properties to compare with the results obtained with the single fibres. These results also complement the sartorius experiments, and are presented as an addendum at the end of this chapter.

The aim of this series of experiments is to try to isolate the underlying mechanism of thixotropy. This involves investigating whether the connective tissue (especially the sarcolemma) contributes to thixotropy, or whether it is purely the result of the interactions of the contractile material.

The contribution of the cross-bridges thixotropy. Can be investigated by the use of fibres in rigor, in which the normal cycling of the cross-bridges is curtailed with at least 70 % of the myosin heads bound to the actin filament permanently.

FIBRE PREPARATION AND MOUNTING

R. temporaria were kept in tanks under virtually natural conditions for a few weeks before being used.

Single fibres were dissected from the iliofibularis muscle, using a dissecting microscope (KYOWA optical x 10), with the aid of jeweller's forceps and finely pointed scissors. A small bundle of fibres was removed from the twitch portion of the whole muscle under isotonic Ringer, and a single fibre isolated, care being taken not to damage the fibre structure. Fibres demonstrating signs of damage, such as a cloudiness on the fibre surface, were excluded from the experiments. To minimize the damage from the mounting procedure small amounts of the surrounding fibres were left attached to the fibre at both ends. These fibres provided a better grip for the clips of the apparatus. The fibre was mounted vertically in a smaller version of the chamber used for the whole muscles; two miniature stainless steel clips held the fibre in place. When the fibre was installed, the sides of the chamber were raised and filled with Ringer solution (5 ml).

APPARATUS B

FIGURE 4a shows a diagrammatic representation of apparatus B.

The top clip was attached to a light 4 cm aluminium lever arm, connected to a motor (ESCAP 16C11-205). The motor formed part of a torque servo system. The mechanical dither was reduced to a minimum, reducing noise in the system. A backstop prevented the fibre from shortening and another stop

FIGURE 4a: A diagrammatic representation of apparatus B

The set up is much the same as for apparatus A (FIGURE 1a).

A Adjustment screw for micrometer, allows changes in the muscle length.

T Transducer measures length changes of the muscle

L Lever arm

M Motor

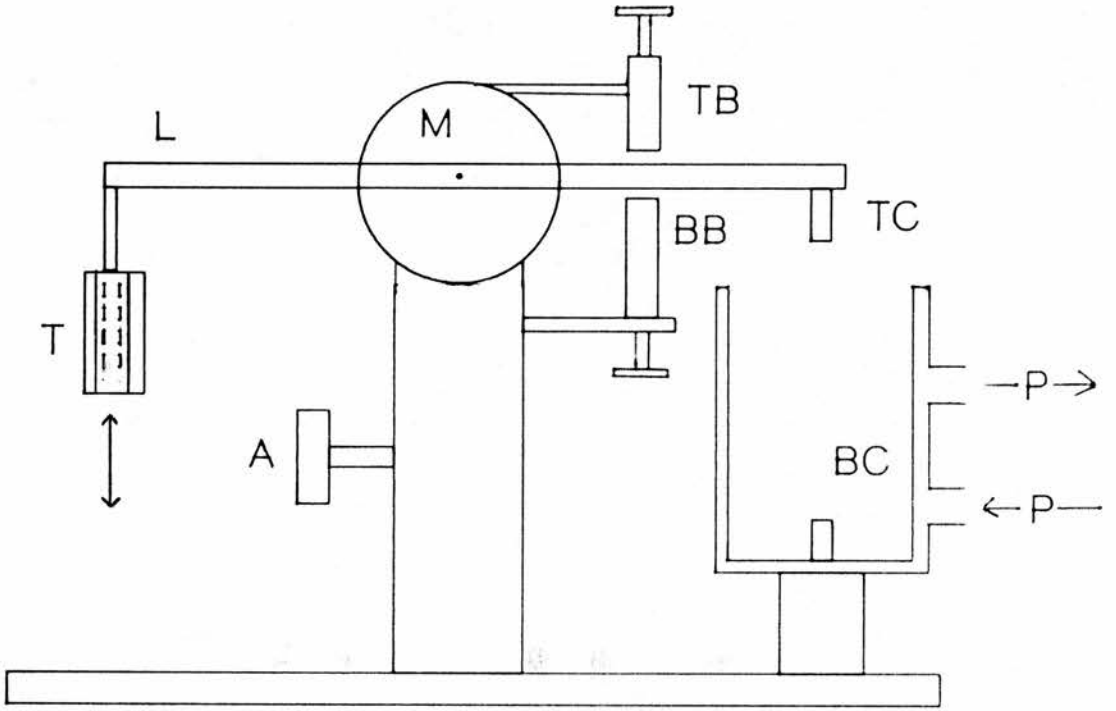
TB Top stop prevents muscle/fibres/bundles from being over stretched

BB Bottom backstop prevents shortening during fixation or during the experiment

TC Top clip for the muscle preparation

BC Bottom clip for the muscle preparation

P Direction of cooling water circulation


L
M
TB
TC
BB
T
A
BC
→P→
←P←

above the lever prevented the fibre from being over stretched during the experiment. A tube in the bottom of the bath allowed the fast removal of one solution and replacement by another. Displacements were recorded using a linearly variable differential transducer (L.V.D.T. type SM1; Sangamo Transducers, resolution 2 μm limited by the inherent noise of the apparatus). All data was fed via a CED 1401 interface (Cambridge Electronic Design) into an IBM AT computer and stored on disc for later analysis.

TEMPERATURE

The temperature was maintained at 3°C for all experiments.

PROTOCOLS

-THIXOTROPY-

The fibre, when positioned in the muscle bath, was allowed 10 to 15 minutes to equilibrate. Very small forces were applied (0.03 g), in the form of rectangular stretches. Each trial consisted of three test stretches; S_1; 3 s of stirring either 1 mm, or 0.5 mm in size; S_2, precisely 200 ms after the stirring stopped. The S_3 stretch was applied at various times after stirring had finished (1, 2, 3, 5, 10 and 30 s). The trial cycle length was set to 3 mins from the end of the stirring, to the following S_1 stretch, on a continuous cycle.

-THE EFFECT OF VARYING THE AMOUNT OF STIRRING-

The protocol was basically the same as used for the earlier thixotropy experiments but with some modifications; S_3 was set to a fixed interval of 3 s after stirring, the S_1

and S_2 stretches were at their usual times; the number of stirring stretches was varied, 1, 2, 3, 6, 12 and 18 stretches.

-THIXOTROPY IN THE SARCOLEMMA-

The fibres were gently squeezed between a pair of forceps to disrupt the fibre structure. A slight touch was sufficient. Clots of contractile material formed within a few seconds of the damage being inflicted, clearly observable without the aid of a microscope, appearing as droplets along the fibre surface. FIGURE 4b shows the timings of the test stretches within the trials, S_1 every 3 mins from the end of stirring, 6 stirring stretches, S_2, 200 ms and S_3 set to a constant 3 s after stirring had stopped. The fibres were tested both before and after damage had been inflicted. After the fibre was damaged, 30 s was allowed for the clots to form before recommencing the experiment. Trial cycles were 3 min, measured between end of stirring and S_1.

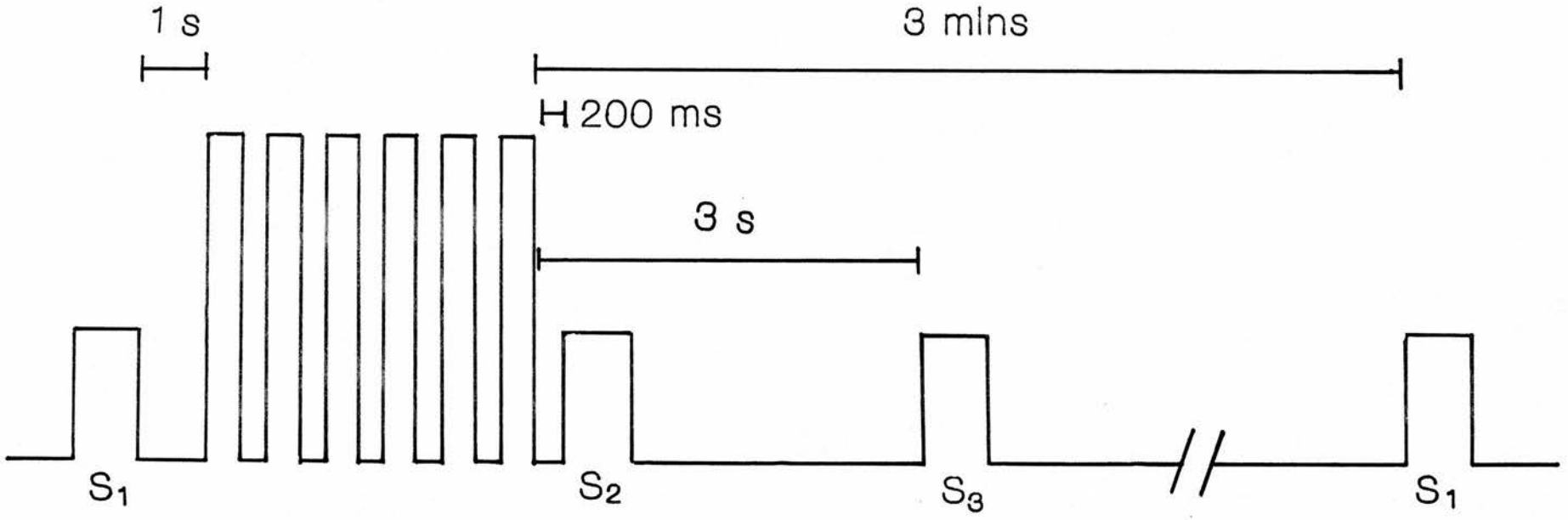
-IAA-

For some fibres the isotonic Ringer was drained and replaced with a 0.2 mM solution of IAA Ringer solution. The effect of the IAA was followed for 24 mins after application. The protocol used was the same as for the sarcolemma experiments (see above for details and FIGURE 4b). The fibres were tested both before and after the application of the IAA solution.

MICROSCOPY

At the end of each experiment the Ringer solution was drained out of the bath and replaced with a solution of formol saline of the following composition:

FIGURE 4b: A schematic diagram of the protocol used (not to scale) for the sarcolemma trials, with a fixed 3 s S_3.


1 s
3 mins
H 200 ms
3 s
S1
S2
S3
S1

Formalin	100 ml
NaCl	9 g
Distiled water	900 ml

This solution was left in the bath for several hours to fix the fibre. The fibre was then removed and mounted under glycerol for observation of the fibre structure. Sarcomere length was measured (some shrinkage of the fibre was caused by fixation). Shrinkage in the vertical plane was kept to a minimum by the muscle undergoing fixation while still clamped in the apparatus, there was some unavoidable lateral shrinkage of the fibres. The largest diameter (a) and the diameter perpendicular to this (b) were measured at different places along the fibre with the aid of an ocular scale. Area (A) was calculated according to the formula:

$$A = \left(\frac{a.b}{2}\right).pi$$

The sarcomeres per unit distance (usually 20 or 30 μm) were counted at two or three places in the middle region of the fibre and the mean value for the sarcomere length calculated. A light microscope (Olympus CHC) with a x80 objective and an ocular micrometer were used for the determination of sarcomere spacing by direct microscopy at x800.

RESULTS

Mean sarcomere length was calculated to be 2.02 μm (S.D. +/- 0.38 μm) and the average fibre width was found to be 85 μm (S.D. +/- 9.2 μm).

SIZE OF STIRRING WAVEFORM

A stirring waveform of 1 mm produced a clear thixotropic effect from the fibres but had the tendency to damage the fibres when extended periods of stirring were used (over 3 s). Fibres damaged by excessive stirring produced inconsistent results, with thixotropy reduced considerably. Under the microscope these fibres resembled those used in the sarcolemma experiments with empty areas of sarcolemma and isolated clots of muscle material. When stirring was reduced to a maximum movement of 0.5 mm, thixotropy was observed consistently throughout the lifetime of the fibre; when the fibre was observed at the end of the experiment the fibre structure remained intact, with the sarcomeres running continuously along the fibre, showing no distortions in the pattern produced by the Z discs.

THIXOTROPIC RECOVERY

The recovery of the fibre's stiffness was recorded over a 3 min trial cycle. FIGURE 4c shows the results of 8 fibres (mean +/- SD). The recovery shows two distinct phases, the first phase lasting for up to 5 s is a rapid recovery of stiffness, followed by a slower recovery extending over the remaining trial period. The percentage decrease in stiffness after stirring was approximately 60 %, 15 % more than with the whole sartorius muscle. This is not a great difference, but the decrease varied greatly between the fibres ranging

FIGURE 4c: The recovery of stiffness for single fibres of the iliofibularis muscle over a 3 min period, means and SD. Temperature 3°C, force 0.03 g, n=8.

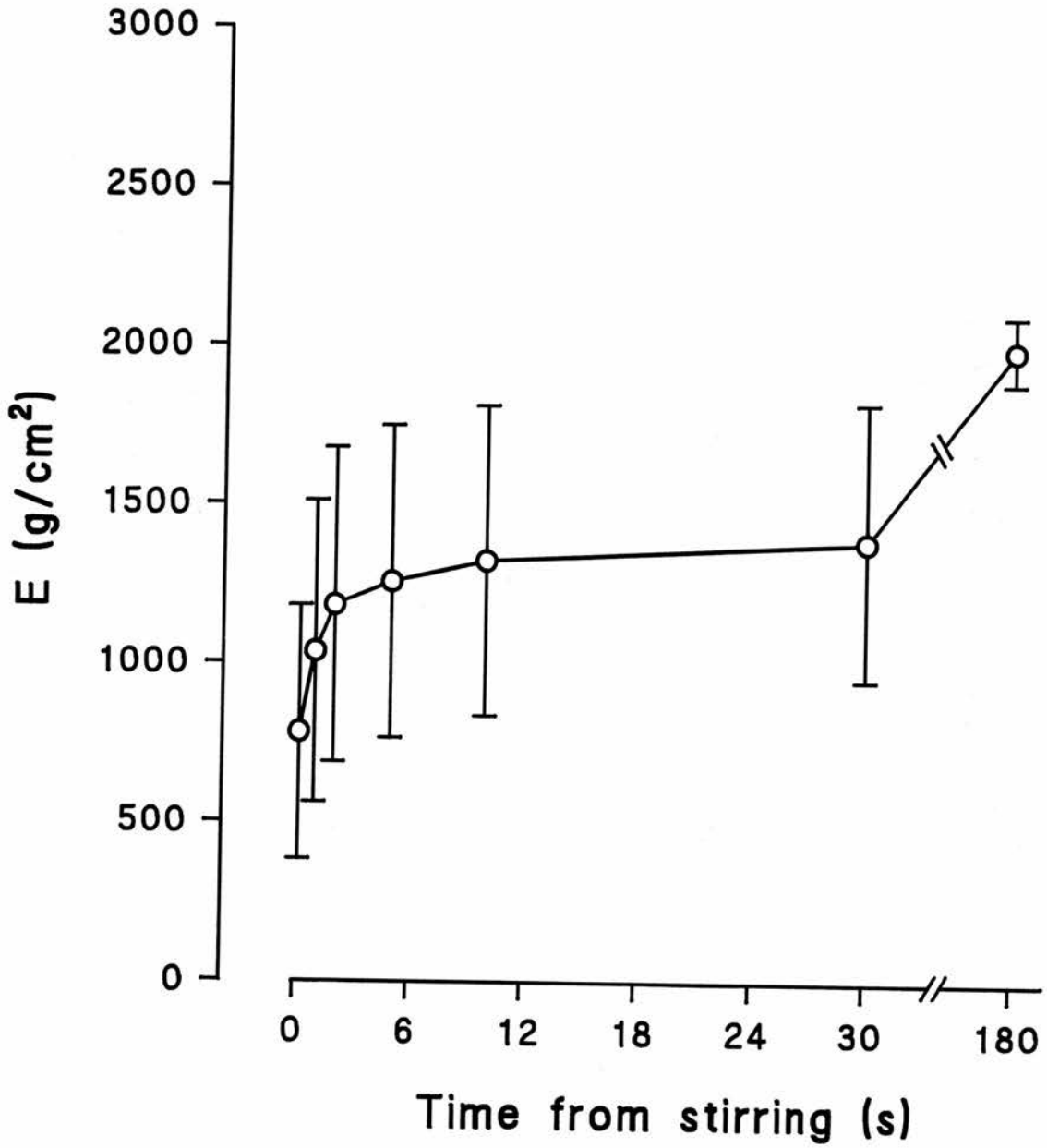

3000
2500
2000
1500
1000
500
0
E (g/cm²)
0
6
12
18
24
30
180
Time from stirring (s)

FIGURE 4d: The raw data for the recovery of stiffness following stirring for one iliofibularis fibre. S_1 every 3 mins; S_2 every 200 ms after stirring and S_3 variable, traces A - E; A, 1 s; B, 2 s; C, 3 s; D, 5 s and E, 10 s after stirring. Temperature 3°C, force 0.03 g and the stirring waveform displacement, 0.5 mm.

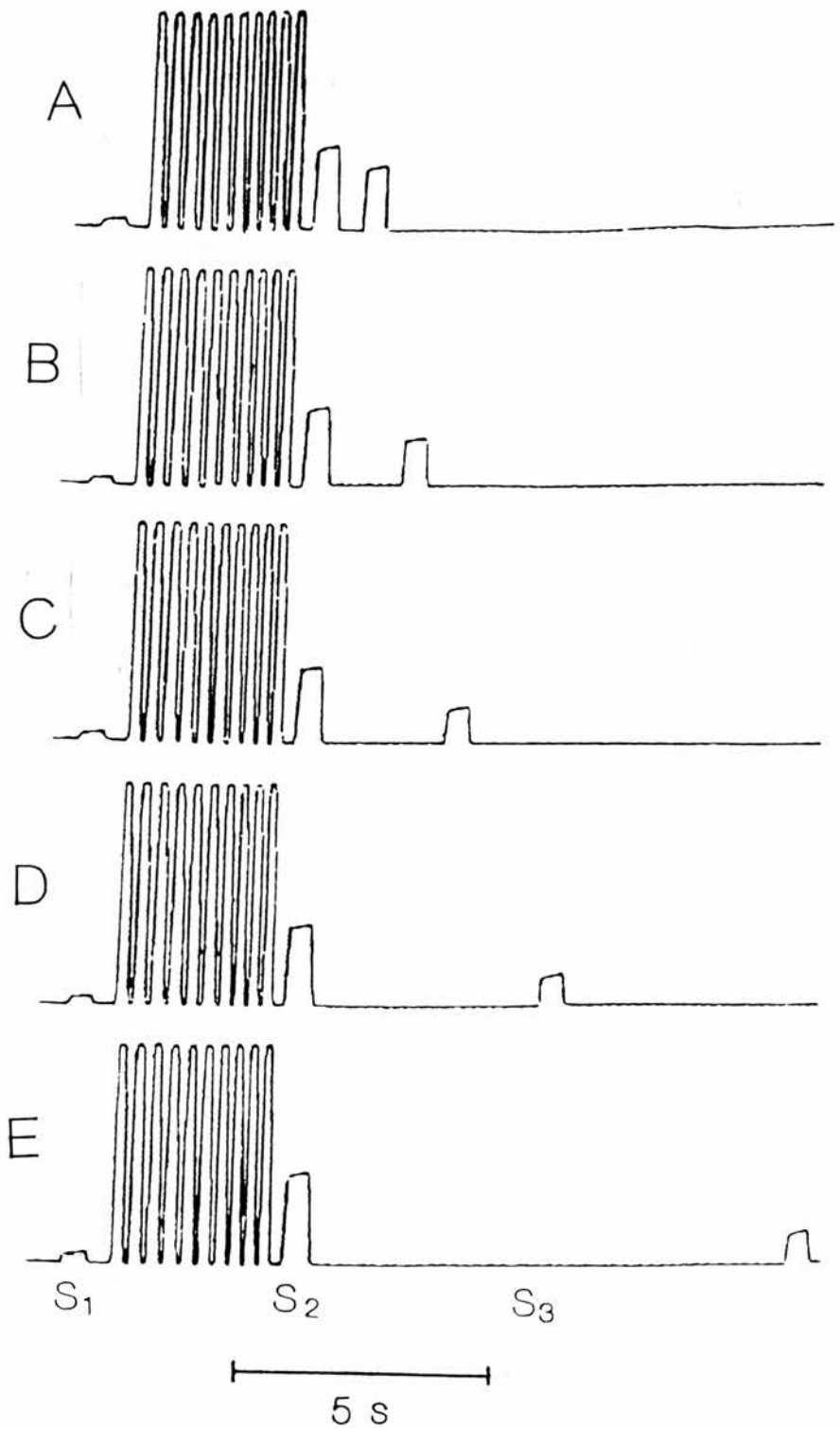

A
B
C
D
E
S1
S2
S3
5 s

FIGURE 4e: The E values for the recovery of stiffness for the same fibre shown in FIGURE 4d.

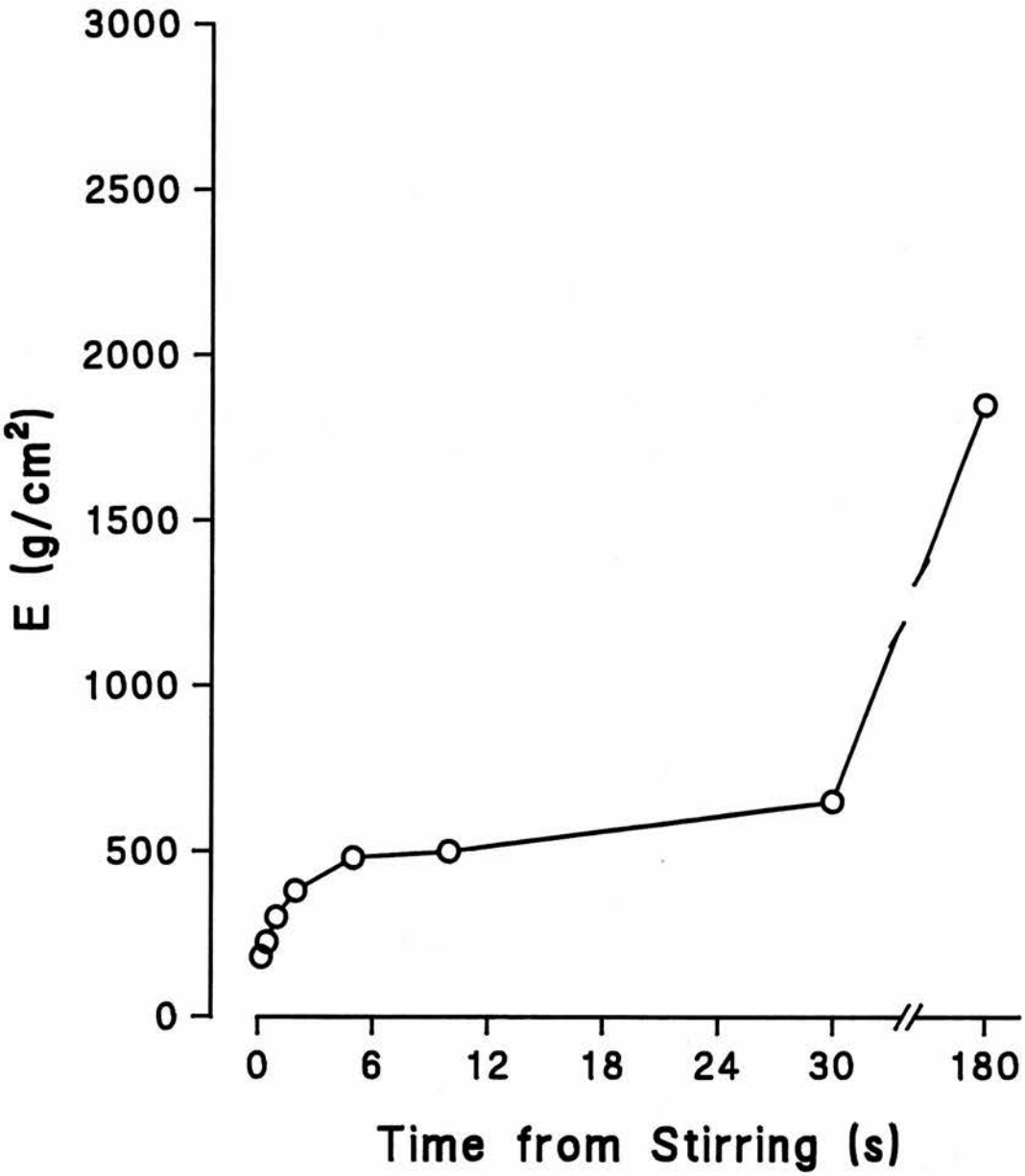
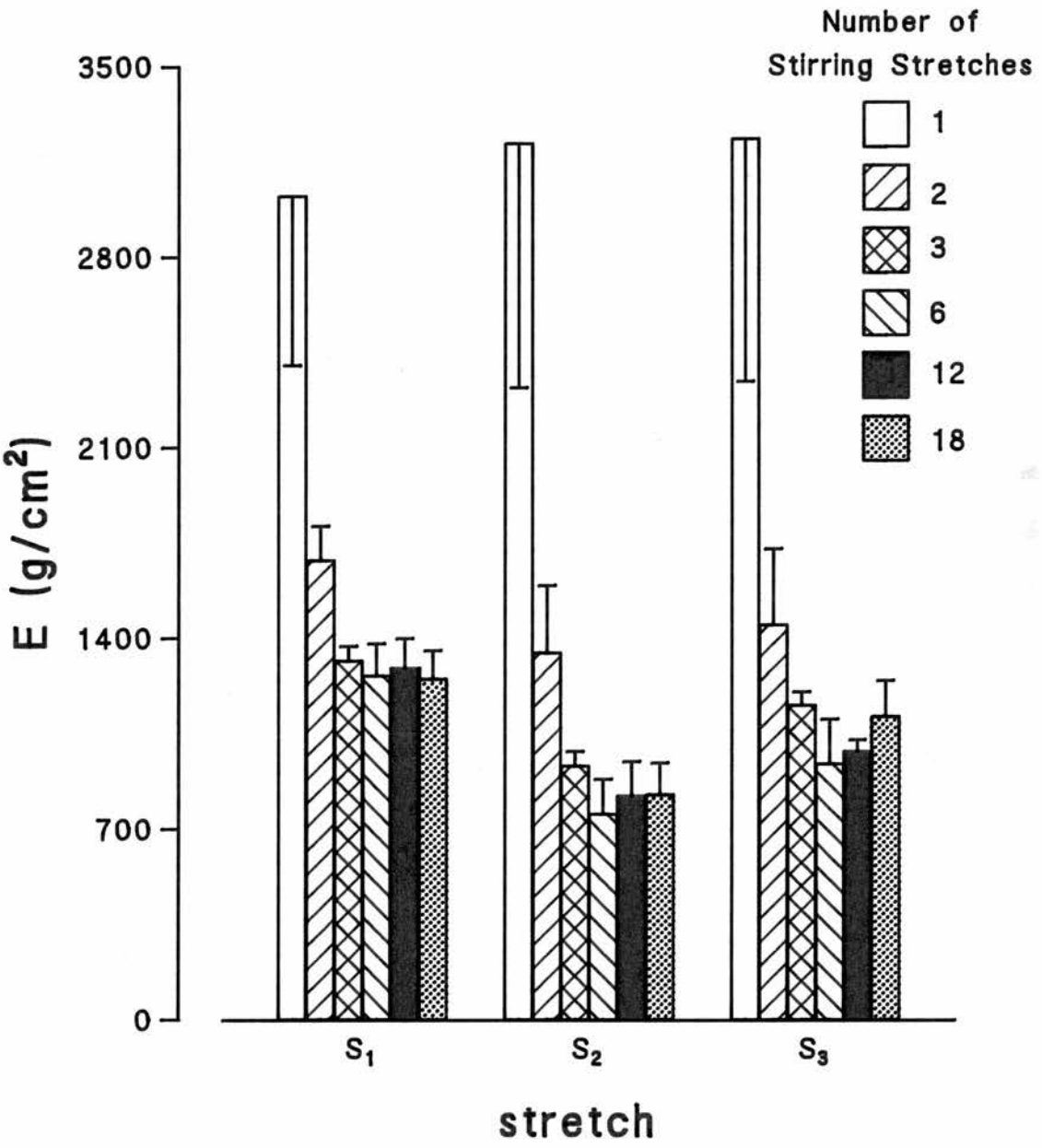

3000
2500
2000
1500
1000
500
0
E (g/cm²)
0
6
12
18
24
30
180
Time from Stirring (s)

FIGURE 4f: The effect of varying the amount of stirring on the mean (plus SD) stiffness values for single fibres. Temperature 3°C, force 0.03 g and n=4.


Number of
Stirring Stretches
1
2
3
6
12
18
3500
2800
2100
1400
700
0
E (g/cm²)
S1
S2
S3
stretch

from 40 - 80 % (results from the fibre showing a decrease of 80 % are shown in FIGURES 4d and 4e). Recovery after 30 s is approximately 70 % complete which when compared to the whole muscle is less than has been reported for the sartorius muscle where 85 % had been recovered.

FIGURE 4d shows a series of raw data records obtained from one single fibre. The S_1 displacements are strikingly consistent in size, between trials, as are the S_2 stretches. These S_2 displacements are roughly eight times the size of the movements caused by the S_1 stretches. The S_3 responses are variable in their size and are dependent on the time elapsed since stirring, decreasing as this time becomes longer.

FIGURE 4e is a graph of the calculated E values from the raw data shown in FIGURE 4d. These results show the apparent discontinuity in the stiffness recovery curve more clearly, than in the whole muscle.

AMOUNT OF STIRRING

FIGURE 4f shows E values for different degrees of stirring. One stirring stretch produces very high stiffness values with no significant change in the fibre's level of stiffness over the three test stretches. Two stirring stretches have lowered the stiffness values by approximately 40 %, and a very small thixotropic response is seen. The three and six stirring stretch trials have the lowest stiffness values, 50 % of the S_1 values with the one stirring stretch trials, and also the greatest degree of thixotropic change. There is no significant difference between these two trials in both size of response and in the time course of the recovery. Further amounts of stirring do not produce any

FIGURE 4g: Three raw data traces for a fibre before (A), 30 s after (B) and 6 mins (C) after damage to the contractile material. Temperature 3°C, force 0.03 g, stirring displacement 0.5 mm.

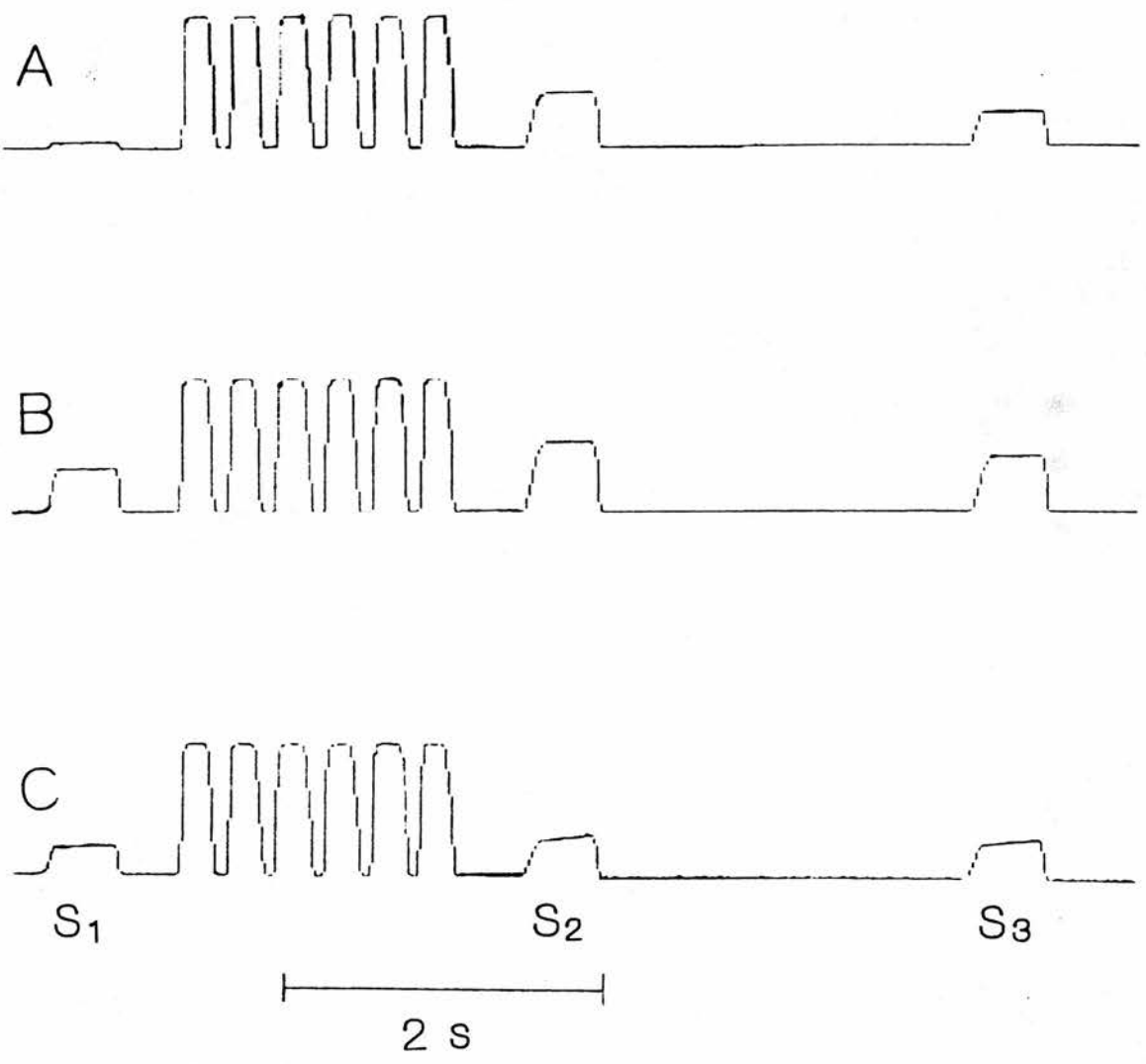

A
B
C
S1
S2
S3
2 s

FIGURE 4h: The mean (plus SD) stiffness values for 6 fibres before and after damaged by gentle squeezing to form clots of contractile material and areas of empty sarcolemma. Temperature 3°C, force 0.03 g.

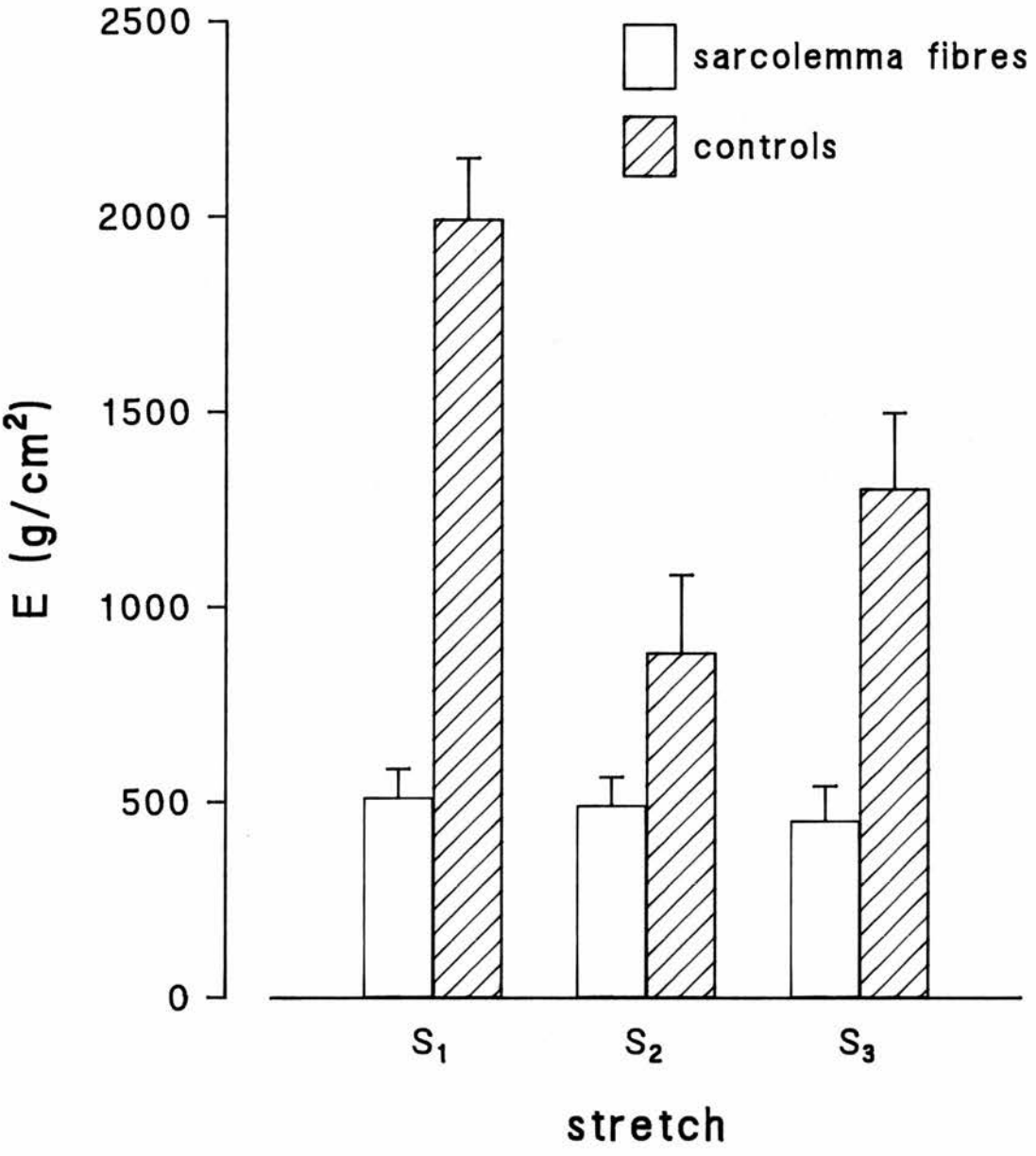
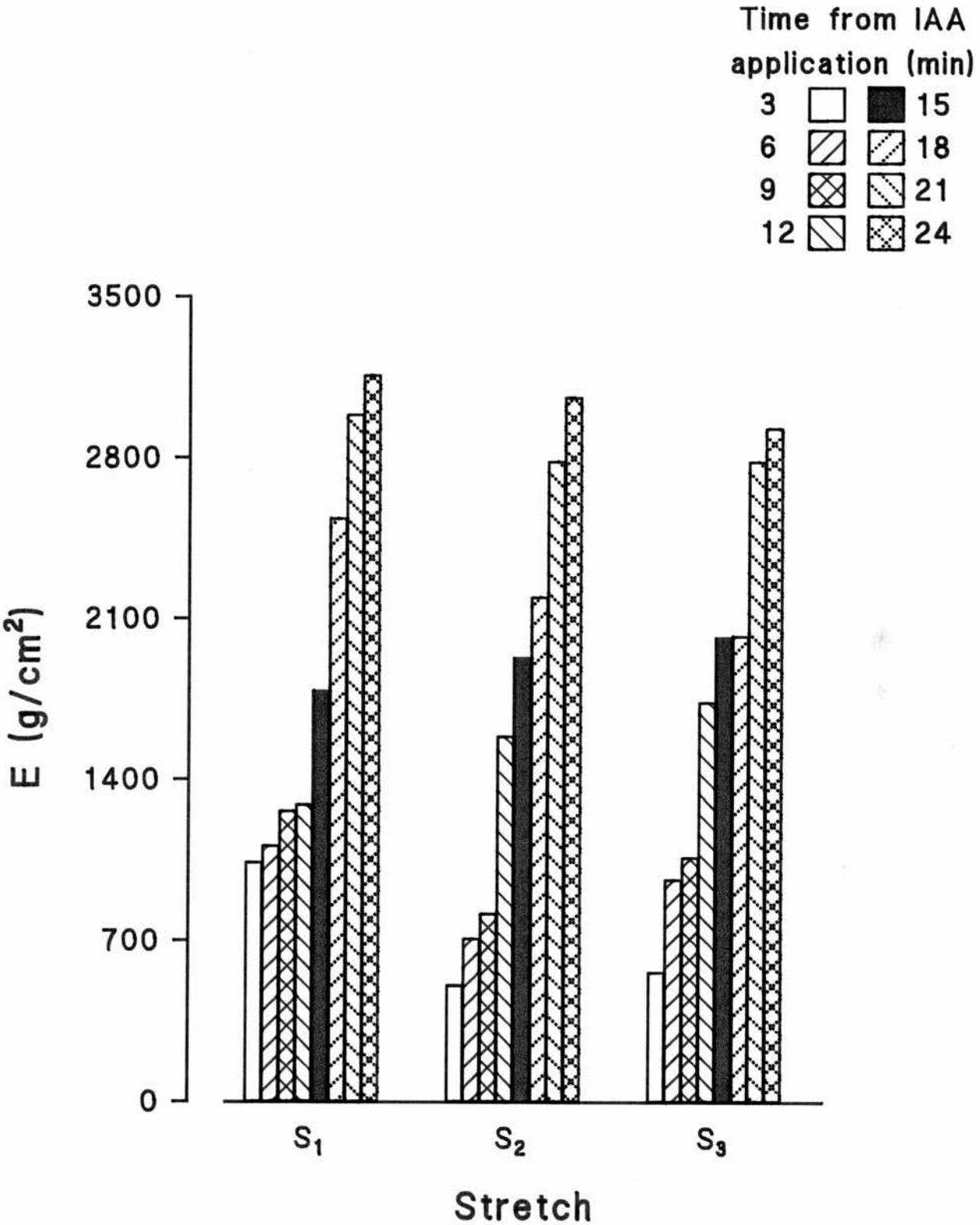

2500
2000
1500
1000
500
0
E (g/cm²)
sarcolemma fibres
controls
S1
S2
S3
stretch

FIGURE 4i: The effect of a 0.2 mM IAA solution on the development of rigor in a single fibre. Temperature 3°C, force 0.03 g.


Time from IAA application (min)
3
6
9
12
15
18
21
24
3500
2800
2100
1400
700
0
E (g/cm²)
S1
S2
S3
Stretch

further decreases in stiffness or increase the size of the thixotropic effect.

SARCOLEMMA

FIGURE 4g is a set of raw data traces from a sarcolemma fibre. The upper trace shows the response before damage (an intact fibre preparation), and shows the typical thixotropic response. The middle trace, taken 30 s after the fibre had undergone damage, shows a fibre with a smaller degree of thixotropy. The S_2 and S_3 stretches now give similar sized responses. The S_1 stretch in (B) is larger than in (A). The bottom trace shows the response 6 mins after damage had been inflicted. Thixotropy has virtually been abolished, and all three stretches are now very similar in the size of response. The fibre is no longer demonstrating thixotropic effects; it is not loosening, or recovering, as an intact fibre would.

FIGURE 4h is the mean response of 6 sarcolemma fibres. The stiffness values are much smaller for the sarcolemma fibres than for the undamaged fibres by 75 %. Thixotropy is also greatly reduced in the damaged fibres, with only very small changes in the stiffness values following stirring and after 3 s at rest.

When the fibres were observed under the light microscope they showed clots of contractile material, with empty areas of sarcolemma on either side.

IAA

FIGURE 4i is the calculated E values from one fibre treated with IAA, followed for 24 mins. The S_1 stretches get stiffer progressively. By 24 mins the fibre is almost three times stiffer than the initial S_1 value. The same trend is

seen for the S_2 and S_3 stretches; the muscle gets stiffer after the application of the IAA solution. Thixotropy is decreased with IAA. Prior to IAA there was a 52 % decrease after stirring whereas after 24 mins with IAA this was reduced to 6 %.

The same general trend, with a reduction in the size of the thixotropic effect and an increase in the stiffness of the fibre, was observed with other fibres treated with IAA, although the onset of the rigor was very variable.

DISCUSSION

Single fibres are thixotropic, and the extent of the loosening after stirring is not significantly different from that seen with the whole muscles. Variation in the extent of the response is greater in the single fibres than with the whole muscles. This can be explained if the whole muscle is regarded as consisting of a population of fibres that are not homogeneous, whereas single fibres represent the mechanical features of a single muscle unit, and therefore a much more difficult experiment to perform repeatedly. The dissection of single fibres is a very difficult procedure to perform and it is possible that some "healthy" fibres may in fact have been damaged, and that their results were included in the data presented here.

The whole muscle's fibre population is also made up of fibres at differing lengths, one fibre's sarcomeres exerting an effect on its neighbours, stretching the weaker ones until an equilibrium is reached for the whole muscle. The strength of the sarcomeres may also vary within a fibre with some sarcomeres providing a weak spot along the fibre (Morgan and Proske 1984). The re-development of the fibre's stiffness will also be affected by the degree of non-uniformity of the sarcomeres in the number of potential attachment sites for the myosin heads at the start of the recovery period. If the muscle is allowed to relax then the sarcomeres will readjust themselves so as to regain their original pattern of non-uniformity (Morgan and Proske 1984). This would suggest that changes in muscle stiffness are the result of the number of cross-bridges in the resting fibre, which break, then reform, with some sarcomeres more labile than others.

The S_1 stiffness values obtained from the single iliofibularis fibre are in general agreement with the corresponding values from the whole sartorius and iliofibularis. The whole iliofibularis has stiffness values of around 2400 g/cm^2; using forces producing comparable sized displacements from the single fibres an average value of 1846 g/cm^2 was obtained; Lannergren (1971) gave a value for iliofibularis fibres of 2280 g/cm^2 with a sarcomere spacing of 2.2 μm compared to 2.02 μm here. It has been found that stiffness values are fairly consistent over a wide range of sarcomere lengths and certainly up to the sarcomere length of 2.5 μm (Lannergren 1971).

When the time course of the stiffness recovery is plotted for the single fibres it is remarkably similar to that of the whole muscle preparations. However certain differences could be seen. It would seem that there are two distinct recovery phases, an initial fast phase can be followed for the first 5 s following the end of stirring, followed by a much longer and prolonged recovery which seems to occupy the remainder of the 3 min trial. Although there is an indication that recovery in the whole iliofibularis muscle involves a fast and a slow process, the transition is unclear. Where it is seen it appears to be shifted to the right - i.e somewhat later, around 12 s rather than 5 s for the iliofibularis fibres and sartorius. This is discussed in greater detail in the addendum to this chapter. This difference in the recovery curve could be the result of removal of extraneous influences such as other fibres of the muscle, and connective tissue. The two phase recovery might be present in the whole muscle but is blunted by the differences in transition time of

individual fibres of the whole fibre population, whereas in the single fibre a simple response is seen. Lannergren (1971) reported a similar recovery curve from a drop in tension after a series of stretches. The time course revealed that the recovery was 50 % complete after 10 s with the residual recovery taking at least another 3 mins. Lannergren (1971 FIGURE 6) fitted a curve to his data; however, when looked at closely, individual fibre's data are consistent with the two phase recovery presented here.

Work on the recovery of stiffness in humans (Lakie *et al* 1983, and Huffschmidt and Schwaller 1987) has also revealed similar recovery curves for stiffness which could equally follow a continuous curve, or two straight lines.

This supposed two phase recovery may simply be the result of a single process with two rate constants, or could be two entirely different processes. Which of these two theories (one, or two processes), is correct can not be stated with any degree of certainty. The biophysical basis for these two phases has still not been clarified and could equally be the result of cross-bridge re-formation, or other interactions between the actin and myosin filaments.

The amount of stirring the fibre receives has a pronounced effect on the extent of thixotropy. A single large stretch is not sufficient to cause a loosening of the fibre and no thixotropy is seen. The optimum amount of stirring was found to be around 6 large stretches; more than this produces no further loosening by the fibre. Presumably, this is due to the fact that stirring ruptures the bonds between the actin and myosin filaments, with no further bonds left to break. The applied force may cause different "length"

changes in different sarcomeres. Thus sarcomeres that have many cross-bridges attached are not effectively loosened by one stretch and may require repeated efforts. A fibre given vigorous stirring tends to show damage to the fibre structure. Any weakness in the sarcomere structure caused either during dissection, or by any latent weakness in this fragile preparation, could with large perturbations, result in these sites giving way totally. Such damage would result in localized contractions with sarcomeres becoming isolated, separated by empty portions of the sarcolemma. This damage was confirmed by light microscopy of the fixed fibres.

Results from these inadvertently damaged fibres led to a closer investigation into the role of the sarcolemma in the fibre's thixotropic properties. The sarcolemma of such damaged fibres behaves mechanically in the same way as in intact fibres and whole muscles (Street and Ramsey 1965).

The sarcolemma is made up of four indentified layers and is about 0.1 μm thick. During damage to the fibre the plasma membrane gets torn but the other three layers remain undamaged (Street and Ramsey 1965).

Reed *et al* (1966) suggest the sarcolemma forms links with different parts of the myofibrillar surface, by some specialized mode of attachment to the membrane. The sarcolemma originally covers the whole surface of the adjacent myofibrils, with portions which attach themselves to the myofibrillar material. These regions remain attached even when the fibre has gone into rigor. These areas appear to be between the H zone and the Z line. Franzini-Armstrong and Porter (1964) confirmed that the sarcolemma does attach itself to myofibrillar structures but only at the Z membrane

by the delicate network of transverse tubules.

The sarcolemma plays a highly dynamic role in the physiological role of the myofibrils. At certain times the membrane could be quite free from the myofibrils with no links, and at other times the complete reverse with regions firmly attached.

The sarcolemma is known to contribute some active tension to the fibres. Street and Ramsey (1965), found after injury seven fibre bundles had an active tension that varied between 30 - 100 % of the original tension. Injured fibres are an unstable and failing preparation, and certainly in this study very variable results were obtained from damaged fibres. In all cases where damage was identified after fixation, thixotropy had been small, and inconsistent. The sarcolemma is an elastic material with some non-linearities of the tension elongation (Fields 1970 and Fields and Faber 1970), but this can be removed by stretching.

The residual thixotropy seen in the damaged fibres could well be due to a few intact sarcomeres that are still viable. Areas of clot formation may represent zone of attachment to the myofibrillar material. Observations by electron microscope, show that fibres with sarcomere damage rupture at the A and I bands and appear as if they have completely separated (Mulvany 1975). A part of the fibre's resting elastic modulus is derived from the sarcolemma, a very small amount at sarcomere lengths used in these experiments. This finding is in agreement with the work by Podolsky (1964), and Rapoport (1972), who showed that below sarcomere lengths of 3.2 μm the sarcolemma does not play a significant part in the fibre's elastic modulus. For sarcomere lengths beyond 3.2 μm

the elastic modulus of the contractile system is reduced by a factor of five by the removal of the sarcolemma. Fields and Faber (1970) found that the value for the sarcolemma's elastic modulus is in approximate agreement with collagen's elastic modulus, and this was confirmed by Fields (1970), suggesting that the principal rigidity of the sarcolemma is in fact due to the collagen fibril layers. Fields (1970) proposed a spring like helical arrangement of these fibrils.

The sarcolemma is not thixotropic, or at least it is not the major source of the thixotropic properties so far observed, suggesting that the contractile material itself is the biophysical basis for the thixotropy. Further evidence for this supposition comes from muscles poisoned with IAA.

Electron microscope work has concluded that the myosin heads are permanently attached to the actin filament in rigor. To reach this state the heads go through part of the normal contractile cycle (Reedy *et al* 1965). In rigor various muscles have been studied to find the proportion of the myosin heads attached, with between 70 and 95 % of them attached (Cooke and Franks 1980 and Lovell *et al* 1981). Mulvany (1975) also provided evidence for a physical attachment in the IAA induced rigor state. He showed that the rigor resistance to stretching was proportional to the overlap between the contractile filaments.

In the present study of the single fibre it was found that 5 to 10 mins following application of IAA the fibres had become very stiff and non-thixotropic. In the rigor state the number of attached cross-bridges is increased from that of the resting state. Thus it can be assumed that the increase in stiffness is the direct result of the formation of these

extra connections. As thixotropy is reduced as rigor develops in the fibre, this suggests that the disconnection of the cross-bridges is a vital process in thixotropy. In rigor fibres the majority of the myosin heads are permanently attached, therefore there should be no loosening, or thixotropy. However even in the rigor state there may be at least 5 % or more of the myosin heads still unattached, which are capable of going through a normal cross-bridge cycle.

For IAA rigor to develop completely it is best to use an active muscle or fibre (Mauriello and Sandow 1959). In these fibres no stimulation was used, so rigor may not have been complete at the time of study.

Once rigor had been established, a larger than usual stirring waveform caused a dramatic reduction in the fibre's stiffness, falling to values below those of the controls, but stiffness does not subsequently increase. This is comparable to humans where movement during rigor loosens the muscle but there is no subsequent restiffening. When fibres were observed under the microscope, damage similar to that seen with the sarcolemma fibres was observed. A massive destruction of the fibre's structure had occurred. Mulvany (1975) stated that weak sarcomeres rupture and are pulled apart to leave the sarcolemma with clots of sarcomeres on either side. The clots may represent areas where the sarcolemma had been attached to the myofibrillar material.

These experiments help to clarify the mechanism underlying thixotropy. The stirring waveform has not only to be of a certain magnitude to take the fibre beyond a critical limit, it has also to be repeated a number of times. Once the optimum amount of stirring has been reached any further

increase does not produce a further drop in the fibre's stiffness, presumably because the majority of the resting cross-bridges have then been disconnected. It is repeated movements beyond the SREC range that produces the greatest thixotropic effects.

The sarcolemma has no role to play in the thixotropy of the single fibres. It contributes a very small amount to the elastic modulus, but its own elastic properties appear to be linear in behaviour at normal sarcomere lengths.

The basic mechanical component for thixotropy seems to lie in the contractile material itself, rather than the tendons and sarcolemma. The most likely candidates are the cross-bridges, with other forms of bonding, inter and intra filamentary, possibly playing a small role in the observed thixotropy.

CHAPTER FOUR ADDENDUM

THIXOTROPY: IN WHOLE ILIOFIBULARIS MUSCLES

INTRODUCTION

This addendum is a report on the behaviour of the whole iliofibularis. It provides a basis from which to compare the stiffness values obtained from single fibres with those for intact muscle.

FIBRE TYPES IN THE AMPHIBIAN MUSCLE

Amphibian muscle contains two major fibre types, twitch and slow fibres. These two fibre types differ with respect to innervation, fine structure, histochemical properties and mechanical performance (Peachey 1961). The sartorius and iliofibularis have differing fibre populations. Histological investigation of the sartorius shows it to be composed of twitch fibres exclusively; with no evidence of any slow fibres (Gray 1958). By contrast, in the iliofibularis, Lannergren and Smith (1966) described a mixed fibre population, composed of twitch fibres, with a distinct bundle of slow fibres (the tonus bundle: Sommerkamp 1928, Peachey and Huxley 1962).

MECHANICAL DIFFERENCES IN SLOW AND TWITCH FIBRES

The slow fibre population of the iliofibularis might be expected to affect the mechanical properties of the muscle. The formation of cross-bridges is slower in slow fibres than in twitch fibres (Costantin, Podolsky and Tice 1967, Proske and Rack 1976, and Gregory, Luff, Morgan and Proske 1978). Slow fibres are also stiffer than twitch fibres, but the SREC is not significantly different in the two fibre types, nor are the mechanical properties of the cross-bridges. The main difference lies in the fact that the turnover of cross-bridges during contraction is lower in slow fibres, and

is too slow to allow any significant break down during an imposed movement. Twitch fibres have a higher turnover of cross-bridges, allowing sufficient time for some break down of cross-bridges to occur, making the muscle more compliant.

This short study investigates the effect of the slow fibre bundle on the thixotropy of the iliofibularis muscle.

METHOD

WHOLE ILIOFIBULARIS

Whole iliofibularis muscles of *R. Temporaria* were dissected for these experiments. The muscle's anatomy dictated that it was removed with no bony attachments. Before dissection the length of the muscle was measured with the leg at 90^{o} (L_{o}). The muscle was transferred to the muscle chamber (FIGURE 4a apparatus B). The tibial and pelvic tendons of the muscle were attached with stainless steel clips to the bottom of the bath, and to the lever arm. The bath was filled with isotonic Ringer. The experiments were conducted in November.

PROTOCOL

The protocol was the same as used for the single fibres observations of thixotropy. The 10 stirring stretches had a maximum movement of 2 mm. The force of the stretches was adjusted to give a displacement that was equivalent to those seen from the single fibres experiment. This was found to be 0.25 g. Temperature was maintained at $3^{o}C$ throughout.

RESULTS

Results from the whole iliofibularis muscles are plotted in FIGURE 4sa. The thixotropic recovery following stirring is remarkably similar to that already plotted for the single fibres, reported in the main chapter, and also from the whole sartorius muscle.

The stiffness following stirring was reduced by 74 % from the values of S_1. Recovery of stiffness is rapid for the first 10 s after stirring, with almost 60 % recovered. After 30 s at rest, recovery is 80 % complete, with the remainder returning in the following 2.5 mins. There is no significant variation in the values of E for the three different types of frog muscle preparation tested.

TABLE 4sa. Shows the S_1 stiffness values recorded from the whole sartorius (minimum, maximum, November and July), whole iliofibularis and single twitch fibres (iliofibularis) that were used in this work. The values for the whole sartorius in November are slightly smaller than for the whole iliofibularis tested at this time, whereas the single fibres tested in July are less stiff than the corresponding month's values from the sartorius. However the values for the iliofibularis do fall within the range of values recorded from the sartorius muscle.

FIGURE 4sa: The mean (SD) stiffness values for the thixotropic recovery for the whole iliofibularis muscle. Temperature 3°C, force 0.25g, n=4.

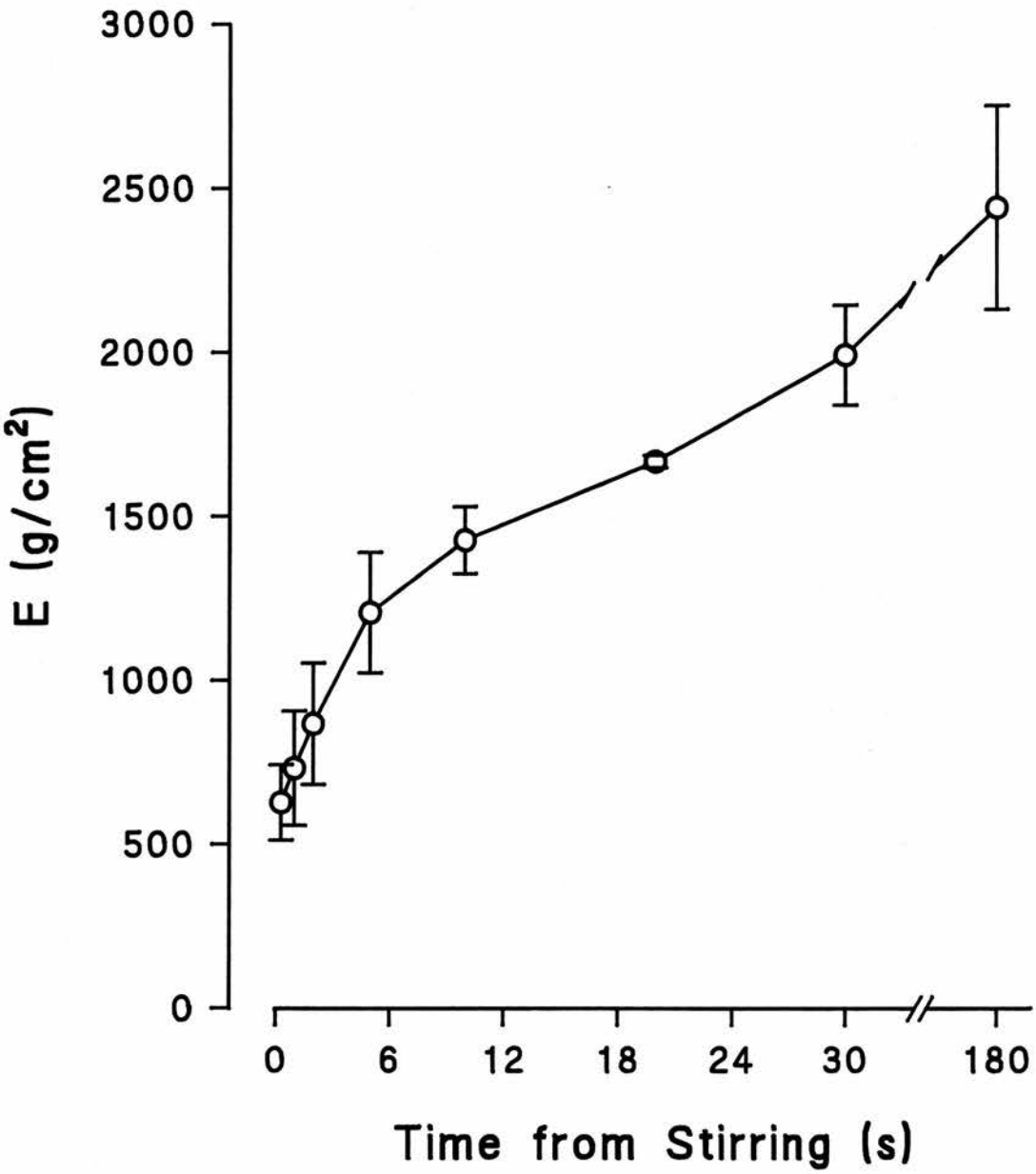

3000
2500
2000
1500
1000
500
0
E (g/cm²)
0
6
12
18
24
30
180
Time from Stirring (s)

TABLE 4sa: The mean E values (plus SD) for S_1 stretches obtained from whole sartorius, whole iliofibularis and single fibre preparations. The sartorius muscle has four values because of its apparent seasonal variation in stiffness, showing its maximum and minimum and also the value for November as a comparison for the iliofibularis muscle tested at this time of year.

MUSCLE	E (g/cm^2)	S.D.+/-	n=
Whole Sartorius:			
minimum (December)	1275.2	183.0	10
maximum (May)	2552.1	81.8	4
November	1692.2	95.8	8
July	2362.0	165.7	4
Whole Iliofibularis (November)	2442.1	631.4	6
Single Fibres Iliofibularis (July)	1992.5	104.4	8

DISCUSSION

The S_1 stiffness values for the three muscle preparations show that the whole iliofibularis muscle is stiffer than the average single fibres taken from it. This difference of 449.6 g/cm^2 is not greatly different, and may reflect some seasonal variation in the mechanical properties of the muscle fibres of the iliofibularis (single fibre experiments conducted in July, whole iliofibularis experiments conducted in November). The difference may however be a reflection of the differing mechanical properties of a single isolated muscle fibre to that of the whole muscle.

The stiffness values from the whole iliofibularis and the single fibres are both very similar to the values quoted by Lannergren (1971), of 2280 g/cm^2, for single twitch iliofibularis fibres. It may be that the difference is a consequence of the increased connective tissue in the whole muscle compared to that found in the single fibres, that adds to the stiffness of the whole muscle.

The decrease in stiffness after stirring is greater than that for the whole sartorius muscle or, of the single fibres by about 15 %. The recovery of stiffness for the whole iliofibularis is more of a continuous curve than the two distinct phases that were found for the recovery of the single fibres. There is a suggestion that the first phase of the recovery has been prolonged, seemingly lasting two or three times as long as for the sartorius and single fibres. This is possibly as a result of the mixed fibre population in the whole iliofibularis with the slow fibre population producing this slower return because of their inherently lower cross-bridge cycling rate (Costantin *et al* 1967, Proske

and Rack 1976, and Gregory *et al* 1978).

In the whole muscle some of the fibres will be under a greater degree of strain than others and their sarcomeres will not all respond to a stretch in the same manner. Certainly in the slow fibres non-uniform sarcomeres are more common, and when a stretch is applied some of the sarcomeres are already lengthening, reaching their yield point sooner and at a lower tension than those that remain isometric, or that are even shortening (Morgan and Proske 1984). Fibres with a lot of non-uniformity will have lengthening distributed among a few sarcomeres thus increasing the lengthening velocity. Sarcomeres which have not reached their particular yield point will add to the extra series compliances already supplied by the tendon (Flitney and Hirst 1978).

The total amount of stiffness change over the 3 min trial is not significantly different for the three types of muscle preparation. The mean drop in stiffness after stirring is approximately half of the starting value in all cases, although the decrease varies widely for the single fibres. Where the sartorius, iliofibularis and single fibres do differ from each other is in the rate of return of the initial stiffness values. After 5 s of rest the sartorius and the single fibres of the iliofibularis have both recovered almost 70 % of the initial stiffness and by 30 s after the end of stirring this has only increased to about 80 %. This indicates that the majority of stiffness recovered for these preparations is regained within the first 5 s of rest. The whole iliofibularis however has by 5 s after stirring only recovered 50 % of the initial value, but by 30 s it too has

recovered 80 % of the initial stiffness. For the whole iliofibularis the recovery of stiffness takes the same total time but the transition from fast to slow recovery takes about twice as long as for the sartorius and single fibres. This could again be a result of the mixed fibre types that make up the iliofibularis, and of its slow fibre bundle in particular.

The stiffness of the sartorius muscle alters with the time of the year, being stiffer in winter and early spring than in summer. The iliofibularis muscle may also experience seasonal changes in stiffness, which may even be greater than, and not synchronous with, that shown by the sartorius muscle, as the population of slow fibres in the iliofibularis may play a major role in seasonal variation.

The iliofibularis is thixotropic and the changes reported in stiffness are similar in size to the whole sartorius and to the single iliofibularis fibres that have been investigated, although the rates for these changes appears to be altered. It is proposed that the same underlying mechanism is at work in these two different muscles.

CHAPTER FIVE

THIXOTROPY: THE EFFECT OF NATURAL AND INDUCED RIGOR

INTRODUCTION

RIGOR MORTIS

Bendall (1951), and White (1970) described rigor as a very slow irreversible contraction, with the disappearance of ATP from the muscle a fundamental prerequisite for the shortening and increased stiffness that occurs. Rigor appears after death, and was originally conceived as a slow form of contracture (Sandow and Schneyer, 1955; Mauriello and Sandow 1959). But it became clear that rigor muscle, unlike contracting muscle, produced only a small recovery from a quick release, and it was therefore a static state. Structural evidence gave support to this view.

Reedy *et al* (1965), found that in rigor muscle the myosin heads became permanently attached to the thin filaments, by normal cross-bridge cycling. The number of attached myosin heads ranges between 70 to 95 % (Cooke and Franks 1980 and Lovell *et al* 1981).

DEVELOPMENT OF RIGOR

The time course for the development of rigor in rabbit muscle is determined principally by the glycogen reserve at the time of death, with rapid glycolysis starting immediately after death (Bate-Smith and Bendall 1947 and 1949). Creatine phosphate (CP) is also depleted as time from death increases, the CP acting as a reserve of energy rich phosphate for the muscle. Bendall (1951) found that the CP levels were high immediately after death, but decreased rapidly thereafter, being reduced to less than 30 % of their initial level before there was any appreciable loss of ATP. The ATP then declines at a steady rate until the ATP stores have reached 20 % of

their starting value. At this point glycolysis is almost at a standstill, and the rate of ATP disappearance declines markedly. The rate of these events is determined by the breakdown of ATP by the ATP-ADPase systems in the muscle which yields adenylic acid (normally built up to ATP by the reaction with CP), also inorganic phosphate, used to initiate glycolysis, and hence the re-synthesis of ATP and CP.

MECHANICAL PROPERTIES OF RIGOR MUSCLE

There is no change in the extensibility of the muscle until the ATP has fallen below 80 % of the initial level. Extensibility then decreases, reaching its lowest level when the ATP content of the muscle has fallen below 20 % (Bate-Smith and Bendall 1947, 1949). The onset of rigor can be speeded up by starvation or by violent exercise of the animal before death. In such cases the ATP begins to disappear much earlier.

Mulvany (1975) demonstrated that only muscles put into rigor with a degree of filament overlap developed a resistance to stretch, and that the resistance was approximately proportional to the amount by which the filaments overlapped when the muscle went into rigor. This is analogous to the linear relationship between tetanic tension and overlap seen in living muscle (Gordon, Huxley and Julian, 1964), which suggests that the rigor resistance to stretch is determined principally by the properties of the cross-bridges.

CROSS-BRIDGES IN RIGOR

Rigor muscle displays several structural differences from live, relaxed muscle. The most interesting observations

concern the changes in the conformation of the cross-bridges. As the muscle moves from the relaxed to the rigor state there is a change in the orientation of the cross-bridges. In the relaxed state, the bridges are at right angles to the filament axis, whereas in rigor they are at an angle. In gycerol extracted lethocerus flight muscle, the cross-bridges appeared to be detached from the actin filaments in the relaxed muscle, but probably attached when in rigor (Reedy *et al* 1965).

IAA RIGOR

Depletion of ATP occurs "*in situ*" following death, or it can be provoked "*in vitro*", either by poisoning the muscle metabolism or, by rupturing the fibre membrane. Metabolic poisons have proved to be the most reliable means of inducing rigor while still maintaining the muscle structure. Sandow and Schneyer (1955) used IAA to inhibit glycolysis, preventing re-phosphorylation of first CP, and then ATP, irreversibly, with the latter especially occurring in rough parallelism with the growth of rigor. Thus the energy of CP is concerned with a purely chemical recovery processes, while ATP serves to directly energize the contractile material for mechanical work (Bendall 1951).

Mauriello and Sandow (1959), found that IAA rigor was further advanced and developed quicker if the muscle had been subjected to a burst of activity (tetanus or a series of twitches). The depletion of the ATP stores was faster than in the non-active muscle.

IAA rigor in single twitch fibres of the iliofibularis muscle was studied in Chapter 4, and showed decreasing thixotropy as the time from IAA administration increased.

The fibres displayed structural damage of their sarcomeres, with in some cases, total sarcomeric disintegration.

In this Chapter the effect of naturally occurring rigor on the mechanical properties of frog sartorius muscle, over a normal experimental period of approximately 8 hr, and over a 48 hr period are studied, and compared with IAA poisoned muscles. The possible implications of rigor on the thixotropic results described in the preceding chapters are discussed.

METHOD

Experiments were conducted on frogs in July and August. The IAA used for these experiments was supplied by Sigma Chemical Co. Ltd..

NATURAL RIGOR (CHANGES IN STIFFNESS)

The stiffness of the sartorius muscle was measured over a 48 hr period; the muscle can be kept viable for a number of days after excision. The surrounding isotonic Ringer solution was changed for a fresh solution every 12 hrs (no antibiotics were added and it was unaerated) .

INDUCED RIGOR (CHANGES IN STIFFNESS AND THIXOTROPY)

The muscle was mounted in isotonic Ringer solution, for the first part of the experiment, which consisted of a control set of eight 3 min stretch trials. After the control trials were completed, the isotonic Ringer solution was drained out of the muscle chamber, and replaced with a Ringer solution which contained 0.4 mM of IAA. The muscle was left to rest and equilibrate with this new solution for 30 mins. The muscle was then given single bipolar shocks (10 v), every 5 s for 90 mins, or until such time as the stimulation no longer elicited a contraction. This stimulation was the preliminary treatment that was used to put the muscles into rigor.

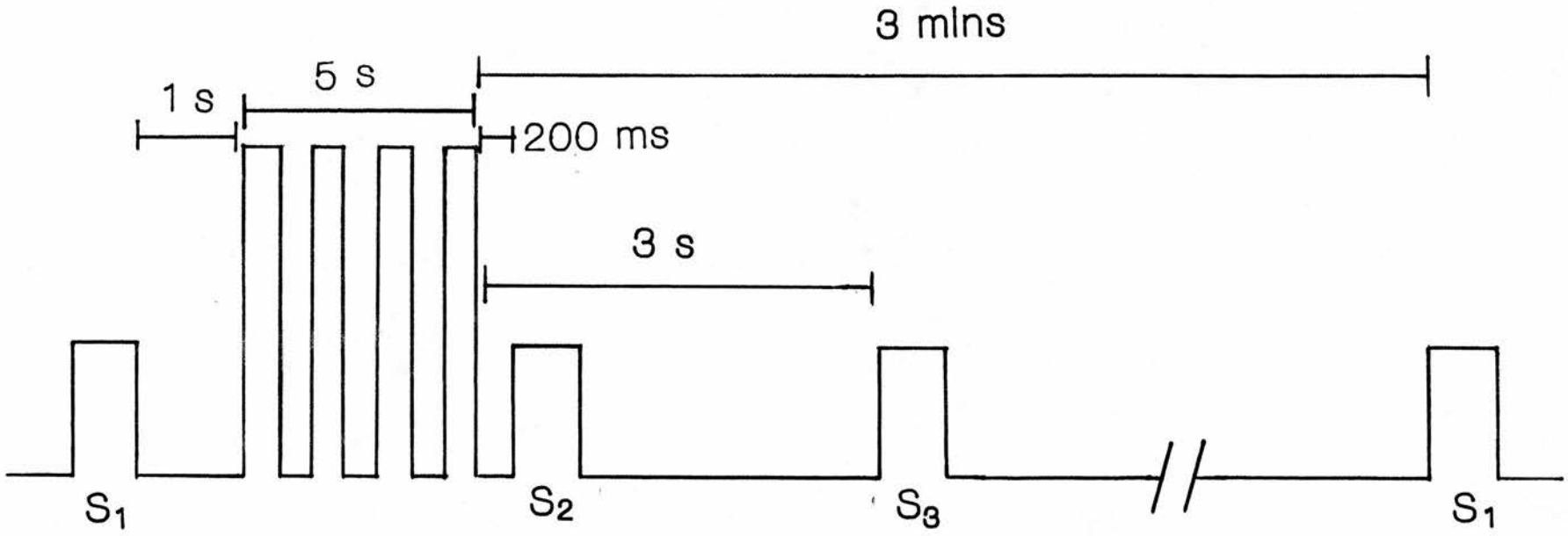
PROTOCOLS

All experiments were conducted at $3^{o}C$.

-NATURAL RIGOR-

For the naturally induced rigor trials five muscles were tested.

FIGURE 5a: A schematic diagram of the protocol (not to scale) used for the induced rigor trials, with a fixed 3 s S_3.


3 mins
5 s
1 s
200 ms
3 s
S1
S2
S3
S1

Group one: Tested for 48 hrs; three muscles; received 2 s of maximal tetanic stimulation (8 v, 30 Hz), followed 1 s later by a single stretch, stimulation and stretches were repeated every 3 mins for a period lasting 8 hrs, followed by 16 hrs of rest before the next 8 hr period.

Group two: Tested for 48 hrs; two muscles; received no stimulation and only a single stretch every 3 mins throughout the 8 hr periods followed by 16 hrs of rest.

Measurements of stiffness were taken at the beginning and the end of each 8 hr period (5 readings were taken in all). The experiment was stopped when the muscle was 48 hrs old. The test stretches were composed of a single rectangular stretch (duration 500 ms, force 0.3 g). All the muscles were in good condition at the start of the experiment.

These muscles provided an indication of the amount of rigor that could be expected, in the muscles used throughout this work, in a typical 8 hr period.

-IAA RIGOR-

Following the preliminary treatment with the IAA Ringer the following protocol was used. The stretch was a rectangular force, of 0.3 g, lasting for 500 ms. Each trial consisted of three stretches, S_1, followed 1 s later, by 5 s of stirring (3 Hz and 3 mm maximum movement). S_2 occurred 200 ms after the end of stirring. S_3 always appeared at 3 s following the cessation of the stirring waveform (FIGURE 5a). Each of the trials were 3 mins apart. The control stretches were the same as above.

RESULTS

NATURAL RIGOR

Results shown in FIGURE 5b, indicate that both muscle groups have a similar degree of stiffness initially, and up to 24 hrs after dissection there is no significant difference. As rigor develops the rate of stiffness development differs for the two groups, depending on the experimental protocol. The muscles receiving stimulation, develop a higher degree of stiffness (increasing by 57 % from the starting value after 32 hrs). Muscles which were not stimulated had lower stiffness values (increasing by only 35 % from the starting value after 32 hrs). After 48 hrs the stimulated muscles have increased their stiffness by 87 % from the starting value, the un-stimulated group has increased by 48 % only.

Throughout this thesis rigor development was not a serious problem. With careful handling of the muscle, consistent recordings could be made for periods that extended over the normal 8 hr experimental period, without serious deterioration of the muscle.

INDUCED RIGOR

FIGURE 5c shows the mean results from the pre-IAA and the IAA rigor muscles. The most dramatic difference is the increase in the stiffness of the muscles after the application of IAA. After the preliminary IAA treatment the muscles had mean S_1 stiffness values approximately 600 % greater than the control trials, compared with the maximum increase in stiffness of 87 % recorded with the natural rigor experiments.

FIGURE 5b: The development of natural rigor followed over 48 hrs for two muscle group regimes (means and SD). Temperature 3°C, force 0.3 g.

Pre-stretch Treatment

stimulation n=3,

no stimulation n=2.

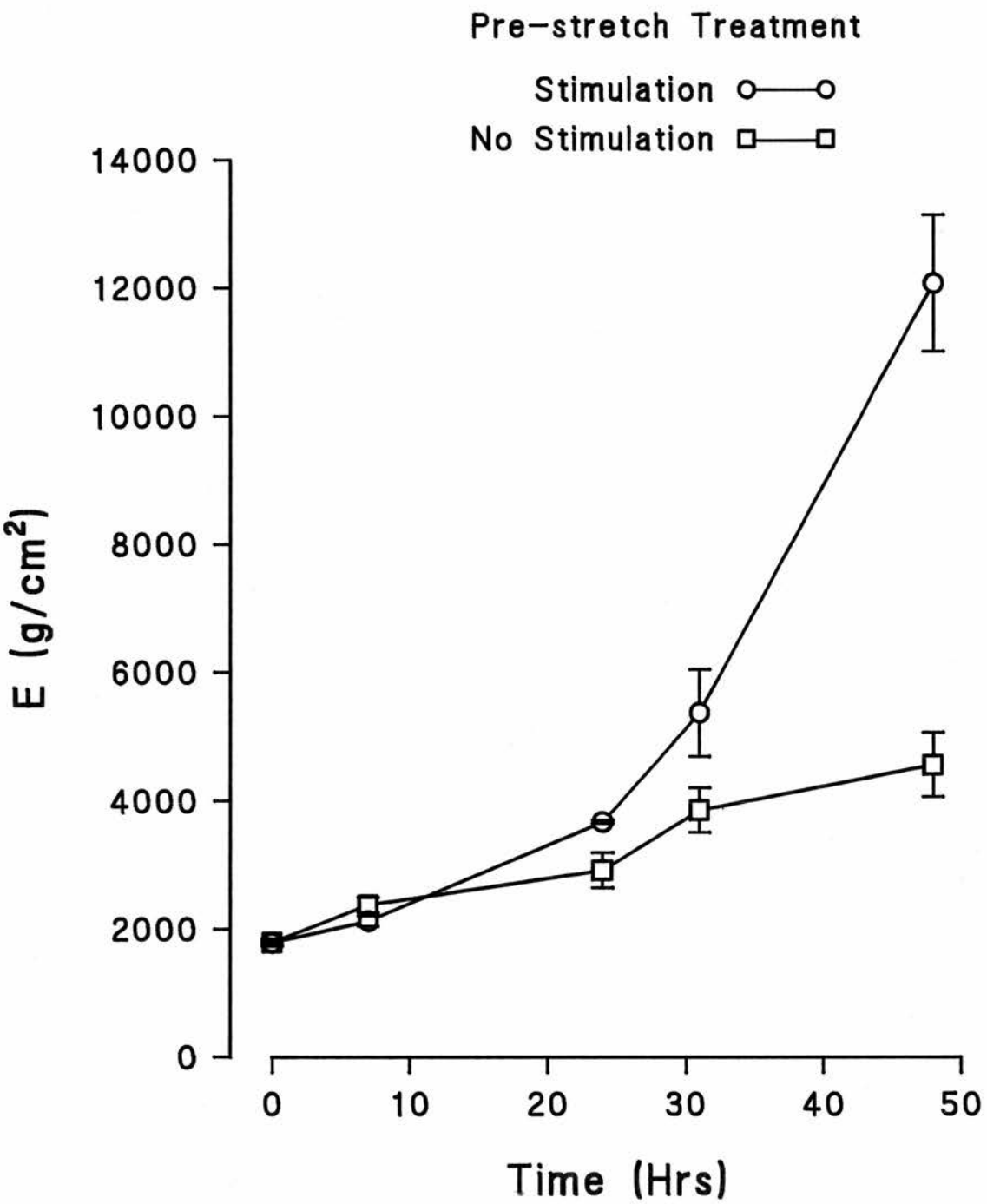
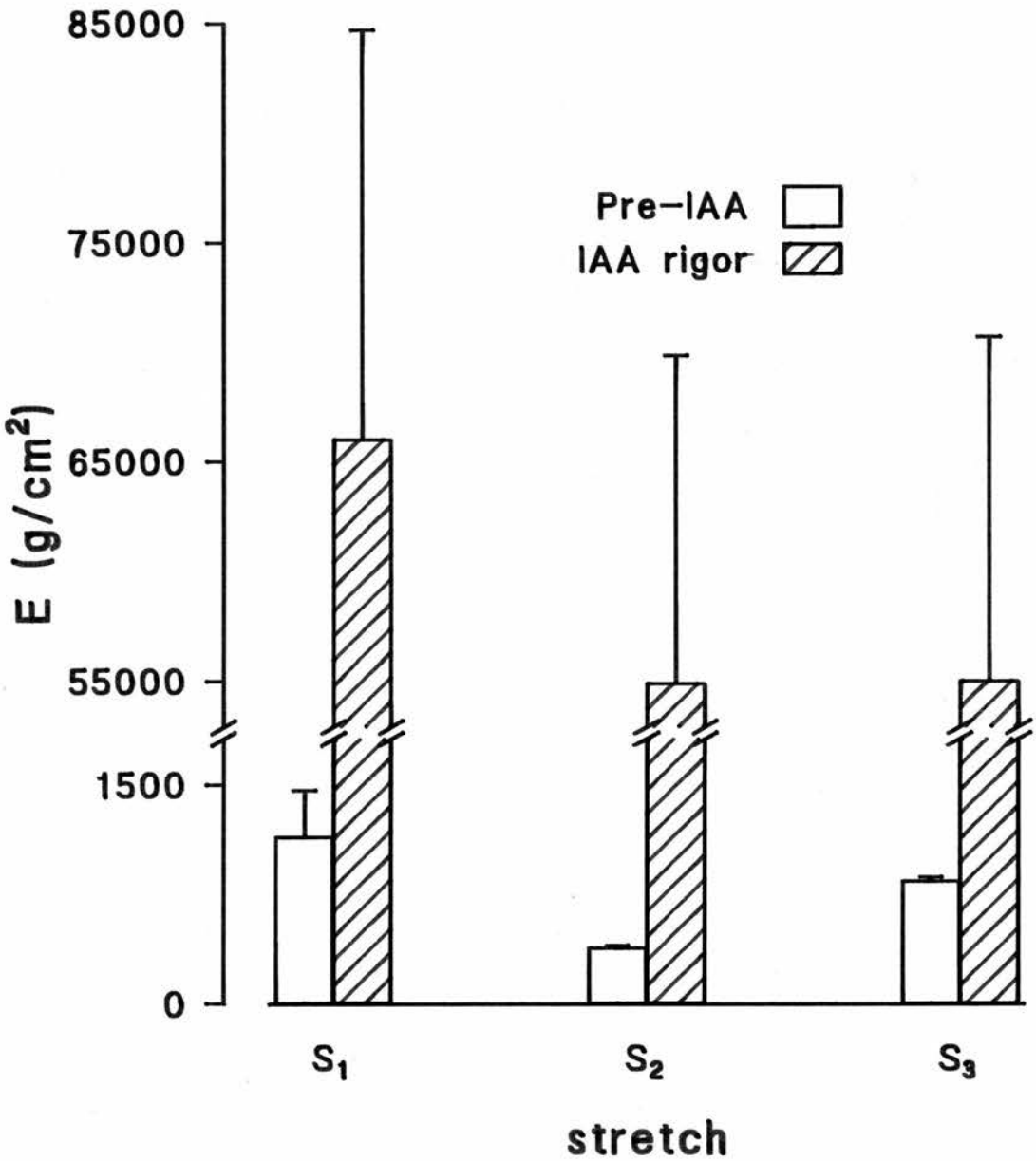

Pre-stretch Treatment
Stimulation
No Stimulation
14000
12000
10000
8000
6000
4000
2000
0
E (g/cm²)
0
10
20
30
40
50
Time (Hrs)

FIGURE 5c: The mean (SD) stiffness values for the controls, and after development of IAA rigor, for 11 trials. Temperature 3°C, force 0.3 g, n=4.


85000
75000
65000
55000
1500
0
E (g/cm²)
Pre-IAA
IAA rigor
S1
S2
S3
stretch

Thixotropy prior to the application of the IAA is within the range that has been reported earlier, with a decrease after stirring of around 60 %, and after 3 s the recovery is 74 % complete. Thixotropy following the application of IAA is greatly reduced, with a small reduction in the muscle's stiffness after stirring of about 15 %. Recovery of stiffness after 3 s of rest is negligible and there was also a wide variation in the E values. The changes in stiffness after the application of the IAA are very small, and are in fact are little more than what can be considered as "noise" in a very variable signal. The appearance of the thixotropy has also been altered with the application of IAA. Whereas with the pre-IAA trials the stretches were predictable in size from trial to trial, with the IAA induced rigor the stretches became very unpredictable in size.

DISCUSSION

DEVELOPMENT OF RIGOR

In natural conditions, the development of rigor is very much slower than in the metabolically poisoned muscles. Even 48 hrs after death the muscles could still be stimulated with single shocks, or a prolonged burst of activity (tetanus), and develop a tension sufficient to cause a deflection of the lever arm. Stiffness values increased from those obtained immediately after dissection, but the maximum stiffness recorded was considerably smaller than the maximum stiffness in the IAA rigor muscles, and these muscle had not probably achieved a complete rigor state.

Active stiffness achieved for frog muscles during maximal tetanic stimulation is of a similar level to that in IAA rigor muscles. Halpern and Moss (1976) and Bressler and Clinch (1974) gave a value of 85.3 Kg/cm^2 for active stiffness of the frog sartorius, and for toad sartorius a value of 140 Kg/cm^2 respectively. This can be compared with the mean stiffness value for IAA rigor of 65 Kg/cm^2 (range 40 - 120 kg/cm^2). It can be assumed that active tetanic stiffness involves the attachment of about the same number of cross-bridges as there are in rigor, so that in resting muscle between 1 - 3 % of the cross-bridges are attached, compared to the 70 - 95 % in rigor. Other values for the active stiffness for various muscle are given in Chapter 2.

The speed of development of natural rigor varied considerably, dependent primarily on the protocol that the muscle had been subjected to during the period of study. Individual variation between the muscles used could be the result of the muscle's condition at the time of death.

Stimulated muscles developed rigor faster and to a greater degree than muscles receiving passive stretches only. This is presumably because their stores of ATP are depleted quicker than in the passive muscle.

Bate-Smith and Bendall (1947) observed that in animals which were passive before stunning and death, rigor was delayed. Thus any factor which has an effect on the metabolic rate of resting muscle can influence the rate of rigor development. The condition of the animal at the time of death will have consequences for the energy stores of the muscle. Some frogs are naturally larger and better fed than others, so that electrical stimulation will have varying effects on the speed of depletion of the energy stores.

The onset of rigor has been linked to the utilization of the muscles store of CP and ATP. In a fresh sartorius muscle there is about 25 μ moles per gram of CP plus a low level of inorganic phosphate. When extracted in alcohol it has been found that an average 0.3 μ moles per gram of CP is hydrolysed for each isometric twitch, with no change in the ATP content of the muscle, until the CP has been depleted to below 30 % of its starting value. If the contractions are isotonic extra CP is hydrolysed in proportion to the physical work performed (Wilkie 1968). Stimulation was performed under isometric conditions, so the former rate of CP hydrolysis can be regarded as the rate of depletion for the CP. The stores of ATP are between 4.35 - 5 m moles per gram, in rabbit psoas muscle (Bate-Smith and Bendall 1949 and Bendall 1951). The turnover rate varies from 0.08 μM/g to 0.006 μM/g (Bate-Smith and Bendall 1949 and Davies, Kushmerick and Larson 1967), depending on the rate of

stimulation.

Rigor develops quickly when the ATP level in the muscle falls below 0.5 mM of the initial concentration (White 1970). Sandow and Schneyer (1955) found that after only 40 or 50 single contractions a muscle poisoned with IAA is exhausted, the contractions decrease rapidly and simultaneously the relaxation becomes more incomplete.

ATP may be lost by hydrolysis or by diffusion into the external solution. Values for the activity of the ATPase of relaxed muscle are about 40 p mole/cm fibre per minute. Loss of ATP by diffusion can also lead to the development of rigor, and it can be accelerated by agitation of the surrounding solution. White (1970) concluded that both hydrolysis and outward diffusion contribute to, and are together sufficient to explain, the loss of ATP by the fibres.

Uneven distribution of ATP between the centre of the muscle and the periphery as a result of the ATP diffusing into the surrounding solution, may result in a fully developed rigor state at the centre, and a live periphery. As ATP passes from the centre to the periphery, the ATP will be depleted sooner at the centre.

Sandow and Schneyer (1955) showed that, over the first 24 hrs with no IAA, a resting unloaded muscle does not develop a significant degree of stiffening. By 16 hrs the muscle has shortened by only 5 %, compared to the 60 % in IAA poisoned muscles. The IAA poisoned muscles display a more complete form of rigor than that seen in the muscles where rigor developed naturally. These IAA muscles have had their ATP stores almost, if not completely, removed with the action of

the IAA preventing glycolytic recovery of ATP.

It is likely that the majority of the cross-bridges are detached from the I-filaments for a large portion of the cross-bridge cycle. The high stiffness seen during rigor would therefore suggest that the cross-bridges are permanently attached (Reedy, *et al* 1965). White (1970) noted that tension is generated in rigor because the inhibition of actin-myosin interaction by troponin-tropomyosin becomes ineffective when the ATP concentration reaches a sufficiently low level (less than 0.5 mM and possibly as low as 5 μM).

RESIDUAL THIXOTROPY

Mulvany (1975) demonstrated that a large proportion of the available cross-bridges had formed in the rigor state and remained attached, increasing the stiffness. This increased resistance to stretches would also have the effect of decreasing thixotropy, as the cross-bridges would not detach and re-form as they do in a live muscle but are tightly bound to the actin filament. Even in a rigor muscle there may be 5 % or more of the myosin heads still unattached to the actin filament and capable of going through a normal cross-bridge cycle.

In a recent paper by Reedy, Beall and Fyrberg (1989), *in situ* cross-bridges were seen to retain a high degree of torsional freedom. Under certain mechanical conditions, rigor cross-bridges can reverse their angle of attachment to actin. There seems to be considerable flexibility around the head-tail junction *in vitro*. The small, unpredictable thixotropic changes that have been observed in the present work may well be the result of a change in the orientation of the cross-bridges. Recent observations of the movement of

actin filaments on substrate-bound myosin fragments suggest that when all heavy meromyosin are tethered in the same orientation, they are able to move actin filaments in either direction (Reedy *et al* 1989). The two other main contenders for producing thixotropic changes are hydrogen bonding between the actin and myosin filaments, and folding of the actin filament. The contributions from these forms of bonding may not provide a repeatable level of stiffening and loosening. Within non-rigor muscles the cross-bridges may mask the variations from the contributions of these other forms of bonding. Thus the thixotropic effect occurs reliably, possibly as a result of pre-determined rates of cross-bridge turnover in non-rigor muscles.

In rigor the majority of the cross-bridges can be assumed to be attached firmly to the actin filament; as thixotropy is reduced and unreliable, it suggests that it is a property of the cross-bridges. Thixotropy seems to require the cross-bridges to be able to attach and detach in a normal cross-bridge cycle. Once the normal cycling of the bridges stops (as in rigor), then thixotropy also ceases to be observable. Variable thixotropy in IAA rigor muscles could be the result of a few cross-bridges still free, and of other forms of bonding that become observable. In conclusion it seems to be the cross-bridges that are the main underlying mechanism for thixotropic changes in the living muscle.

CHAPTER SIX

PLASTICITY: THE EFFECT OF STRETCH DURATION ON RELAXED FROG AND MOUSE MUSCLE

INTRODUCTION

Relaxed muscle has malleable characteristics, responding differently to stretches of varying durations. Preliminary experiments used triangular stretches where the velocity decreases with increasing stretch durations. Later experiments used rectangular stretches which reduced this variation in velocity as the length change was almost instantaneous.

FORCE / VELOCITY

Jewell and Wilkie (1958) found that active muscle shows a relationship between force and velocity. The velocity of shortening at any one moment is a function of the force at that time.

Joyce and Rack (1969) showed that the force / velocity relationship at any length and stimulus rate clearly depends on the movement that preceded the measurement. Blix (1893) demonstrated that muscle did not obey Hooke's law, and that its elastic properties were markedly time dependent, leading to a hysteresis and after extension. He attributed this behaviour to some internal viscosity, and experimented with visco-elastic model systems. The time dependent property has been explained two ways:

1: Resting muscle contains a number of linearly damped elements with different time constants (Buchthal and Kaiser 1951).

2: That there is a single damped element, which has highly non linear properties.

MUSCLE PLASTICITY AND CREEP

The stiffness of relaxed muscle with stretches of

physiological size and of varying durations has apparently not been investigated before.

Hill (1968) showed that the relaxed muscle's tension response is not linearly related to any imposed change in length. Earlier workers had described similar results but had not commented on them in any detail (Blix 1893, Denny-Brown 1929, and Buchthal and Kaiser 1951). Hill (1968) showed a clear yield point, before which the muscle was stiffer and after which the muscle became markedly less stiff. He called this property the SREC, now linked to the thixotropic properties reported in the earlier Chapters. To see the SREC clearly required the tonicity of the muscle to be raised by bathing the muscles in hypertonic solutions.

The SREC has been attributed to the existence of a few cross-bridges in relaxed muscle which have formed bonds with the actin filament, which abruptly yield after a certain amount of movement. The range of movement that these locked-on cross-bridges work over is small, only amounting to about 0.2 % of the muscle length (Buchthal and Kaiser 1951, Denny-Brown 1929, Hill 1968 and Huxley 1960). Stiffness values which are high may reflect distortion of these attached cross-bridges, reaching a maximum when stretched by 10 - 20 nm/half sarcomere, suggesting that the cross-bridges remain attached to the actin filaments over this distance. Flitney and Hirst (1978) in active frog muscle measured the range of movement that cross-bridges remained attached to actin during a stretch as being between 10 - 12 nm.

If the attached cross-bridges are displaced beyond 10 - 20 nm/half sarcomere they begin to slip along the actin filament detaching so that the number of cross-bridges is

reduced, for a period before they reform further along the actin filament (Sugi and Tsuchiya 1988).

Rack and Westbury (1974) found the SRS to be independent of the velocity of the stretch, but noticed that it lasted longer for the faster movements than for a slow one. The muscle's overall stiffness was dependent on the speed of the movement. For the fast movements the cross-bridges remained attached, while for the slower movements the cross-bridges were able to break down and reform further along the actin filament at least once during the lengthening.

Creep is the deformation of a muscle at a constant stress rate over a period of time. Buchthal and Kaiser (1951), described creep as a:-

'smooth variation of length obtained
as a function of time.'

These authors showed that there was a plateau in the creep appearing at between 2 - 4 s, and that the elongation was approximately proportional to the logarithm of time. The extent of the creep was also practically independent of temperature.

The investigation of creep by muscle has been neglected. Recently there has been a study of creep in actomyosin extracts but not in whole muscle (MacGinnis 1989).

RECOVERY RATES FOR OTHER MUSCLE PROPERTIES

The recovery of muscle stiffness after stirring has been related to the re-formation of the cross-bridges, broken by the movement. Stiffness recovers with an initial fast phase followed by a slower recovery 2 - 5 s after the stirring has ceased (Lakie and Robson 1988b). Single fibres also gave the same two phase response, with a more pronounced change point

at around 5 s (Lakie and Robson 1990). Following a burst of isometric stimulation stiffness is reduced, returning to the initial stiffness value with a time scale that is similar to that after a series of the passive stretches (Lakie and Robson 1988d).

Lannergren's (1971), observations on the recovery of tension after a movement in single fibres of the iliofibularis also revealed an initial fast phase and then, after about 5 s, a slower recovery to the starting values.

Maughan and Berman (1984) found that for the recovery of force after a rapid release, the time constants remained remarkably similar no matter what the release amplitude had been. They fitted a double exponential curve to the data and found the time constants to be 0.16 s and 2 - 3 s.

Studies on creep in actomyosin extracts have reported a recovery that appears to have two distinct phases the first phase lasting 3 - 4 s, and the second lasting minutes (MacGinnis 1989).

CROSS-BRIDGE MECHANICS

The cross-bridges in relaxed muscle after movement will be in a state of constant attachment and detachment proceeding at set rate constants, with the equilibrium of the reaction favouring the attachment of cross-bridges thus increasing the muscle's stiffness over time.

Maughan and Berman (1984) claimed that the 2 - 5 s change point was the result of some cross-bridge property. Using Dextran T500 which reduces the fibre width they found that if the time course reflected the effects of the 'hindered' cross-bridges, one or both of the time constants would be expected to depend upon the fibre width, but there was no

such relation between the time constants and the fibre widths that they used.

MAMMALIAN MUSCLE

Mammalian muscle is structurally made up from the same family of proteins as the amphibian muscle. Unlike the frog, where each muscle is generally made up of either fast or slow fibres, mammalian muscle exists as a mixture of fibre types or, as almost exclusively fast twitch or slow fibre muscles. The soleus muscle is a muscle that in the mouse is made up almost exclusively of slow fibres. The basic underlying cross-bridge properties in the frog are the same for mammalian muscle.

The aim of this Chapter is to investigate if there is a time dependent property and possible temperature dependence of the cross-bridges in frog sartorius muscle. The plastic properties of frog muscle will be compared to mammalian muscle (mouse soleus).

METHOD

MATERIAL AND APPARATUS

The experiments were conducted on frogs in December, with the muscles mounted in apparatus A (Chapter 1, FIGURE 1a), surrounded by isotonic Ringer at temperatures of 3 and 17^{o}C.

PROTOCOL

The applied force changes were either in the form of a triangle: the force rising linearly to a preset maximum, then falling linearly to the resting level; or a rectangle: where the force rose immediately to the preset maximum and remained there for the duration of the stretch before returning instantaneously to the resting level. For the triangular stretches the time to reach peak force will be referred to as the rise time (half the duration of the full stretch). The equivalent times for the rectangles will refer to the full stretch duration. The rise times and durations used were 0.15, 0.225, 0.5, 1, 2, 5, 20, and 40 s. Stretch durations were randomized in the trials, and one stretch duration was generated every 3 mins. The peak forces for these stretches were 0.2, 0.25 and 0.3 g, (with a few trials of 0.4 g, included to compare with the mammalian muscle later). FIGURE 6a shows a schematic representation of the protocols used.

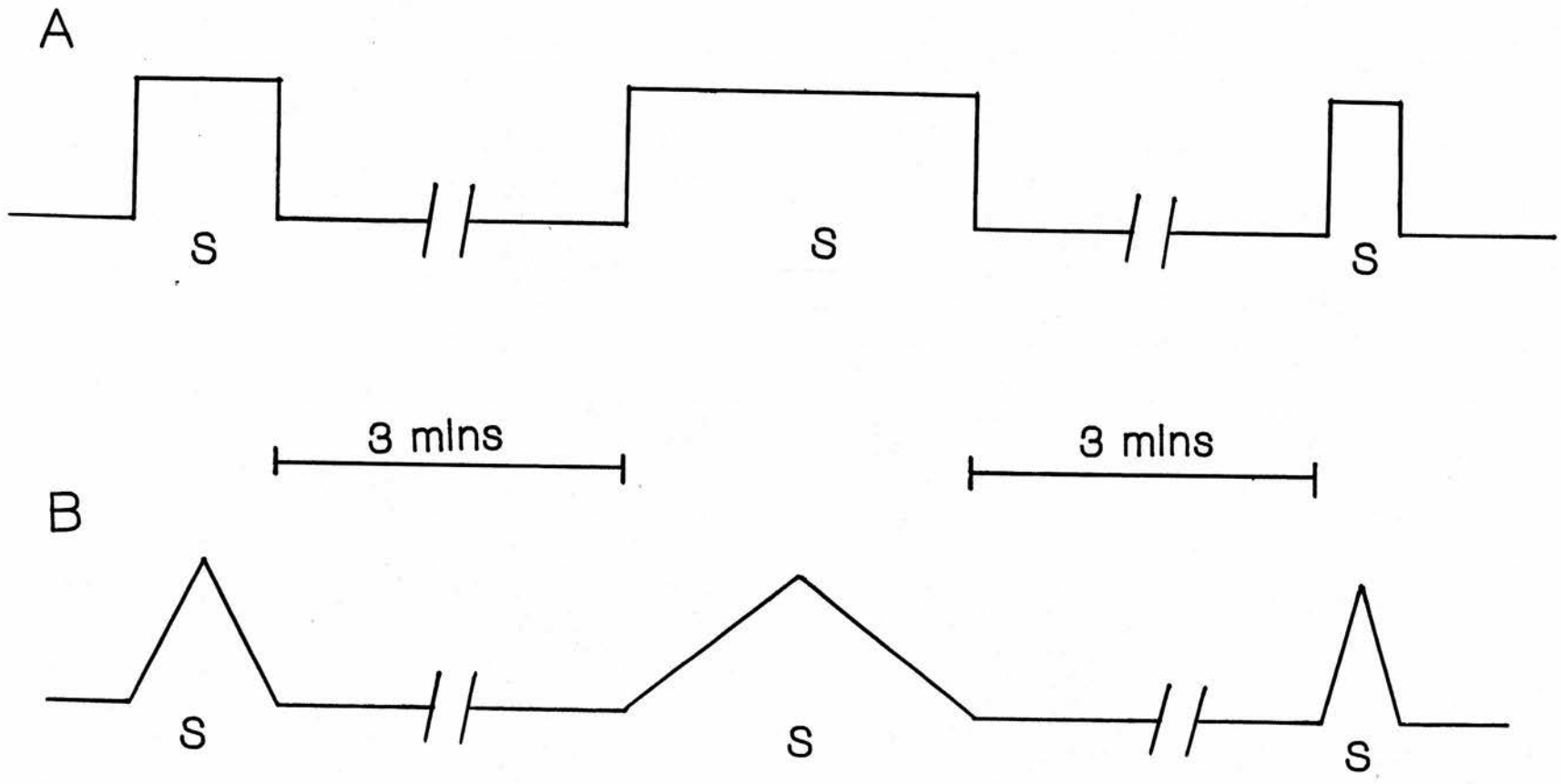
-TRIANGULAR STRETCHES-

One stretch was applied to the relaxed muscle. The rise time was altered randomly from trial to trial. The 0.2 - 0.3 g peak forces were used and only 3^{o}C tested.

-RECTANGULAR STRETCHES-

The problem with triangular stretches is that the

FIGURE 6a: A schematic diagram of the two protocols used to test the effect of varying the stretch duration (not to scale). (A) rectangular test stretches and (B) triangular test stretches.


A
S
S
S
3 mins
3 mins
B
S
S
S

velocity changes with the duration of the stretch. This could be obviated by switching to rectangular stretches which allow an almost instantaneous change in the force exerted on the muscle. A comparison of the two types of stretches was conducted using one muscle with several trials with each type of stretch, and force, so that an average response to the two stretch protocols was obtained. The triangular and rectangular trials were interspersed with each other so that any effect of a change in the muscle's properties as a result of each stretch type over the experimental period was reduced (triangular 0.2 g, rectangular 0.2 g and so on).

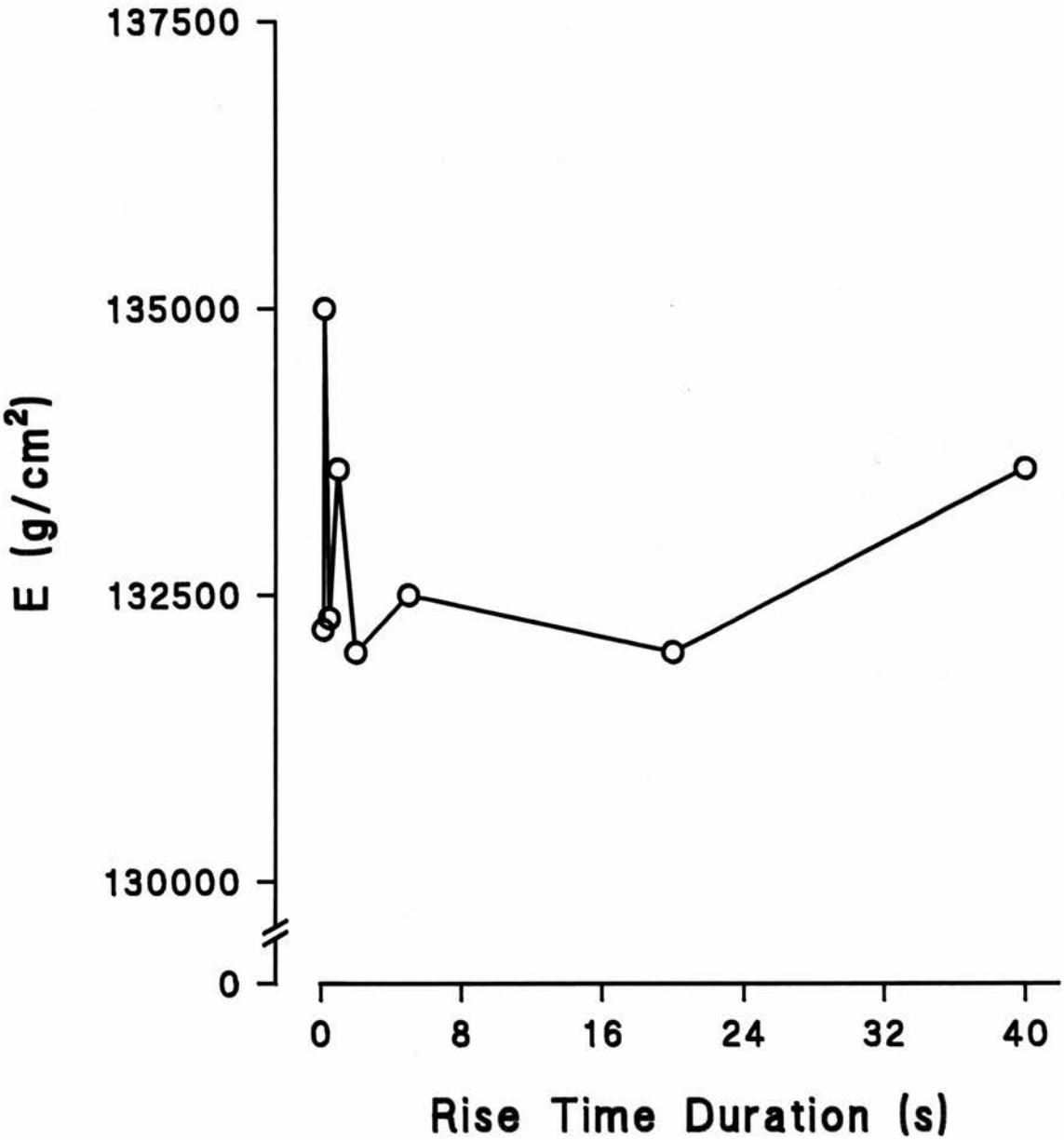
Individual stretches were used in preference to a single long stretch, as more independent measurements could be obtained, and direct comparisons to the triangular stretches made.

A number of studies were undertaken with the muscle receiving either a single 40 s stretch or the individual stretches. Readings were taken along the single 40 s stretch at the same times used for each of the individual stretches. Readings were taken just before the force was removed.

MAMMALIAN SOLEUS MUSCLE

The mice used in this part of the study had previously been killed by cervical dislocation, before dissection of the soleus. The mice (all female, of CD-1 strain) were inbred in the University of St Andrews animal house. Mice were housed six to a cage and fed and watered *ad libitum*. The soleus muscles were quickly dissected out and tied at both tendons by small pieces of stainless steel wire. They were mounted into the apparatus at L_o and surrounded by an aerated isotonic Kreb's solution (composition and aeration TABLE 6a).

FIGURE 6b: The stiffness values for an elastic band to stretches of varying durations. Temperature 3°C, force 0.25 g.


137500
135000
132500
130000
0
E (g/cm²)
0
8
16
24
32
40
Rise Time Duration (s)

The temperature of the bath was maintained at 37°C thermostatically.

PROTOCOL

The protocol for the mammalian muscle was the same as reported above using rectangular stretches, but the force was 0.4 g. There was one stretch duration per trial, and this duration was randomized from trial to trial.

CONTROLS

The sartorius / soleus muscle was replaced by an elastic band of approximately the same dimensions as the muscles. The protocol employing the rectangular stretches was used, but the force was increased to 1.3 g. Both temperatures were investigated.

Results from the elastic band are shown in FIGURE 6b. There is no significant difference between the stiffness values for the 8 stretch durations used. Values of stiffness obtained with the elastic band are significantly greater than any obtained from the muscles. The elastic band is about 100 times stiffer than the average frog sartorius muscle (with the fastest stretch). It can be concluded that any differences with the muscle in place are a result of some muscle property.

TABLE 6a: Composition of the Krebs Hienseleit solution that was used to bath the mammalian muscles (mouse soleus).

Salt	mM
NaCl	118.4
$NaHCO_3$	24.9
KCl	4.7
KH_2PO_4	2.2
$MgSO_4.7H_2O$	1.2
$CaCl_2$	2.5
Glucose	10.0

The solution was buffered by Tris base and aerated with 5% CO_2 and 95% O_2

RESULTS

STRETCH DURATION EFFECTS

-TRIANGULAR STRETCHES-

FIGURES 6c and 6d show the effect of varying the rise time on the displacement and stiffness. FIGURE 6c shows the displacements produced with the 0.2 and 0.3 g peak force stretches. Naturally the larger forces produced bigger displacements from the muscles. The muscle behaves in a plastic manner, giving more to a stretch with a slower rise time. FIGURES 6c and 6d show that there is an inflection in the curves appearing at the same time for both the forces plotted of between 2 - 5 s. This inflection is independent of the displacement as it appears at 0.5 and 1.35 mm for the 0.2 and 0.3 g peak force stretches respectively. The stiffness values decreased in a steep linear fashion up to about 2 s. After this stiffness does not decrease to the same extent for the longer stretches (FIGURE 6d).

-TRIANGULAR 'v' RECTANGULAR STRETCHES-

FIGURE 6e shows that calculated stiffness values are less for rectangular stretches than for triangular stretches of the same peak force, with the stiffness for the rectangular stretches on average being 20 % smaller than the triangular stretches. However the inflection for both curves appears between 2 - 5 s and the shape of the response as the stretch durations increased was qualitatively the same for the two stretch types. The results for the other two forces (not shown here) showed a similar disparity in calculated values of stiffness.

FIGURE 6c: Typical displacements obtained from a muscle tested with triangular stretches of varying rise times.

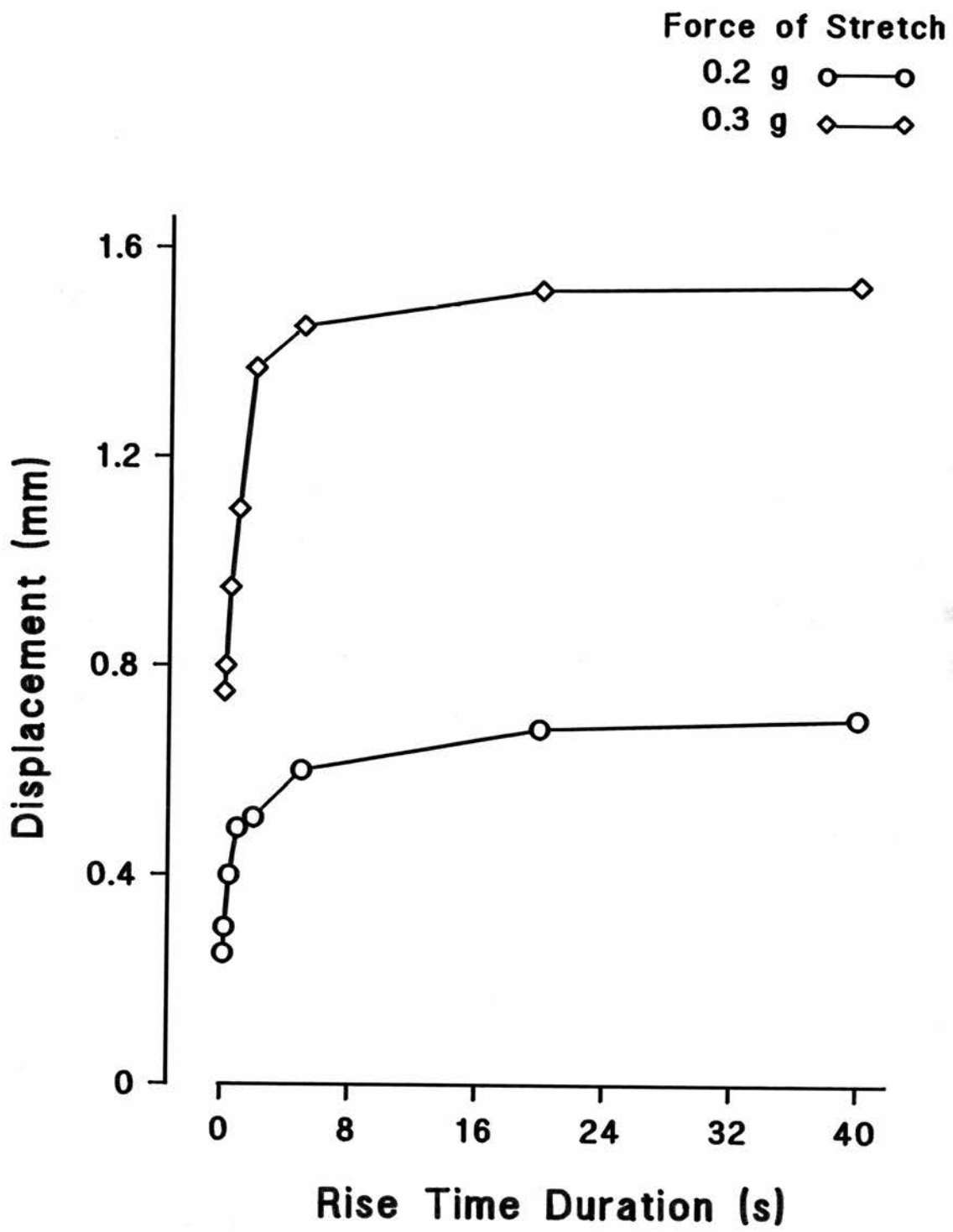

Force of Stretch
0.2 g
0.3 g
1.6
1.2
0.8
0.4
0
Displacement (mm)
0
8
16
24
32
40
Rise Time Duration (s)

FIGURE 6d: The mean (SD) stiffness values at two force levels (triangular stretches), on the discontinuity in the muscle's response. temperature 3°C, n=4.

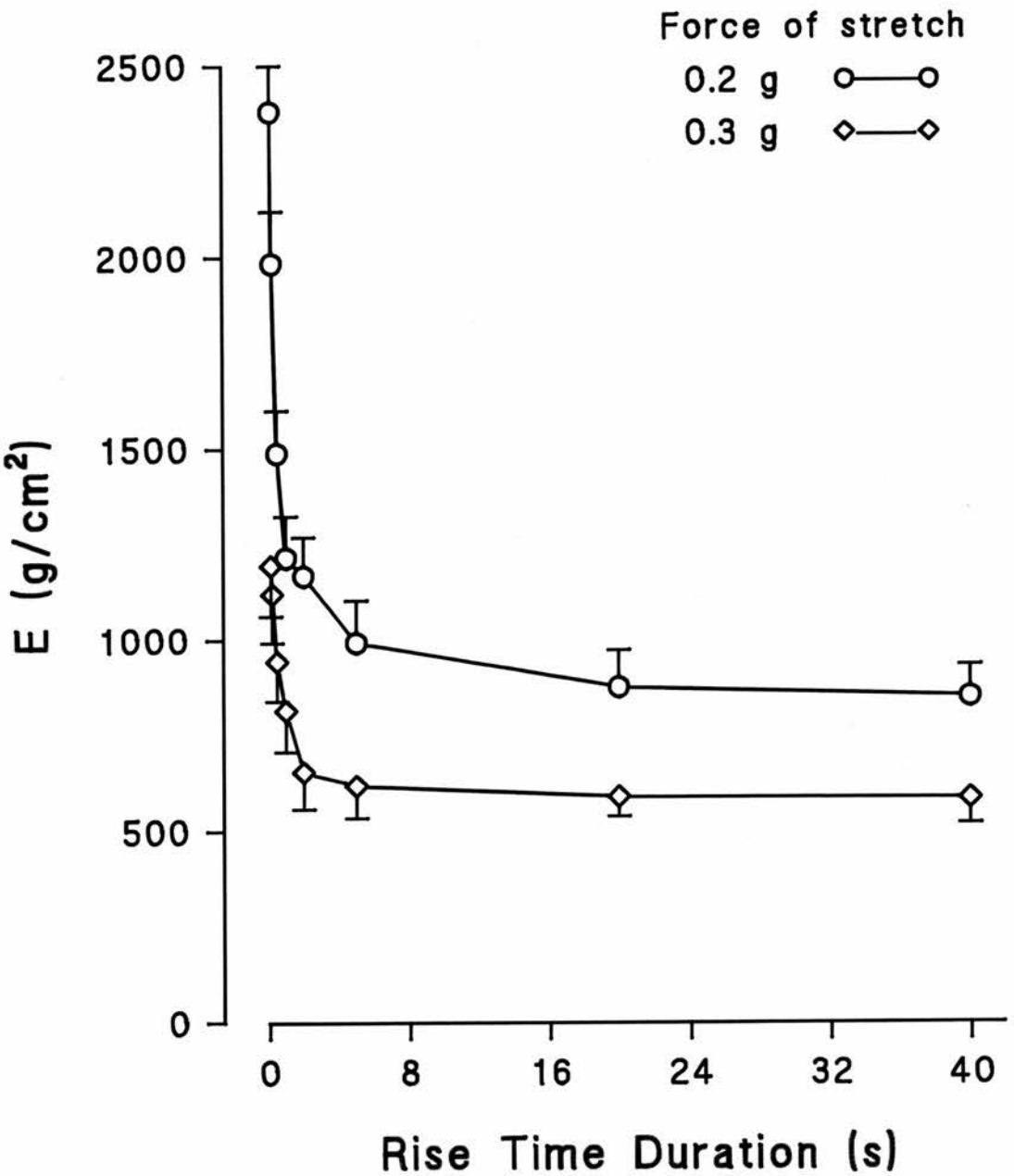

Force of stretch
0.2 g
0.3 g
2500
2000
1500
1000
500
0
E (g/cm²)
0
8
16
24
32
40
Rise Time Duration (s)

FIGURE 6e: The effect of changing the stretch type (triangular/rectangular) on the muscle's stiffness values. Temperature 3°C, force 0.25 g.

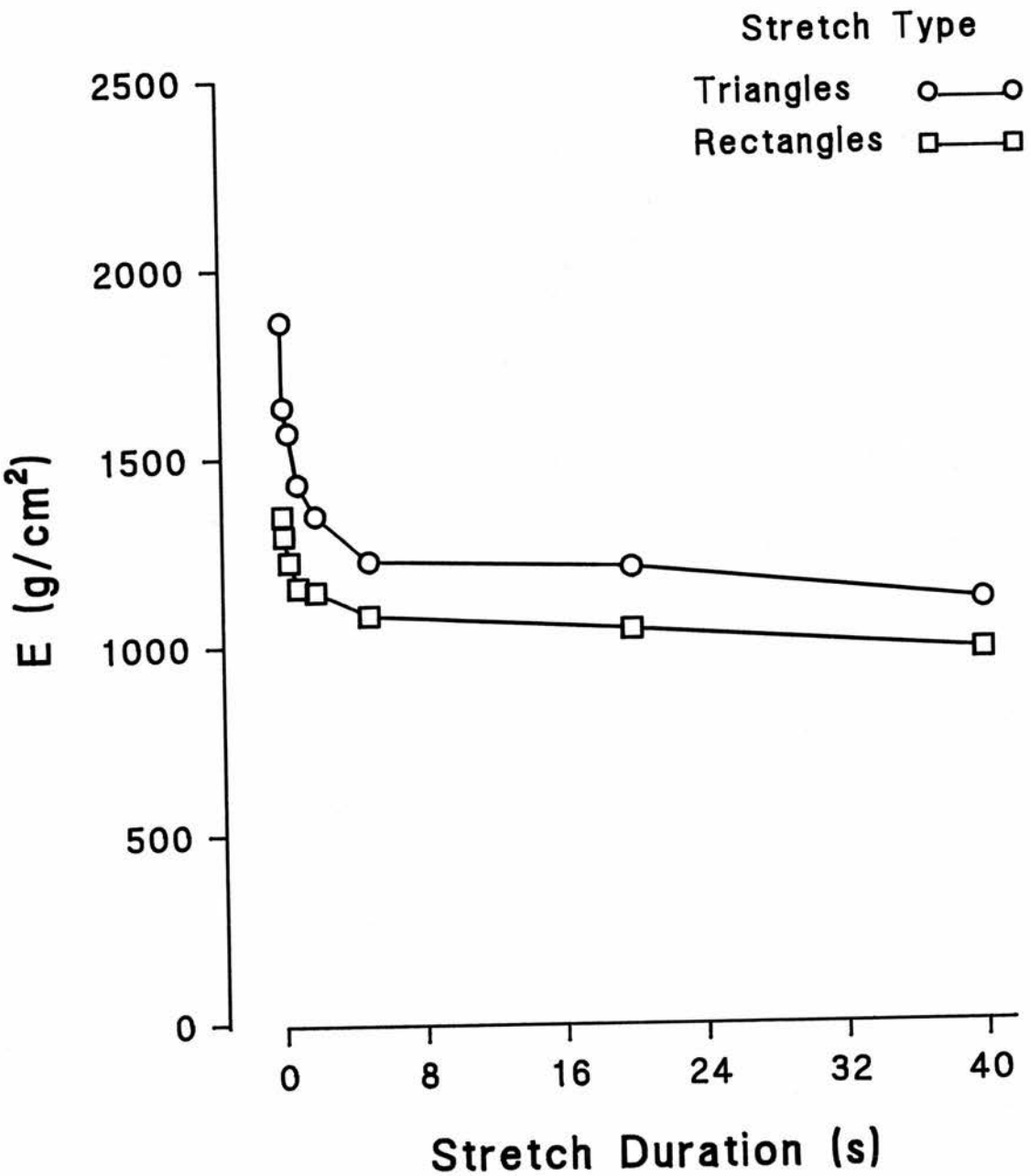

Stretch Type
Triangles
Rectangles
2500
2000
1500
1000
500
0
E (g/cm²)
0
8
16
24
32
40
Stretch Duration (s)

FIGURE 6f: The effect of using one long stretch or separate rectangular stretches on the stiffness values recorded for one muscle. Temperature 3°C, force 0.25 g only shown.

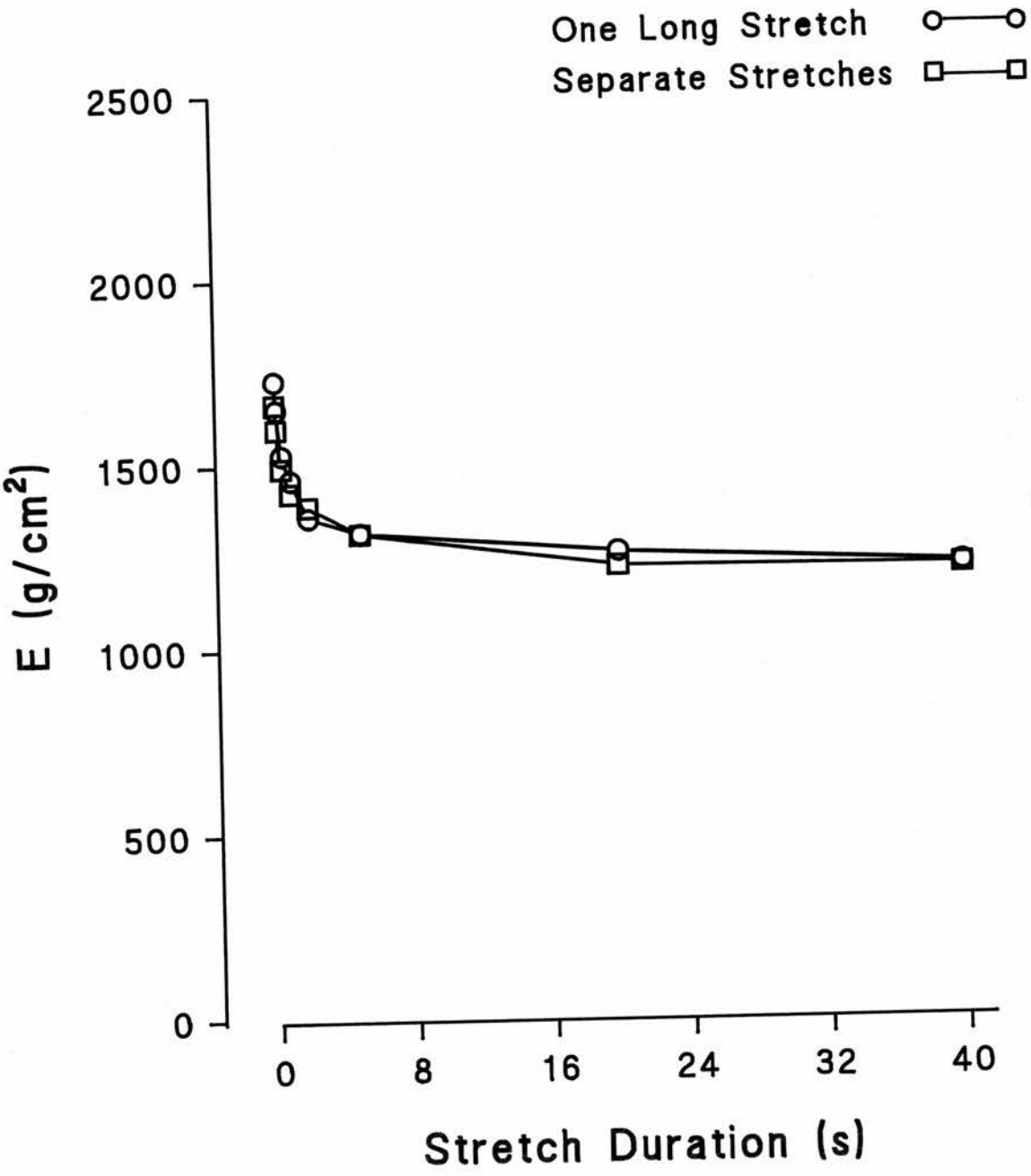

One Long Stretch
Separate Stretches
2500
2000
1500
1000
500
0
E (g/cm²)
0
8
16
24
32
40
Stretch Duration (s)

FIGURE 6g: The effect on the appearance of the discontinuity and the stiffness values of raising the temperature from 3 - 17°C using frog sartorius muscles and rectangular stretches of varying durations (means and SD). Force 0.25 g only shown.

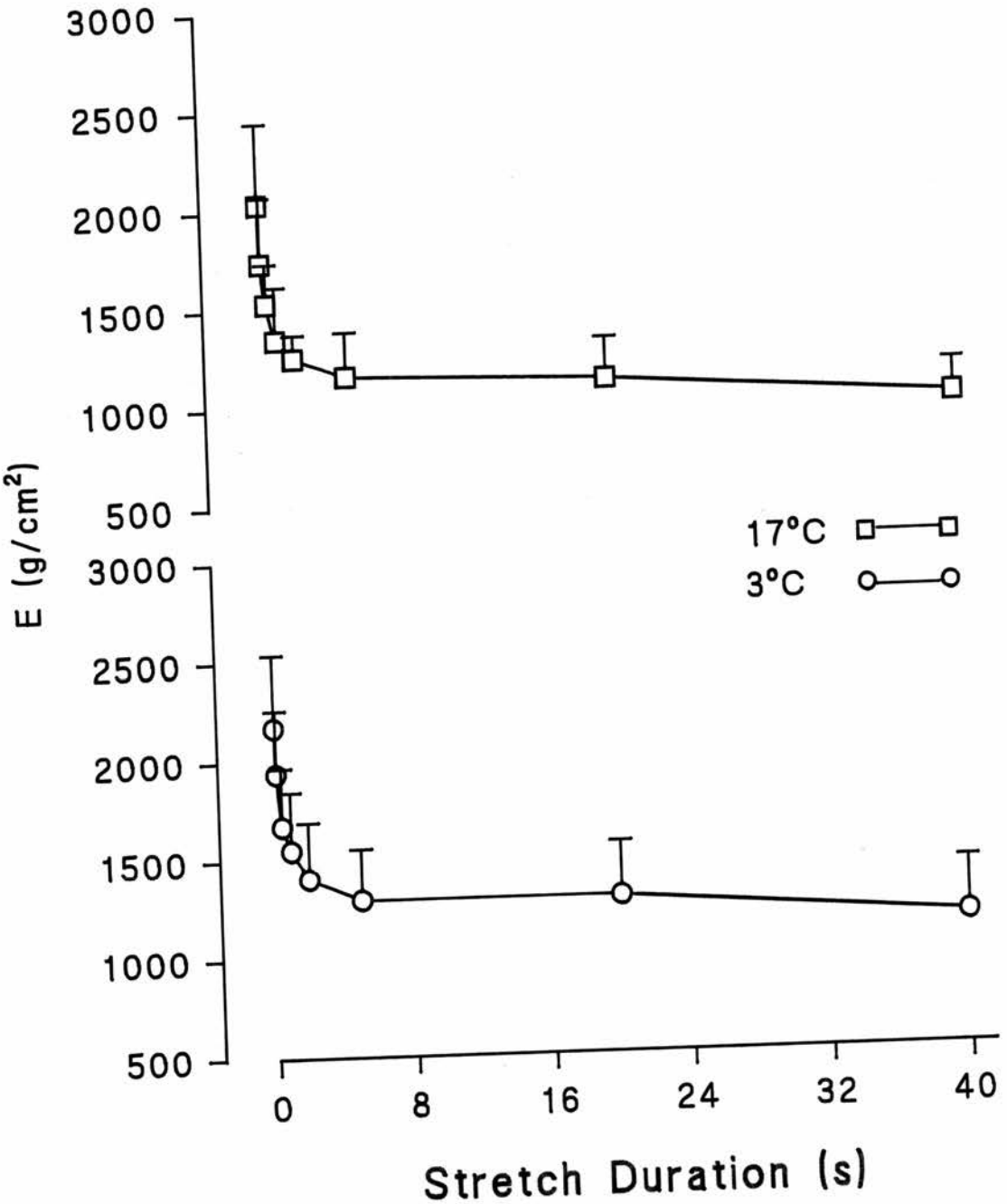
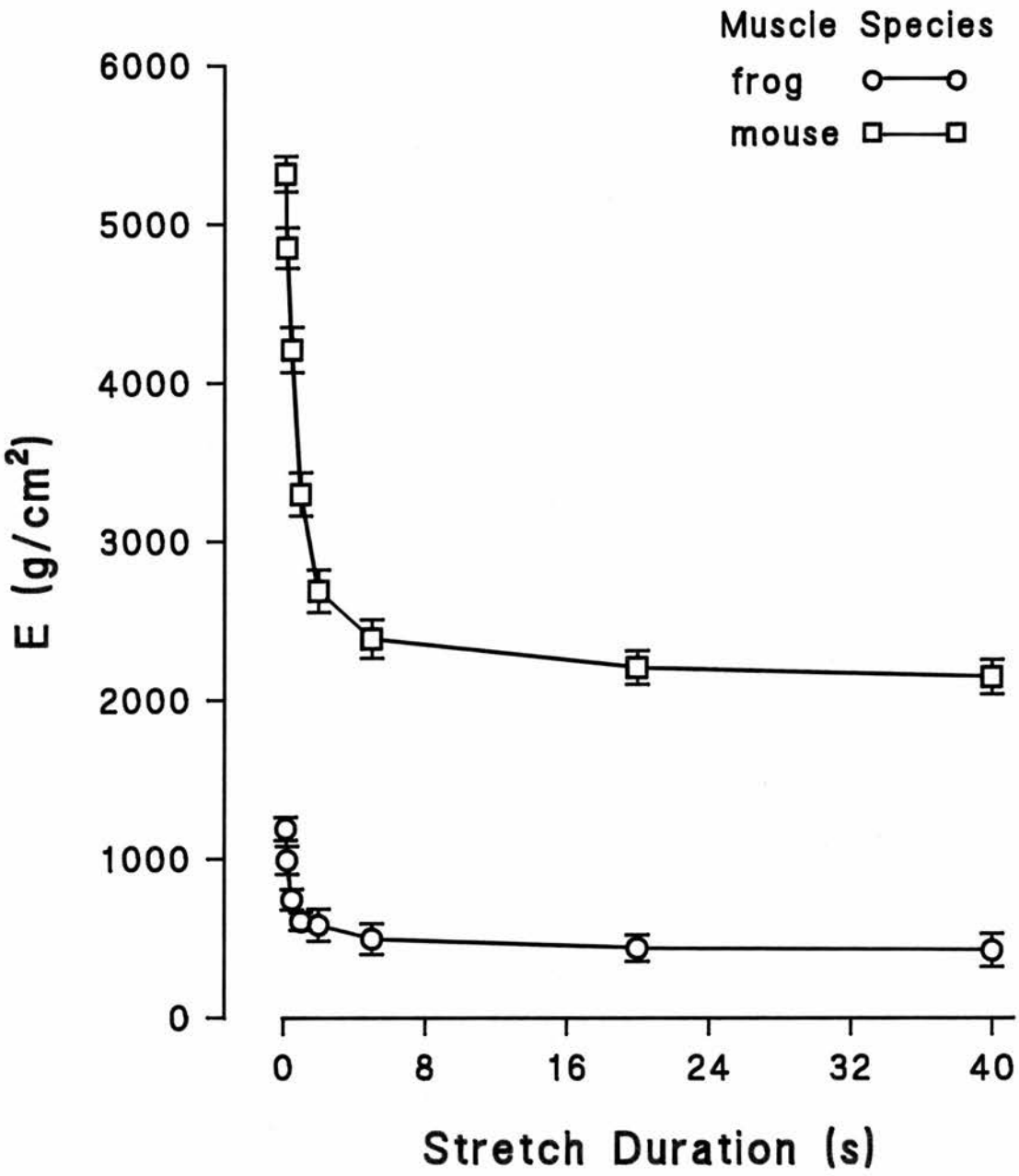

3000
2500
2000
1500
1000
500
E (g/cm²)
17°C
3°C
0
8
16
24
32
40
Stretch Duration (s)

FIGURE 6h: The stiffness values (mean and SD) for mouse soleus muscle (37°C), and frog sartorius muscle (3°C) for rectangular stretches, force 0.4 g, mouse soleus n=6, frog sartorius n=6.


Muscle Species
frog
mouse
6000
5000
4000
3000
2000
1000
0
E (g/cm²)
0
8
16
24
32
40
Stretch Duration (s)

-ONE 40 s RECTANGULAR STRETCH 'v' SEPARATE RECTANGULAR STRETCHES-

FIGURE 6f shows that for the two protocols the yield point appears at between 2 - 5 s and the two sets of readings are almost superimposable on each other. The results for the other two peak forces and for the higher temperature (17^{o}C) show the same pattern of response.

-RECTANGULAR STRETCHES and TEMPERATURE-

FIGURE 6g show the mean and SD of calculated stiffness with individual rectangular stretches (0.25 g only). The yield point of 2 - 5 s can clearly be seen at both temperatures. The rate of stiffness decline over the first five stretches (up to the 2 s stretch) was around 40 % for both temperatures, and over the full 40 s the decline was about 50 %.

MAMMALIAN MUSCLE

FIGURE 6h shows that the soleus muscle is stiffer than the sartorius muscle, when measured with the same force (0.4 g), but the shape of this muscle's response with increasing stretch duration is very much the same for the frog sartorius muscle. The decrease in stiffness over the first 5 stretches (up to the 2 s stretch) was 37 % and over the full 40 s the decrease was 60 %.

DISCUSSION

Blix (1893) demonstrated that relaxed muscle's elastic properties were markedly time dependent. He attributed this to an internal viscosity. Results obtained from both the triangular and rectangular stretches seem to show the same phenomenon, a discontinuity in the response of the muscles to stretches of a duration longer than 2 - 5 s. There was an initial fast decline in the stiffness of the muscles to increasing stretch durations from 0.15 s to 2 s. Durations longer than this produced a slower decrease in stiffness. The stiffness of the muscle decreased on average by 40 % for the fast stretches, with only a further 10 % reduction up to 40 s.

Plasticity of muscle has been studied almost exclusively in active muscle. Active muscle has very different properties compared to relaxed muscle, so comparisons of this work to results presented here are naturally tentative. Previous studies have used stretches with velocities that were much faster than the stretch velocities used in this section. Another aspect of the previous work was that it measured tension changes of the muscle in response to fast length changes, rather than looking directly at yielding with applied tension. Sugi (1972) stretched frog fast fibres during tetanic stimulation to full isometric tension with the stretches at velocities ranging between 0.1 to 15 cm/s. Tension rose abruptly at first, and then started to fall quickly before the end of the stretch, taken to indicate 'slip' by the contractile component. The extent of the fall in tension was more marked as the stretch velocity was increased.

Sugi (1972) did conduct some experiments on resting muscle fibres. Unlike the active fibres the tension always continued to rise irrespective of the stretch velocity, only decreasing after the stretch was complete. But no comment was made as to the significance of these results. Sugi's explanation of the results obtained from active muscle was that the force (and therefore presumably the stiffness) exerted by a distorted cross-bridge is likely to be much larger than that exerted by a cross-bridge in its normal position.

Flitney and Hirst (1978) found that the amount of stretching required to induce sarcomere "give" in active frog muscle is an index of the range of movement over which a cross-bridge can remain attached to actin during a stretch (about 1.2 % of the sarcomere length). At the end of this displacement the heads of the myosin would have been rotated backwards. For the faster duration stretches the cross-bridges may not detach during the movement, remaining attached to the actin filament, resulting in them becoming distorted, with the elastic portion of the myosin head getting twisted from its true alignment. Each cross-bridge is made up of two myosin heads forming the attachment area of the cross-bridge. These heads are attached to the myosin backbone by an elastic portion just behind the head area. It is this portion that allows the head to appear in a number of alignments (Eisenberg and Greene 1980, Tregear and Marston 1979 and Reedy *et al* 1989).

Relaxed muscle with its small proportion of attached cross-bridges, which are constantly breaking and reforming at set rate constants, will react differently to fast and slow

stretches. With the faster stretches the cross-bridges do not have enough time to respond to the stretch. The cross-bridges will remain attached to the thin filament, the myosin heads becoming distorted, with the result that the stiffness measured for these stretches will be high. As the stretch duration increases the rate constant for the cross-bridge cycling in relaxed muscle will mean that a larger proportion of the cross-bridges will be able to respond to the stretch, by being able to let go and re-attach further along the thin filament. This will result in a larger displacement and thus a lower stiffness value.

For stretches of less than about 2 s duration, extension is approximately proportional to the duration of the stretch, proceeding rapidly. With longer stretches the relationship is still approximately linear, but extension proceeds much more slowly. An analogy may be useful. Consider the escapement on a weight driven clock. The weight descends at a constant rate controlled by the making and breaking of the mechanical escapement. The rate of descent is not affected much by the size of the weight. In relaxed muscle there appears to be two such escapement mechanisms, working at different rates. The first one is rapid but works for only 2 s or so and the second is slower but appears to work for at least 40 s. It is possible that at least one escapement is the action of making and breaking of actomyosin bonds.

Increasing the force had no effect on the timing of the inflection in the stiffness curves. The time at which this happens is the result of the rate constants for the formation and break down of the cross-bridges.

It is interesting that mytilus byssus retractor and

octopus mantle muscle strips (Dr M Lakie, personal communication) also respond to stretches of increasing duration with the same discontinuity in yield seen with the frog sartorius muscles. Therefore the same underlying principle must be at work.

Thus the cross-bridges in relaxed muscle seem to have the same rate constants for cycling in a variety of muscle types. This is probably different to the rates of cross-bridge cycling during activation (Proske and Rack 1976, and Gregory *et al* 1978).

The point of the inflection was not related to the SREC (FIGURE 6c) which in these muscles was about 0.049 mm. Rack and Westbury (1974) found the SRS was independent of velocity of stretch in tetanized cat muscle, but noticed that the SRS lasted for more of a fast movement than for a slow one.

Huxley (1971) proposed that there is an extension of the myofilaments without the cross-bridges themselves breaking, as seen by extension of the A-band when a muscle is extremely stretched during rigor or tetanus. The extension of muscle structures can not be disregarded, folding of the actin filaments does occur and these attachments break after a critical elongation, but the present results show no critical length responsible for the discontinuity. Also with relaxed muscle it is unlikely that the force is ever great enough to produce such elongation (Helber 1980, Gillis and O'Brien 1975 and O'Brien *et al* 1975).

The cross-bridges of active muscle may behave as though they were 'locked on' for small displacements (fast stretches) but would be broken rapidly when the stress exceeds a critical limit with slower duration stretches, but

which is velocity independent (Rack and Westbury 1974). In a stretch of tetanized frog muscle the maximum stiffness value reached during the stretch was also found by Tsuchiya and Sugi (1986), to be independent of the stretch velocity. Sugi and Tsuchiya (1988) found that the initial stiffness values which are high may reflect the synchronized distortion of attached cross-bridges, before cross-bridge slippage. Podolsky, Nolan and Zaveler (1969) have also reported that the turnover of cross-bridges for a given contraction distance was independent of the speed of the motion.

The recovery of stiffness following passive stretches also shows a discontinuity in the results at around the 2 - 5 s point, with an initial quick recovery of the stiffness followed by the slower return. This recovery has been linked to the re-formation of cross-bridges that have been broken by the perturbations imposed on the muscle. In relaxed muscle there are some cross-bridges that are attached and confer on the muscle a resting level of stiffness (Brenner *et al* 1982 and Schoenberg *et al* 1984). The same time scale of events has been described by Lannergren (1971) for tension return after a movement. Maughan and Berman (1984) looking at force redevelopment following a rapid release in a fibre treated with Dextran T500 to reduce the fibre width, showed that the time constants appear to be relatively insensitive to the release amplitude. The yield point of the muscles was around the 2 - 3 s point for the transition from fast to slow recovery of force redevelopment. Godt and Maughan (1981), also found that there was a change point appearing at approximately 3 s for force development in the rabbit soleus fibres at *in situ* widths.

Actomyosin extracts have shown that the creep over a 1 min period increases rapidly at first and then levels off, rising at slower rate (MacGinnis 1989). The recovery appears to have two distinct phases the first phase lasting 3 - 4 s, and the second lasting minutes. Increasing the stress applied to the muscle did not alter these times.

Early work by Buchthal and Kaiser (1951) demonstrated that creep was a smooth variation of length as a function of the time, with what appeared to be a discontinuity, at around the 2 - 4 s period. They however reported that even after 15 mins, elongation continued to be approximately proportional to the logarithm of time. They also reported that a change in temperature made no difference to the extent of the creep as has been confirmed by these present experiments where raising the temperature to 17^{o}C had no affect on the appearance of the 2 - 5 s discontinuity.

This phenomenon has benefits for the animal, in that the muscles will resist fast movements, but yield to long lasting forces, also after movement the muscle will remain less stiff for a while, until the cross-bridges can re-form. This implies that a warm up routine should be included before any form of physical activity, with the routine made up of slow / held stretches as these have a greater effect on loosening the muscles than a fast movement. A loosened state would be advantageous for a coming bout of exercise, as the probability of damage to the muscle or other structures would be reduced.

CHAPTER SEVEN

SEASONAL CHANGES IN THE STIFFNESS OF RELAXED FROG SARTORIUS MUSCLE

SEASONAL VARIATIONS IN THE FROG

The frog is a cold blooded animal, unable to maintain itself in the same condition in winter and summer, unlike warm blooded animals. The activity of the frog is altered markedly by the temperature of the environment, to which it must adapt itself. There are periodic changes in the frog metabolism, due to the ripening of the reproductive cells, which especially in females make extensive demands on the stores of nutrients in the body (review Holmes 1906).

In autumn the frog's body has become a rich store of nutrients, in the form of fat and glycogen, accumulated throughout the summer months while food was plentiful. During the winter months the frogs hibernate and the stores of nutrients maintain the temperature of the body, furnishing it with the energy necessary to carry on the various activities of the organs, and also contributing to the growth of the reproductive cells.

Some of the glycogen and other nutrients are stored in the muscles, which during the winter months decrease in weight in relation to the rest of the body. Gaule (1900, in Holmes 1906), found that during the summer months the gastrocnemius of female frogs weighed on average 33 mg for every gram of body weight, while in winter this had fallen to 26 mg. In males the weight of the muscles does not vary to such an extent.

Seasonal changes have also been observed in the liver. Its large stores of glycogen entirely disappear by the end of the breeding season. The weight of the organ mirrors these changes in the glycogen, with a minimum during and after

spawning, increasing over the summer months, reaching a maximum in late autumn (Smith 1950).

The blood sugar levels follow an annual cycle with two highs during the sexually active periods, and a low in late summer (Smith 1950). Smith (1954), reported that there was a seasonal hyperglycaemic response which was paralleled by the known thyroid activity. The hyperglycaemia in response to handling, that in spring, summer and autumn was normally absent or reduced, could be induced by prior treatment with thyroxine, suggesting that thyroid hormone inhibits the destruction of circulating adrenergic compounds.

The adrenal gland of *R. Temporaria* is seasonally affected (Van Kemenade and Van Dongen 1965). In Spring and Summer there are compact cells which are more numerous than at other times of the year. There are also cavities which become numerous in April and August - September; the appearance of these cavities reflects the active state of the gland.

Common frogs eat nothing or very little during the winter and spawning, which in northern Finland can last for as long as 7 - 8 months, with spawning ending around the middle of May, leaving only 4 months for the frogs to gather and store nutrients ready for the winter (Pasanen and Koskela 1974).

MUSCLE FIBRE TYPES

Shamarina (1962) described fibres that appeared to have properties intermediate to those of the fast and slow fibres. The validity of these results was questioned by Orkand (1963). The evidence for fast and slow fibres does not preclude sub-types within either group or, an entirely unrecognized group of fibres. Lannergren and Smith (1966) supported the broad division of muscle fibres both on

histochemical and functional grounds. Histochemically and physiologically two major fibre types have been indentified, but fast muscle fibres can further be divided into fast fatigue resistant (Fast Oxidative Glycolytic, FOG or type 2A), and fast fatigue sensitive fibres (Fast Oxidative, FO or type 2B), with the slow muscle fibres designated, type 1 (Burke, Levin, Tsairis and Zajac 1973). The physiological basis for the differences in properties is related to the composition of the myosin heavy chain (MHC).

MYOSIN ISOFORMS

Myosin is comprised of a family of proteins with a similar structure and subunit composition. Adult rat skeletal muscle myosin isoforms have been classified into two major types: first, with a fast rate of ATP hydrolysis, found in fast muscle fibres; second, a low ATP hydrolysis rate, typical of slow muscle. The configuration of the neuromuscular junction is thought to be responsible for the transformation from developing to adult forms (Whalen, Sell, Butler-Browne, Schwartz, Bouveret and Pinset-Harstrom 1981).

Recent work on rat skeletal muscle has now identified a third MHC in adult muscle type 2X fibres. This fibre type has properties intermediate to those of the fast type 2B and slow muscle (Schiaffino, Gorza, Sartore, Saggin, Ausoni, Vianello, Gundersen and Lomo 1989). This MHC isoform can be produced from muscles containing one of the other MHC isoforms by either chemical or electrical interference, indicating that the isoforms are interchangeable. The type 2X fibres can be found in the normal and hyperthyroid diaphragm and the soleus muscle after high frequency chronic stimulation. They display a velocity of shortening intermediate between that of

slow muscles and that of the fast muscles composed of predominantly type 2B (FO).

SEASONAL CHANGES IN THE ACETYLCHOLINE CONTRACTURE

For a general review of the literature on the slow muscles of the frogs and seasonal changes see Peachey (1961). Sommerkamp (1928), using an acetlycholine (ACh) solution to produce contractions from frog muscles, reported the response of the muscles could be divided into three broad classes, dependent on the muscle under investigation:

Class one; gave only a twitch, this includes the sartorius, semimembranosus and the gracilis minor.

Class two; producing a slow, long lasting contraction in which all the fibres seem to participate. This includes the rectus abdominis, flexors and extensors of the shoulder.

Class three; a group of muscles that gave a slow contraction in which only a group of fibres in a localized region participated. This group includes the gastrocnemius, iliofibularis and the semitendinosus.

The distinction between these three classes was sharpest during the summer months. In winter the muscles in class one responded to ACh application like those of the other two classes. This finding was not supported by histological evidence of alterations in the muscle fibre population. Gray (1958) examined eleven sartorius muscles and failed to find any slow fibres.

Wachholder and Nothmann (1931) stressed that the ACh response was most evident in winter, being weak or totally absent in some muscles in summer. The ACh response was stronger in males than females, and seemed to be particularly noticeable in the muscles involved with amplexus. A probable

cause for this seasonal variation in the ACh response, was later proposed by Wachholder and Matthias (1933). Muscles bathed in a summer Ringer's solution (high Ca^{2+} and low K^{+}) gave a weaker response than muscles in winter Ringer (low Ca^{2+} and high K^{+}). Which solution is used affects the response to ACh. A winter frog's response to ACh can be reduced by bathing it in summer Ringer.

ACETYLCHOLINE RECEPTORS

The nicotinic receptor at the neuromuscular junction has recently been found to exist in two forms which are structurally similar but which differ in their kinetic behaviour (Brehm, Kullberg and Moody-Corbett 1984, Dionne 1989, Henderson, Lechleiter and Brehm 1987, Igusa and Kidokoro 1987 and Neher and Sakman 1976).

SLOW STRIATED MUSCLE

Kruger (1929) noticed morphological differences between fast and slow fibres and correlated these with physiological properties. Classified as "Fibrillenstruktur" and "Felderstruktur" fibres, the appearance of felderstruktur fibres confirmed the presence of tonic properties on the muscle (Peachey and Huxley 1960). Tasaki and Mizutani (1944) demonstrated clearly that stimulation of a single motor nerve fibre, isolated from a nerve innervating a toad muscle, resulted in the production of either a slow response or a typical twitch response, but never both. Nerve fibres were classified accordingly by their diameter, a diameter of 4 µm (conduction velocity of 2 - 8 m/s^{-1}), produced a slow response whilst larger nerves (conduction velocity of 8 - 40 m/s^{-1}) always gave a faster response; this was confirmed by

Kuffler and Gerard (1947). Hess (1970) morphologically classified the differences between twitch and slow muscle fibres as:

1) Twitch fibres are the same size, whilst slow fibres are more irregular.

2) Twitch fibres have a more abundant sarcoplasmic reticulum.

3) The T-system in slow fibres is incomplete or absent.

4) Slow fibres do not possess an M-line.

This year long study followed the seasonal changes briefly mentioned in Chapter 3. There are a number of possible explanations for such changes in the muscle's mechanical properties; for example changes in circulating hormone levels, which may affect the contraction of muscles directly, by changing the muscle membrane properties to ions, or indirectly by changing the expression of myofibrillary proteins. The underlying cause will not be investigated here, but each possible explanation will be examined in relation to the results presented in this and the following chapter.

METHOD

MATERIAL AND METHODS

Apparatus A (Chapter 1, FIGURE 1a) was used for these experiments. The frogs were kept in tanks with light and temperature near to natural outdoor conditions. A minimum of four muscles per month were tested, and a maximum of fourteen. No observations were made in June or September.

PROTOCOL

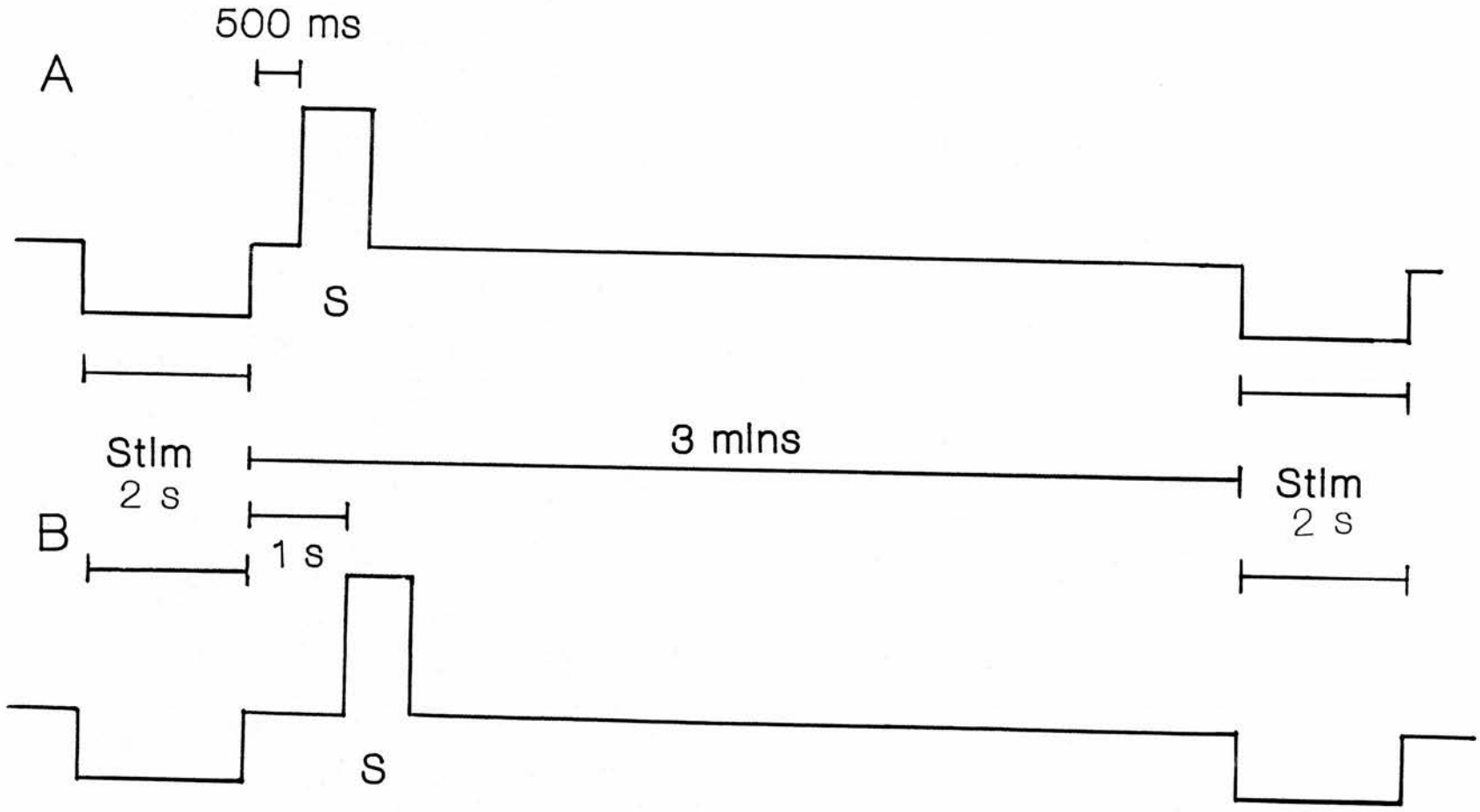
-SEASONAL EFFECTS-

The muscle was equilibrated with isotonic Ringers solution for half an hour following the dissection. A control trial was then begun where the relaxed muscle was subjected to five rectangular stretches of 500 ms duration, 3 mins apart (force 0.3 g). Following the control set of stretches, the experiment was begun.

-ELECTRICAL STIMULATION-

The muscles received a 2 s burst of tetanic stimulation with the muscle under isometric conditions. FIGURE 7a shows the experimental protocol used. Each experimental trial consisted of a single stretch appearing either 500 ms or 1 s after the stimulation had ceased. After 10 trials with prior stimulation, a control trial of five stretches with no stimulation was conducted, before the start of the next stimulation trials. This was done to ensure that the stimulation had no averse affects on the mechanical properties of the muscle, and only if the size of the displacements were within the range of the initial control stretches was the next stimulation trial started. The trials

FIGURE 7a: A schematic diagram of the protocol used for testing the seasonal variation in response to prior stimulation (not to scale): (A) 500 ms and (B) 1 s before the generation of the test stretch


500 ms
A
S
Stim
2 s
3 mins
Stim
2 s
B
1 s
S

were 3 mins in length between the periods of stimulation, on a continuous cycle.

TEMPERATURE

The experiment was conducted at 3 and 17^{o}C.

HISTOLOGY

A collaborative histological study of frog muscle was undertaken from January to March. The iliofibularis, gastrocnemius and sartorius muscles were removed, frozen, sectioned and stained for ATPase activity (to differentiate slow and fast fibre types.

SEASONAL EFFECTS

-CONTROL STRETCHES-

FIGURE 7b is a plot of the stiffness values obtained from the control trials throughout the year, at 3°C. Stiffness increases from January to February by 613 g/cm^2, after which the stiffness values plateau until about July. After July the muscle's stiffness declines gradually, with the lowest stiffness values for the year obtained in December. The largest difference in the stiffness values over the year was 1277 g/cm^2. The results obtained at 17°C, were not significantly different from those obtained at 3°C, following the same pattern of stiffness changes over the year (not shown here) the difference in stiffness values at this higher temperature was 1454 g/cm^2.

-EFFECT OF PRIOR STIMULATION-

FIGURE 7c (A & B) show stiffness values for stretches 500 ms after stimulation at 3 and 17°C throughout the year. FIGURE 7c (A) shows the results at 3°C. For the majority of the year the stretches after stimulation are less stiff than the control values. However in February and March the stiffness following stimulation was found to be greatly increased. In 10 of 14 observations made in February the stretch produced zero displacement; the muscle had evidently not fully relaxed it was therefore impossible to calculate a stiffness value for this month. FIGURE 7c (B), the results obtained at 17°C, were similar. However, it was possible to determine stiffness values for February (February mean control value 2134.5 g/cm^2 +/- 123; 500 ms after

FIGURE 7b: The stiffness values for the control trial stretches over a year at 3°C, force 0.3 g (mean and SD).

	n=		n=
Jan	6	Jul	4
Feb	14	Aug	6
Mar	10	Oct	6
Apr	4	Nov	6
May	4	Dec	10

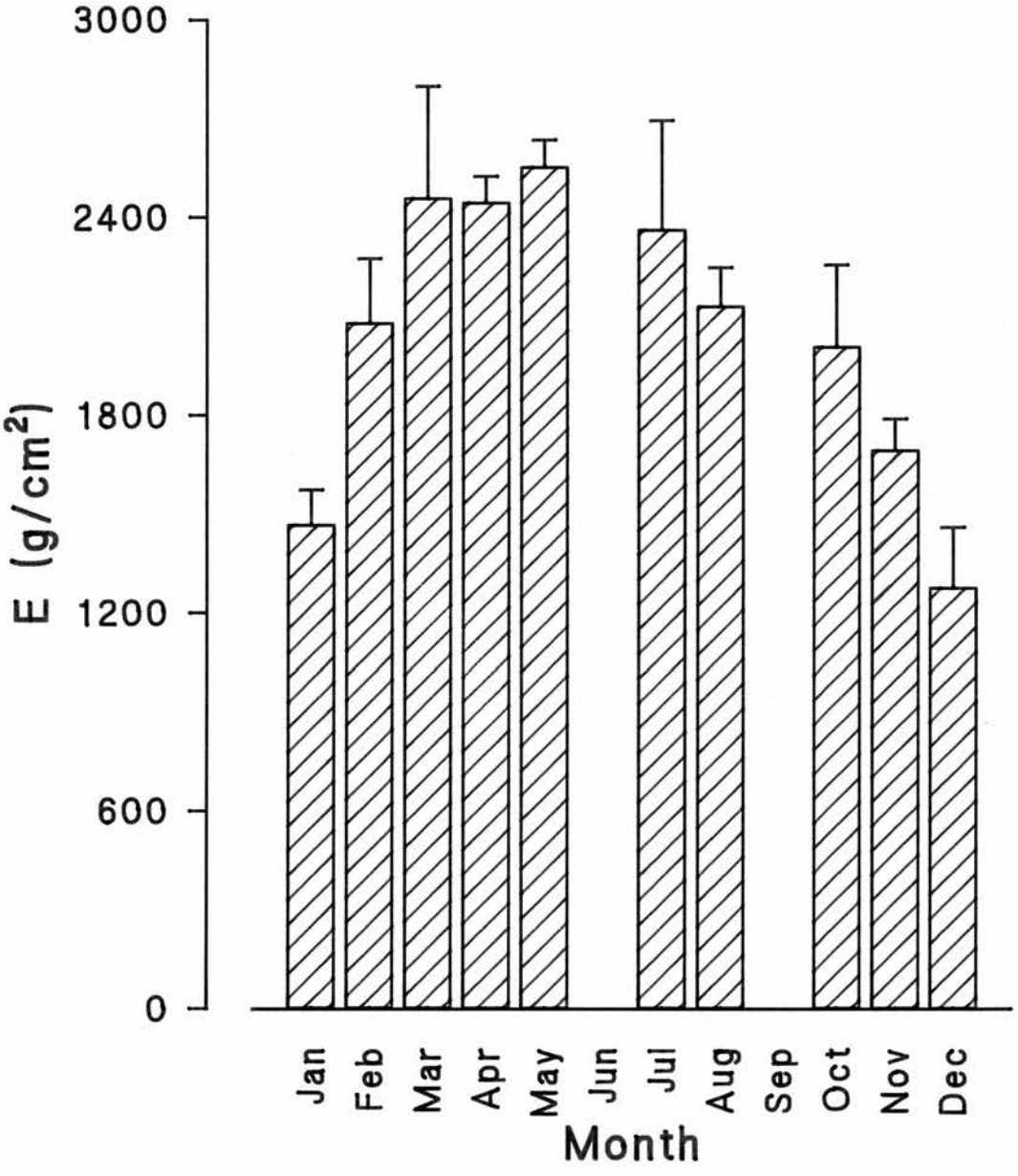
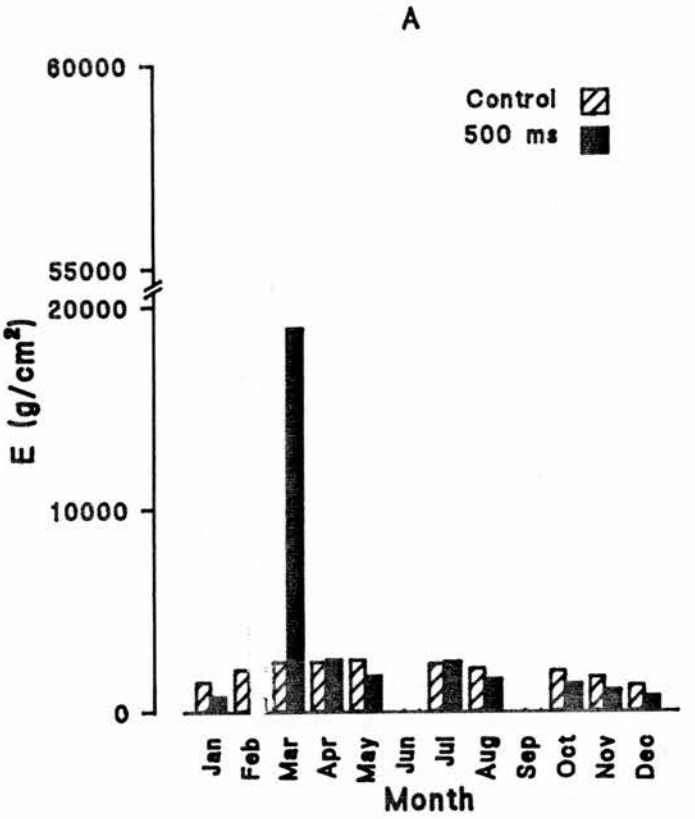
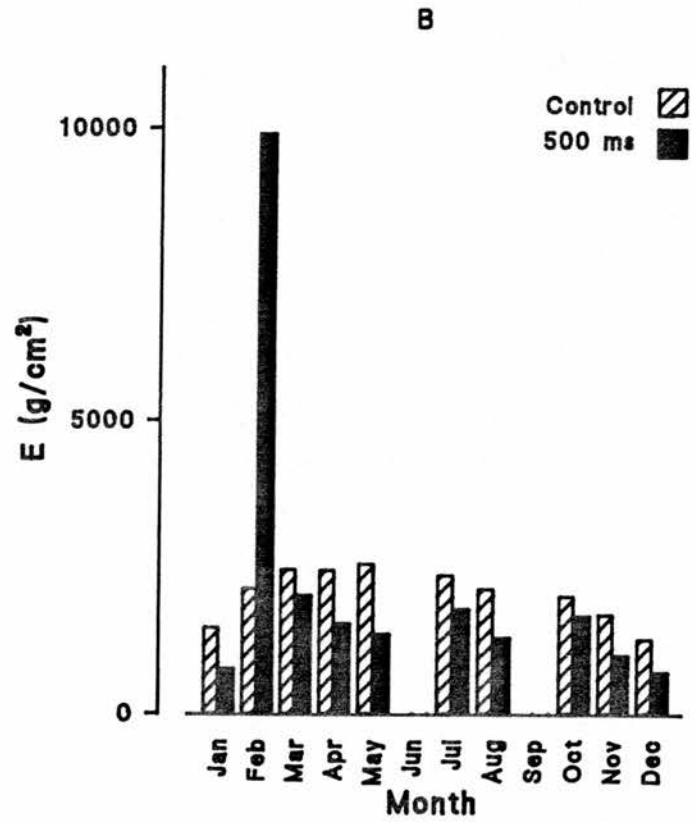

3000
2400
1800
1200
600
0
E (g/cm²)
Jan
Feb
Mar
Apr
May
Jun
Jul
Aug
Sep
Oct
Nov
Dec
Month

FIGURE 7c: The effect of stimulation 500 ms before the stretch on the stiffness values for the year at 3°C (A), and 17°C (B). Force 0.3 g mean (SD not shown for clarity). In February at 3oC (A) no stiffness value could be determined (see text). For number of muscles tested see FIGURE 7b. Note different scales.


A
60000
55000
20000
10000
0
E (g/cm²)
Control
500 ms
Jan
Feb
Mar
Apr
May
Jun
Jul
Aug
Sep
Oct
Nov
Dec
Month


B
10000
5000
0
E (g/cm²)
Control
500 ms
Jan
Feb
Mar
Apr
May
Jun
Jul
Aug
Sep
Oct
Nov
Dec
Month

stimulation mean value 9899.5 g/cm^2 +/- 234).

Stiffness values at the two temperatures 1 s after stimulation show no such increase in stiffness in the early spring and show the usual decrease in stiffness after stimulation.

The results are probably an indication of the amount of relaxation. Relaxation is slowed down by the lower temperature so that at 3^{o}C the muscle may still be in an active state in February and March, explaining the fact that in these months stiffness values are very high or un-measureable.

DISCUSSION

There is a marked degree of seasonal variation in the stiffness values recorded. The muscles are least stiff during the winter months, becoming stiffest in the early spring, coinciding with the frog's mating season. Stiffness gradually returns to winter levels over the summer months.

The increase in stiffness took place over a few weeks from the end of January to mid February. The frog sartorius muscle which up to then had behaved with an apparently fast twitch muscle response, started to respond with a slower response after tetanic stimulation. So that the lack of response to the stretches appearing 500 ms after stimulation may be a direct result of prolonged relaxation time in the spring and early summer. Histological examination of the sartorius muscle failed to reveal any slow fibres in the sartorius muscle, or any change in the fibre populations of the iliofibularis and gastrocnemius muscles. This confirms work by Gray (1958) who found no slow fibres in the sartorius.

During spawning, in early spring male frogs develop amplexus, with muscles able to sustain contractions over days and even weeks. It is not just a temperature stimulus that brings on these changes as frogs kept with no temperature or light stimuli will still display amplexus and spawning in the spring (personal observation). Thibert and Nicolet (1975) investigating the flexor carpi radialis (FCRM), one of the shoulder muscles used in amplexus, found that the proportion of slow fibres in the muscle was 14.6 % but the common classification of frog muscle fibres into twitch or slow fibres did not give a satisfactory explanation of the results

from this particular muscle or from the sartorius and: that an intermediate type, with especially twitch like fibres with a great potential for fatigue resistance were present (FOG fibres). Kuffler and Vaughan-Williams (1953) reported a small nerve innervation to the sartorius, which they regarded as an indication that there were slow / tonic fibres in this particular muscle.

Wacholder and Nothmann (1931) studied seasonal variation in the response of muscles to the application of ACh. They showed that muscles regarded as tonic (iliofibularis, and rectus abdominis) were more sensitive to ACh application during the winter months, while non-tonic muscles (sartorius) were converted to a "tonic" like behaviour. It was concluded that muscle fibres in the non-tonic muscles were, depending on the season, able to give two quite distinct responses. Seasonal variations had earlier been reported by Sommerkamp (1928). As there is no histological change in the fibre population, there must be an alternative mechanism by which the sartorius could adopt some of the properties of a slow fibre muscle.

A few of the possible explanations are now discussed, in the light of these seasonal effects.

ACETYLCHOLINE RECEPTORS

ACh is the neurotransmitter at the neuromuscular junction of the muscle, working via nicotinic receptors. Nicotinic receptors are not homogeneous, but consist as two distinct forms, functionally different (Katz and Thesleff 1957 and Dreyer, Walther and Peper 1976). The two receptor forms were further characterized by Igusa and Kidikoro (1987), in developing Xenopus muscle. Recent findings have shown that

slow muscle fibres of the costocutaneous muscle's of the garter snake have two receptor channels (Dionne 1989). Designated as type-T and S: type-T found on twitch fibre end plates with a high conductance, whilst the type S channels have a lower conductance. Type S channels were slower to desensitize, making them especially suited to maintaining a contracted state for long periods of time. Neher and Sackman (1976) also found these two ACh receptor channels in denervated adult frog muscle, and the low conductance channel remained open for about four times longer than the high conductance channel.

The results presented here might well reflect some proportional change in the number of each kind of ACh receptor, without any alteration in the population of muscle fibres. The receptors are structurally similar, varying only in their aggregation of the ACh receptive units. That there might be some change from type-T to type-S in the winter muscles of the frog is plausible, as in the developing embryonic muscle a mixture of the two types exist, but during development the relative number of each channel alters. The fraction of the type S low conductance channels declines during maturation. Twitch fibres favour the type T receptors whilst slow muscle fibres in the mature state favour the coexistence of both channels (Brehm *et al* 1984 and Henderson *et al* 1987). The fact that a chronic alteration in the temperature can cause a change in the affinities of adrenoceptors in cardiac muscle in both the frog and rat is evidence that the type of receptor may affect the muscles mechanical properties (Benfey 1977).

ISOFORMS

An alteration in the expression of the muscle's myosin isoform to a slower fibre type as a result of some temperature effect or hormonal influence is also a possibility. The type of MHC can affect the muscles mechanical properties. Also, FO and FOG muscle fibres are composed of different MHC (Dalla Libera, Sartore, Pierobon-Bormioli and Schiaffino 1980). There is a transformation of MHC's from developing to adult forms brought about by some alteration in the configuration of the neuromuscular junction (Whalen *et al* 1981). Schiaffino *et al* (1989) have identified a new MHC. This MHC confers on the muscle intermediate velocity of shortening between slow and FO muscles. It is the MHC rather than the light chain that has been implicated in altering the muscle's mechanical properties. Hoh, McGrath and Hale (1978) and Schwartz, Lecarpentier, Martin, Lompre, Mercadier and Swynghedauw (1981) demonstrated that the maximum velocity of shortening induced by either an increase in thyroid hormone levels or by haemodynamic overload, in the ventricular myocardium were correlated with a shift to a high ATPase alpha MHC and low ATPase βMHC with no change in the light chain. This correlation has also been shown to exist in toad skeletal muscle (Lannergren 1987), so seasonal changes in the stiffness properties could be the result of a change in the MHC composition.

Seasonal changes in the myofibrillar structure have been seen in acclimated fishes. In the striped bass the proportion of aerobic fibre types are significantly higher in cold than in the warm acclimated fish (Jones and Sidell

1982). The changes allow the fish to maintain an efficient swimming speed as well as sustaining swimming speeds for longer than the non-acclimated fish. Re-modelling of the myofibrillar material may allow the muscle to utilize ATP better, by altering the MHC to a more favourable configuration, so that the acclimated fish will be able to maintain swimming performance. In *R. Temporaria* there have been no such reported changes in myofibillar structure. However in *R. Pipiens* there has been shown to be a lowering of the metabolic rate in cold acclimated frogs (Shertzer, Hart and Pavlick 1975).

Lowering of the myosin ATPase activity leads to a decreased turnover of the myosin cross-bridges. Therefore, changes to a slow muscle ATPase activity may be an adaptive physiological response, resulting in a more efficient conversion of chemical energy for the frog when its body stores have been depleted after hibernation and spawning (Dillman, Berry and Alexander 1983). The effect of semistarvation on the myosin ATPase activity in rat cardiac muscle was to reduce the Ca^{2+} activated myosin ATPase activity by 28 % (Dillman *et al* 1983). In cardiac muscle the half life of the MHC in the rat is 5 - 6 days (Martin, Rabinowitz, Blough, Prior and Zak 1977). Changes in the proportions of the various types of MHC could well be brought about fairly rapidly, mirroring the changes in the frog muscle's properties.

ELECTRICAL PROPERTIES

The electrical properties of the frog sartorius muscle also show a seasonal variation. Dulhunty and Gage (1973), have shown that the electrical properties of fibres are

different in summer and winter.

	Summer	Winter
Internal Resistance	147 Ω.cm	194 Ω.cm
Membrane Resistance	7.6 K Ω.cm^2	3.9 K Ω.cm^2
Membrane Capacitance	4.7 μF/cm^2	6.7 μF/cm^2

Vaughan, Howell and Eisenberg (1972), found a wide variation in the membrane resistance, that might be associated with the reproductive state of the frogs. Even within one season there is a wide variation (Dulhunty and Gage 1973). The membrane capacitance is considerably lower in summer than in winter and is not related to the fibre diameter. It is possible that there is a seasonal change in the average dielectric constant of the membrane. If the density of ionic channels were less in summer than in winter, then a higher membrane resistance and lower capacitance might be expected. Alternatively the degree of saturation of the membrane lipid might change with season (Freeman, Satchell, Chang and Gray 1968). In winter the metabolism of the toad is largely fat based but in summer there is a switch to carbohydrate metabolism. The diet of the frog changes with the season. In summer they are in the main insectivorous, but in winter they eat nothing or in mild conditions a little grass. The nature of the dietary lipid saturated or un-saturated could may be reflected in the membrane lipid composition. In a lipid layer that contains a high degree of saturated fatty acids the structure would be rigid (Fox 1972), and the dielectric constant lower than in a lipid layer consisting of un-saturated fatty acids, where deformation and consequent fluidity would be greater. This implies that the surface membrane of frog muscle fibres

contains more un-saturated fatty acids in winter than in summer. The membrane capacitance might be expected to be higher in winter, and this was confirmed by (Dulhunty and Gage 1973). Dulhunty and Gage (1973) proposed that another membrane might appear in series with the surface membrane in summer, increasing the membrane resistance and lowering the membrane capacitance. Electronmicrographs do seem to show an apparently denser, more adherent, basement membrane in summer than in winter.

THYROID HORMONE

The triggering of the seasonal variations may be based on hormonal influences, possibly thyroid hormone (T3 and T4). Increased levels of T3 produce changes in fibre composition in mammalian muscle (Fitts, Winder, Brooke, Kaiser and Holloszy 1980). The effect of T3 on the soleus and extensor digitorum longus of the rat have been investigated (Nicol and Johnston 1981). In hyperthyroid conditions the soleus changes from a slow to a fast muscle, while the EDL, originally a fast muscle of the glycolytic type, converts to the oxidative twitch fibres. The hormone apparently decreases the activity of the oxidative enzymes, succinate dehydrogenase, citrate synthase and cytochrome c oxidase (Sillau 1985). Ianuzzo, Patel, Chen, O'Brien and Williams (1977), investigating the effects of long term hypothyroidism and hyperthyroidism on skeletal myosin, reported that alterations in the thyroid state did produce phenotypic changes in skeletal muscle myosin. Six weeks of hyperthyroidism were equivalent to 6 - 15 months of cross-innervation by a fast nerve (Barany and Close 1971). The histochemical procedure for resolving fast and slow

twitch myosin is based on alkali resistance and susceptibility respectively (Samaha, Guth and Albers 1970). Ianuzzo *et al* (1977) found that depending on the thyroid status of rats there were clear differences. Hypothyroid animals had a small percentage of fibres that were alkali stable, while hyperthyroid muscle had a large percentage. The myosin ATPase activity was also altered with the thyroid treatment with the hypothyroid animals showing a lower activity than the hyperthyroid. This may well help in explaining seasonal variations that have been reported. As the thyroid level changes from winter to summer there may a change in the myosin of the muscle. Dillman *et al* (1983) showed that plasma levels of T3 and T4 are reduced by between 45 - 50 % in the cardiac muscle of semistarved rats, with changes in the MHC proportions to a slow muscle type; the normal state was restored if T3 was administered. The thyroid hormone levels are an important determinant of the myosin ATPase activity and the myosin isoform distribution.

In frogs, thyroid hormone plays a vital part in the transformation from tadpole to adult, but in adults it is not thought to play any significant role, yet the thyroid gland remains. It is possible that as yet an un-identified role exists. Although food was available at all times of the year, it is likely that during the spawning period food takes a secondary place to mating, with the frogs living off their body stores. This then may cause a reduction in the circulating thyroid hormones and consequent changes in myosin isoforms to those of a slower type.

CONCLUSION

The results of this study would seem to suggest that the

seasonal variation in the muscle's properties are the result of some internal body clock of the frogs, and possibly hormone linked. The alteration is probably related to some change in the muscle fibres, MHC, ACh receptors or, the electrical properties of the muscle membrane. The seasonal changes are likely to be due to a combination of events, all causing the mechanical alterations reported here. In the following chapter, another possible factor in the seasonal effects will be investigated; adrenaline.

CHAPTER EIGHT

THIXOTROPY: THE EFFECT OF ADRENALINE

INTRODUCTION

The metabolism of amphibians and other animals is greatly affected by adrenal secretions.

ADRENAL GLAND

The anurian adrenal gland consists of tissue strands in which the internal and chromaffin cells are intermingled. In some species a third cell type (summer or Stilling cell) has been indentified. These transitory cells may serve as part of an integrated endocrine mechanism, as they are responsive to pituitary Adrenal Cortical Tropic Hormone (ACTH). The chromaffin cells are responsible for the production of adrenaline and noradrenaline which in the frog are produced in equal quantities. The cortical tissue produces steroids but these have proved difficult to identify.

ADRENALINE: EFFECTS ON MUSCLE

MAMMALIAN MUSCLE

Adrenaline produces different effects in mammalian muscles depending on whether they are slow or fast. Brief descriptions of the main findings follow.

-FAST CONTRACTING MUSCLE-

Oliver and Schafer (1895) demonstrated that extracts of the adrenal medulla increased the tension of indirectly elicited twitches, from non-fatigued gastrocnemius muscle of the anaesthetized dog. This was subsequently confirmed by a number of workers (Bowman and Raper 1964, 1966, Bowman and Zaimis 1958, Goffart and Ritchie 1952, Montagu 1955 and West and Zaimis 1949).

Adrenaline and splanchic nerve stimulation increase the strength and size of twitch contractions in non-fatigued fast

contracting muscle when the stimulation has been applied directly following full curarization or chronic denervation (Bowman and Zaimis 1958 and Goffart and Ritchie 1952). This indicates that the effect is independent of neuromuscular transmission, and is exerted on the muscle fibres themselves. Experiments on isolated skeletal muscles bathed in physiological salt solutions, and on muscles where blood flow was recorded simultaneously with contraction, indicate that there are no concomitant vascular changes (Bowman and Raper 1964, Bowman and Zaimis 1958, Goffart and Ritchie 1952 and Montagu 1955). The smallest dose of (-) adrenaline to cause a detectable increase in the maximal isometric twitch tension of the non-fatigued tibialis anterior muscle of the cat is around 3 µg/kg intravenously and 0.2 µg aterially. According to Bowman and Zaimis (1958) it is unlikely that physiological levels of adrenaline have any effect on contraction dynamics in unfatigued fast muscle.

-SLOW CONTRACTING MUSCLE-

Slow muscles react to sympathomimetic amines and to splanchic stimulation in the opposite way to fast contracting muscles (Bowman and Raper 1962 and Bowman and Zaimis 1955, 1958). The maximal isometric twitch tension can be reduced by as much as 20 % and is associated with decreases in the time to peak tension (about 25 %), and a reduction in the total duration of the twitch (up to 35 %). Bowman and Raper (1962), suggested these were a result of a curtailment of the active state of the muscle.

Cat soleus muscle is sensitive to (-) adrenaline, doses of 0.05 µg/kg intravenously and only 0.003 µg aterially (Bowman and Zaimis 1958). These concentrations are within the

physiological range, so adrenaline probably is physiologically significant in slow muscles (Denny-Brown 1929).

-HUMAN MUSCLE-

Human muscles are less well differentiated into fast and slow contracting muscles than those of smaller animals. Tremors experienced by people under stress or patients with Phaeochromocytoma might well be due to the action of adrenaline, decreasing the fusion of incomplete tetanic contractions in slow motor units. Infusions of adrenaline or isoprenaline enhance physiological tremor (Marsden, Foley, Owen and McAllister 1967 and Marsden and Meadows 1970). The adrenaline infusion effects are blocked by the β - receptor blocking drug (+/-) propranolol, but are not affected by the isomer (+) propranolol, which has only weak β - receptor blocking activity (Marsden *et al* 1967 and Marsden and Meadows 1970).

AMPHIBIAN MUSCLE

In amphibian muscle the experimental results are few and contradictory. Both Hutter and Lowenstein (1955) and Oota and Nagai (1977) reported a positive inotropic effect of adrenaline on curarized muscles stimulated directly; but Corkill and Tiegs (1933), Brown, Goffart and Vianna Dias (1950) and Bowman and Nott (1969) found that adrenergic substances had no effect on the contractile properties of amphibian skeletal muscles.

EVENTS IN MUSCLE CONTRACTIONS

For a muscle contraction to occur several steps must take place, collectively known as Excitation Contraction Coupling

(E-C coupling). The action potential in the plasma membrane leads to an electrical signal, conducted to the interior of the fibre by the "T" tubules. This causes a release of bound Ca^{2+} from the lateral sacs of the S.R. which are in close proximity to the myofibrils. The release of Ca^{2+} initiates cross-bridge formation by its interaction with the troponin/tropomyosin complex releasing the inhibition for cross-bridge formation. Relaxation takes place because of the sequestering property of the S.R. for Ca^{2+}, removing it from the contractile elements, involving the hydrolysis of ATP. It is likely that the effect of adrenaline on the contractile properties of both fast and slow muscles involves changes in the amounts of Ca^{2+} released from the S.R. or, in the rate of its re-uptake.

ADRENALINE RECEPTORS

The direct action of adrenaline in the mammalian muscle fibres, is mediated via β - adrenoceptors, especially the β_2 receptors. Bowman and Raper (1966) concluded that alpha - receptors were pre-junctional, whereas the β - receptors were post-junctional. There is no direct innervation by the sympathetic system of muscle fibres, so that any effect is the product of the circulating vascular catecholamines, able to diffuse into the muscle.

ADRENALINE AND cAMP

It has been suggested that the adrenaline effects are mediated through the adenyl cyclase / cyclic 3'- 5'- Adenosine Mono Phosphate (cAMP) system, which in turn leads to changes in the rates of uptake of Ca^{2+} by the SR, (Bowman and Nott 1969).

The positive inotropic effect of adrenaline could be due to two separate effects (Gonzalez-Serratos, Hill and Valle-Aguilera 1981):

1.) In the heart there is a prolongation of the action potential plateau, coupled with an increase in the inward Ca^{2+}. This could lead to an increase in tension; this may be the same system that is found in skeletal muscle.

2.) It may have some direct effect on the actinomyosin ATPase complex, or on the SR.

Adrenaline causes an increase in the cAMP in skeletal muscle, which may then be responsible for the effects seen in frog skeletal muscle. With a 0.01 mM, +/- adrenaline solution there was an increase in tension of 80 %, the total duration was the same but the maximal rates of tension development and decay also increased by 80 and 50 % respectively. The involvement of a second messenger system is suggested, as the onset of these effects are slow (3 mins) for the positive inotropic effects and 8 mins to reach maximum, and half an hour was required by the muscle to return to the normal level of response (Gonzalez-Serratos, *et al* 1981). Posner, Stern and Krebs (1962), demonstrated that the addition of adrenaline *in vivo* increases cAMP levels, phosphorylase *b* kinase activation and phosphorylase *a* formation in rat skeletal muscle.

Increases in the twitch tension, the rate of tension production and tension decay could be due to stimulation of Ca^{2+} pumps in the SR, thus increasing Ca^{2+} concentration in the SR and increasing the cross-bridge turnover rate (Hoh, Rossmanith, Kwan and Hamilton 1988).

ADRENALINE AND THE Na^+/K^+ PUMP

Adrenaline also facilitates the Na^+/K^+ pump activity in a concentration dependent manner (Kaibara, Akasa, Tokimasa and Koketsu 1985). This works via the β - receptors and is related to the level of cAMP. However, dibutyryl cAMP did not change the Na^+ efflux and the Na^+/K^+ ATPase. Adrenaline increases the affinity for extracellular K^+ and accelerates the kinetic process of Na^+ extrusion. It was shown that, due to a Na^+/K^+ adrenaline interaction, the rate of Na+ extrusion was accelerated by as much as 2 - 5 times.

This series of experiments with adrenaline were conducted to determine if the time course for thixotropic recovery could be altered following administration of adrenaline.

METHOD

MATERIAL AND METHODS

The frog iliofibularis muscle was dissected out and divided into a small bundle of fibres (5 - 10 fibres), separated from the muscle bulk using a dissecting microscope (KYWO OPTICS x10), bundles were taken from the twitch portion of the muscle. The bundles were mounted at L_o in apparatus B (Chapter 4, FIGURE 4a). Solutions were changed quickly by draining the old solution from the bottom of the bath and replacing it from the top, filling the bath each time with the same quantity of liquid. All drugs were supplied by Sigma Chemical Co. Ltd. The experiments were carried out at room temperature (17 - 21^{o}C), and conducted in July and August.

PROTOCOL

-EFFECT OF ADRENALINE CONCENTRATION-

In four fibre bundles the effect of varying the concentration of adrenaline in the bathing solution was tested. The concentrations were 0.001, 0.002, 0.005 and 0.01 mM (+/-) adrenaline. The protocol was the same as that below, except that the S_3 stretch was set to a constant 3 s after the end of the stirring.

-THIXOTROPY-

Three rectangular stretches (force 0.09 g, duration 500 ms) were used, S_1, 1 s before 5 s of stirring (rectangular stretches at 3 Hz and 1.5 mm maximum displacement). Stirring was followed 200 ms later by S_2. The S_3 stretch in each trial was at one of the following times: 1, 2, 3, 4, 5, 10, 20, 30 and 60 s after stirring. The order of this third

stretch was randomized from trial to trial. The trials formed a continuous cycle lasting 3 mins, timed from the last period of stirring.

Once consistent control results for the test stretches had been obtained, the adrenaline solution was introduced into the bath and left to equilibrate for 15 mins. The bundles were then tested with the above protocol, for 5 trials.

After completion of the adrenaline trials the bundle was washed with isotonic Ringers several times, allowed to rest for 15 mins and re-tested in isotonic Ringers and the results compared with the first set of control readings. Only if the response had returned to the level of the previous controls was the next solution tested. If the readings were significantly different, then the bath was flushed again with isotonic Ringers and re-tested. The same protocol was used, when testing the other solutions.

All drug solutions were made up in isotonic Ringers, the concentrations were: (+/-) adrenaline, 0.01 mM; (+/-) propranolol, 0.01 mM and (+/-) adrenaline plus (+/-) propranolol (both 0.01 mM).

THE EFFECT OF VARYING THE ADRENALINE CONCENTRATION

FIGURE 8a shows that the effect on the iliofibularis bundle's stiffness with adrenaline is concentration dependent. There is a small reduction in the stiffness with the lower doses. With the 0.001 mM adrenaline solution there was only a 5 % reduction in the S_1's stiffness from that of the control trials, whereas with the 0.01 mM solution the stiffness was reduced by 50 %. Thixotropy is also reduced with increasing adrenaline concentration. Even the lowest dose used here is not within the physiological range of the frog (10^{-7} - 10^{-11} M).

TIME DEPENDENT STIFFNESS: THE EFFECT OF ADRENALINE AND PROPRANOLOL

FIGURE 8b shows the course of stiffness recovery in the presence of adrenaline (0.01 mM).

The control trials thixotropic recovery follows the same pattern as has already been reported for the whole sartorius, iliofibularis and iliofibularis single fibres. An initial fast phase in recovery, immediately after stirring is followed by a slower recovery, with an apparent change point in the rates at about 5 s. After the administration of adrenaline the stiffness of the muscle is reduced by about half, the final stiffness obtained is quite similar to that of the post-stirring stiffness values in the absence of adrenaline. There is still evidence of a discontinuity in the recovery of stiffness after stirring, however this appears to have been shifted to about 2.5 s. There is also a large percentage of the total recovery taking place within

FIGURE 8a: The effect of increasing the dose of adrenaline on the mean (SD) stiffness values recorded from the three test stretches S_1, S_2 (200 ms after stirring) and S_3 (3 s after stirring), using iliofibularis muscle bundles. Temperature 17°C, force 0.09 g, n=4.

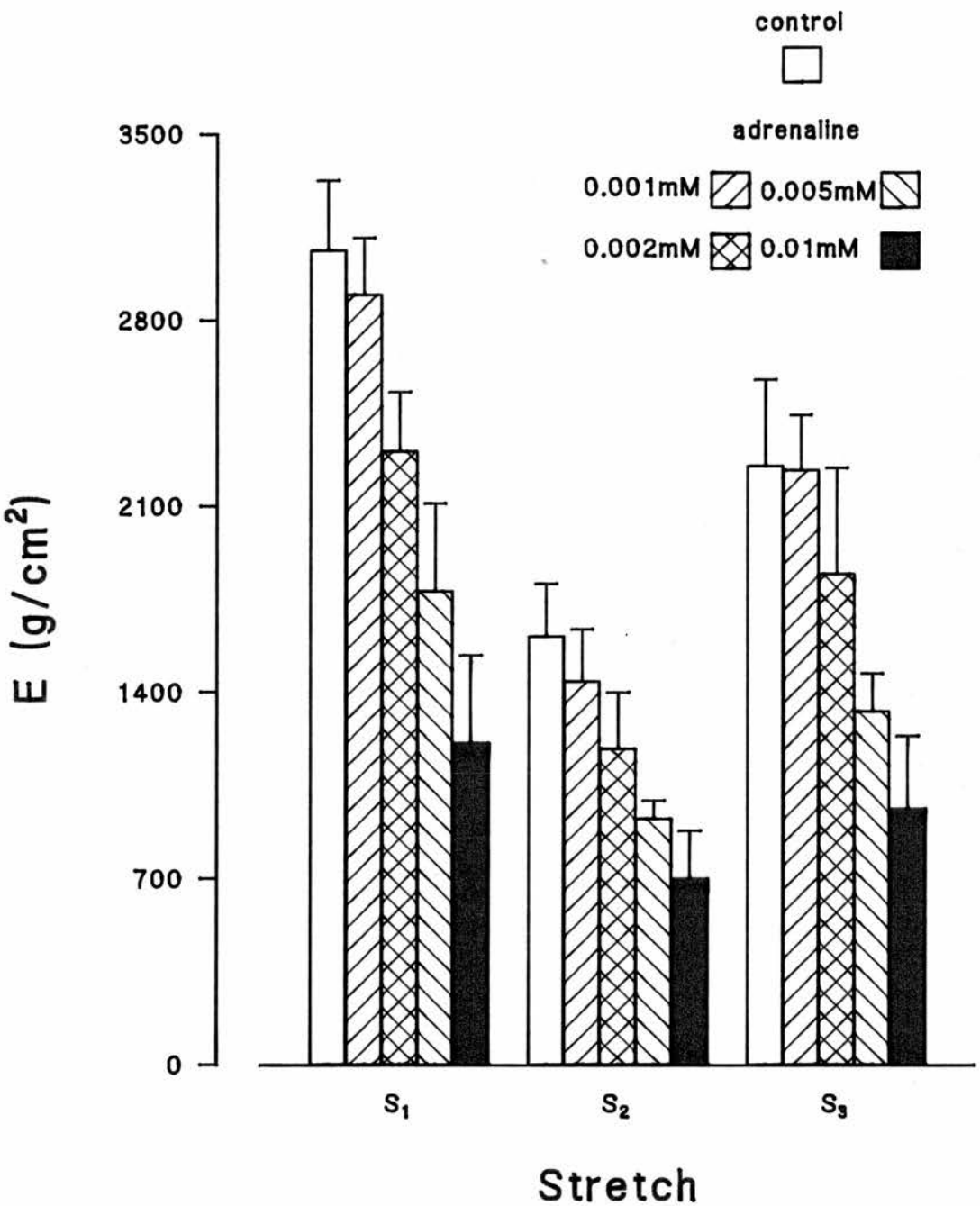

control
adrenaline
0.001mM
0.005mM
0.002mM
0.01mM
3500
2800
2100
1400
700
0
E (g/cm²)
S1
S2
S3
Stretch

FIGURE 8b: The effect on the thixotropic recovery on the mean (SD) stiffness values before and after adrenaline (0.01 mM). Temperature 17°C, force 0.09 g, n=4.

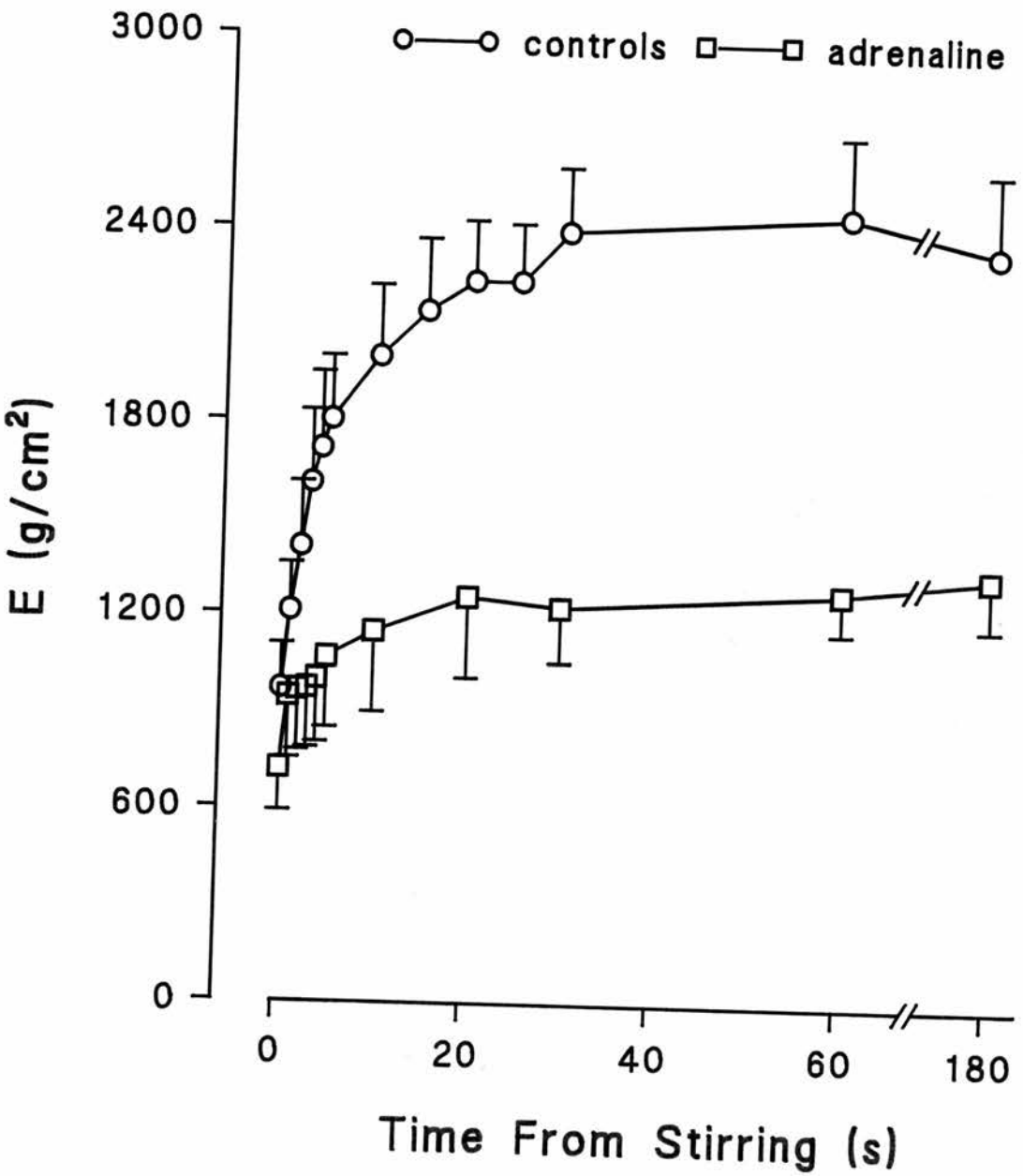
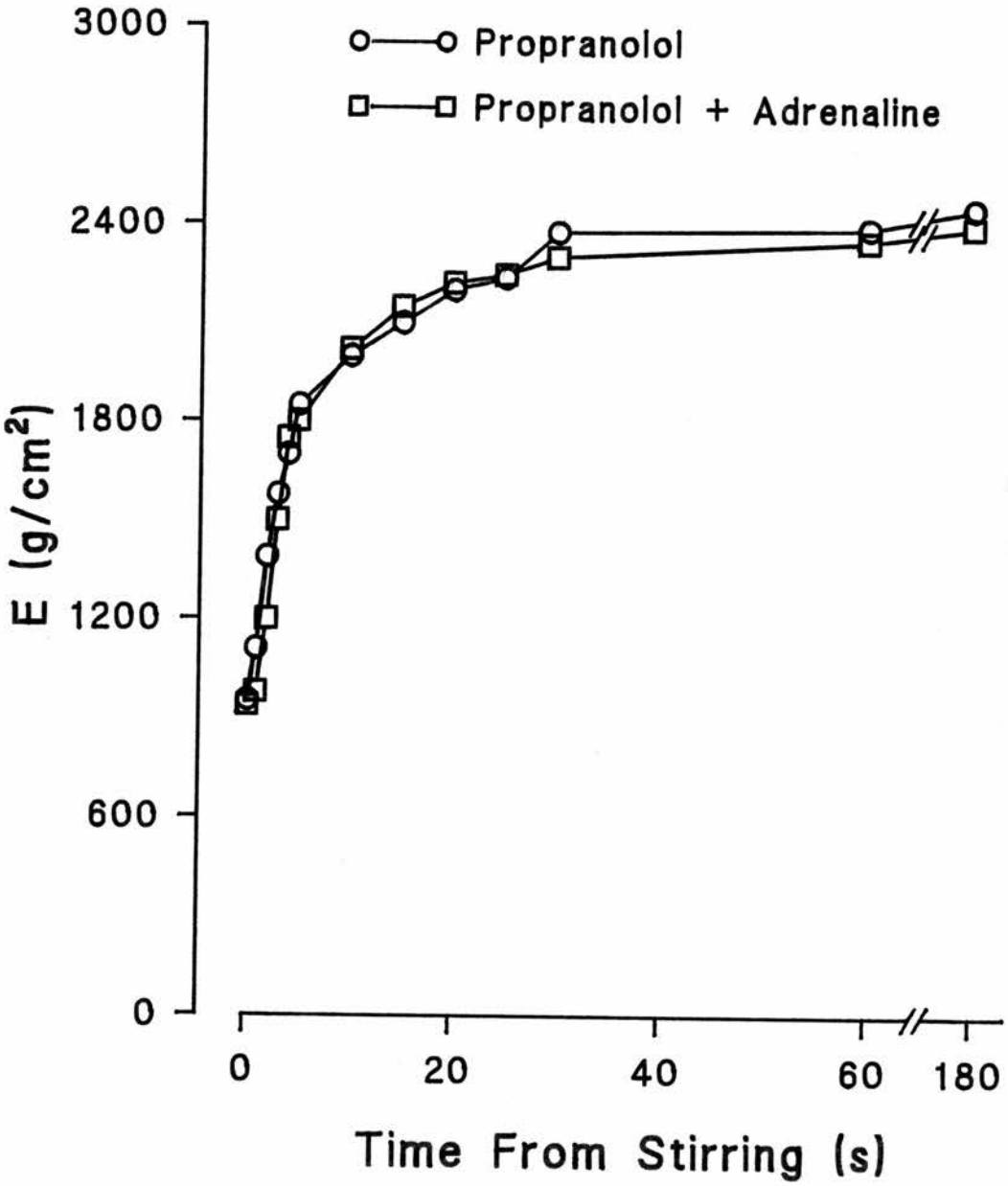

3000
2400
1800
1200
600
0
E (g/cm²)
controls
adrenaline
0
20
40
60
180
Time From Stirring (s)

FIGURE 8c: The effect on the thixotropic recovery (mean, SD's are not shown for clarity) with the administration of propranolol (0.01 mM) and propranolol plus adrenaline (both 0.01 mM). Temperature 17°C, force 0.09 g, n=4.


Propranolol
Propranolol + Adrenaline
3000
2400
1800
1200
600
0
E (g/cm²)
0
20
40
60
180
Time From Stirring (s)

the first few seconds, with 70 % recovered by 1 s after stirring compared to only 50 % in the control trails.

FIGURE 8c shows the effect of the administration of 0.01 mM (+/-) propranolol: these results are not significantly different from those of the controls. The same recovery rate is seen and the stiffness levels are also of the same magnitude. When adrenaline (0.01 mM) is now added the recovery rate is not altered.

DISCUSSION

Adrenaline caused an overall loosening of the muscle so that S_1 values were similar in scale to the values obtained immediately after stirring in the isotonic Ringers. There was a suggestion that thixotropic stiffness recovery, although smaller in extent, was somewhat faster.

In rat cardiac muscle Hoh, *et al* (1988), found that cross-bridge cycling was enhanced by as much as 25 - 50 % with the addition of adrenaline. This increase was mediated via β-adrenoreceptors, with the adrenaline enhancing both the rate of rise of the tension as well as augmenting the rate of relaxation during an isometric twitch. The increased rate of rise of isometric tension has been attributed to a quicker rate of rise in the intracellular Ca^{2+} concentration (Allen and Kurihara 1980). The importance of this increased Ca^{2+} transient in the rate of rise in the tension is offset by the fact that β-adrenergic stimulation reduces the sensitivity of the regulatory proteins to Ca^{2+} through the phosphorylation of troponin-I (Solaro, Moir and Perry, 1976 and England, 1976). It should also be pointed out that the rate of rise of the isometric tension during a twitch may also depend on the rate of attachment of the cross-bridges. Both the Ca^{2+} transient and the rate of attachment of the cross-bridges are probably important in increasing the rate of rise in tension, but the relative importance of each factor is not easy to evaluate without modelling the kinetics of these processes. Although the mechanism involved in enhancing cross-bridge cycling in phasic regulations is at present not well understood, it appears to be a consequence of an elevation in the intracellular cAMP (Bowman and Nott 1969).

An increased cross-bridge cycling rate could explain the changes in the overall stiffness, as it would mean that activated cross-bridges undergo their cycle faster; when the bundle is stretched there is the possibility that a larger proportion of the cross-bridges are in a state of transition, resulting in a reduced stiffness. The quicker thixotropic recovery can also be explained as the rate of cross-bridge attachment is greater. The total number of cross-bridges will be less than normal where cross-bridge cycling is slower. The effects of the adrenaline seem to be mediated via the cross-bridges. However reduced actin folding is known to occur with an increased Ca^{2+} concentration, so that intra filament folds can not be discarded as a possible explanation of the change in stiffness obtained with adrenaline (Gillis and O'Brien 1975 and O'Brien *et al* 1975).

Adrenaline has been implicated in releasing Ca^{2+} from the SR, acting via cAMP, to stimulate the SR-Ca^{2+} pump (Gonzalez-Serratos *et al* 1981). An increase in the concentration of Ca^{2+} released during subsequent activation produces the increase in twitch tension. The results presented here seem to confirm the view of Gonzalez-Serratos *et al* (1981) that adrenaline acts via a second messenger, thus adrenaline has no effect on the thixotropic recovery rate when first administered. The effects become obvious after 10 mins, close agreement to the 8 mins for the maximum inotropic effect reported by Gonzalez-Serratos *et al* (1981).

Oota and Nagai (1977), using frog sartorius muscle, were able to block the adrenaline potentiating effect on the twitch tension and also the potassium contracture by *B*-adrenoreceptor blocking drugs. In the results described

here the effects could also be removed by the application of propranolol.

Adrenaline has facilitatory effects on the Na^+/K^+ pump activity, in a concentration dependent manner, which are blocked by propranolol, and are probably mediated via *B*-adrenoreceptors (Kaibara *et al* 1985). The application of dibutyryl cAMP did not change the Na+ efflux, which leaves in question adrenaline working via cAMP, but does not rule this out completely.

Adrenaline increases the affinity for extracellular K^+ and accelerates the kinetic process of Na^+ extrusion (by 2 - 5 times). In the summer months, the adrenal gland is in an active state producing adrenaline which causes the release of Ca^{2+} by the muscle. Adrenaline also influences the Na^+/K^+ pump causing an inward pumping of K^+ into the muscle, thereby reducing the extracellular level of K^+. In winter the reverse is true, the adrenal gland is inactive, consequently there is no release of Ca^{2+} and there is a higher extracelluar K^+ concentration. Ca^{2+} depletion causes a reduced liberation of ACh, which may make the muscle more sensitive to its administration producing a prolonged contraction in winter (Del Castillo and Stark 1952).

Goffart and Perry (1951) found that in summer there is a reduced loss of K^+ from the muscle itself to the extracellular medium which would seem to confirm the above suggestion that adrenaline acts in the summer months to make the muscle less stiff by releasing Ca^{2+} and preventing the loss of K^+.

Increased cross-bridge turnover is probably the result of increased levels of intracellular Ca^{2+} and also of an

increased intracellular K^+ concentration. It has also been found that the muscles are generally less stiff in summer when the frog and the adrenal gland are active. However, the results presented here were all obtained from highly non-physiological concentrations, and only small effects were seen with the lowest dose (0.001 mM). The implication of these results for the everyday physiology of the frog is therefore highly speculative.

Effects on the cross-bridge cycling rate and muscle isoform composition produced by altered thyroid states tend to be long term; it may be that adrenaline acts in the short term to speed up cross-bridge cycling in times of stress at any time of the year.

CONCLUSION

CONCLUSION

The underlying mechanism of muscle thixotropy reported in this work, as well as by a number of other workers in both human and animal muscles, has been indentified with more certainty (Lakie *et al* 1979, 1983, 1984, Lakie and Robson 1988a-d, Lakie and Robson 1990, Walsh and Wright 1987, 1988, Hufschmidt and Schwaller 1987, Buchthal and Kaiser 1951 and Wiegner 1987).

Thixotropy confers stability on muscle, making it less susceptible to small random movements arising both from within and outside the musculature.

Thixotropy and the SREC have been linked together, giving the first clue as to the origin of the thixotropy. Hill (1968) concluded that the SREC was the product of a small number of cross-bridges present even in the relaxed muscle. These bonds make the muscle appear very stiff for movements over a short range (0.2 % of the muscle's length). Although these cross-bridges are long lived connections they can be broken by a series of large perturbations, with the result that the muscle's stiffness is transiently reduced. The loss of stiffness is transient, as once the perturbations have ceased the cross-bridges re-form spontaneously, resulting in the muscle regaining the previous level of stiffness.

The best thixotropic affects are visible with small stretches (<0.2 % of L_o). Larger stretches rupture the attached cross-bridges, so that the perturbations have no further loosening effect on the muscle.

The time course for the recovery of the stiffness after the perturbations has been mapped out, and found to be highly

non-linear. There is an initial fast recovery within the first few seconds after the movement has stopped, lasting until around 5 s, after which the recovery is slower and may continue for minutes or even hours, if undisturbed. Thus the stiffness of a relaxed muscle is highly dependent on the previous history of movement. Movements will cause a reduction while rest will result in stiffening.

After these initial findings that thixotropy was linked to the SREC, it still had to be confirmed that it was a result of the cross-bridges rather than some other muscle structure. Hill attributed the SREC to a small proportion of the cross-bridge population, however other workers have challenged this supposition. The sarcolemma has been rejected by Rapoport (1972) as the sarcolemma plays no part in the resting tension of the muscle at sarcomere lengths below 3 μm. Actin folding has been another major contender for the origin of the SREC and thus by implication the thixotropic behaviour. Helber (1980), proposed that the actin filament was able to fold back on itself and that these bonds worked over the SREC range, and thus may account, at least in part for the presence of the SREC.

From the work on single fibres it was shown that fibres that had been damaged to remove the contractile material leaving the sarcolemma behind, no longer displayed a thixotropic response, therefore the sarcolemma was rejected as the cause of the thixotropic response.

Both the single fibres and whole muscles poisoned with IAA to induce rigor provided strong confirmation that it was the cross-bridges rather than filament folding that was the

major component of the thixotropic response. Rigor fibres/muscles had higher values of stiffness than the control fibres/muscles, and the thixotropic response was greatly reduced or, in most cases, totally abolished. A small amount of thixotropy remains in rigor fibre/muscles which may be attributed to the small population of un-attached cross-bridges that are still able to go through a normal cross-bridge cycle, and/or the fact that the myosin head is flexible around its neck region, which allows it to change orientation even when attached in rigor (Reedy *et al* 1989).

Prior isometric electrical stimulation of frog muscle causes the muscle to become less stiff. This somewhat unexpected result, can be explained if it is assumed that under isometric stimulation the cross-bridges that are formed during the stimulation are under strain. This would mean that any remaining attached cross-bridges would be in a strained configuration, and that any stretch could detach them and make the muscle then appear to be less stiff than it was before stimulation. The stiffness was recovered with the same time scale as a muscle mechanically perturbed to break these bonds.

Three novel findings have arisen from this study. First, muscle has a time dependent yielding to stretches of varying durations. A discontinuity in elongation was seen in response to stretches of varying durations which could not be attributed to either a critical displacement or a critical velocity. Muscles yield more to stretches of a long duration than to stretches of short duration, with a discontinuity in the response at around 3 - 5 s. This behaviour of relaxed

muscle was also attributed to the cross-bridges. There is probably a small degree of cross-bridge cycling continuously taking place; a fast stretch will be acting against mostly attached cross-bridges, causing distortions but not detachments, resulting in a muscle that is stiff. Longer duration stretches will be acting over several cross-bridge cycles, with detached cross-bridges allowing the muscle to yield, and thus will be generally less stiff as the filaments slip past one another.

This time dependent property can also be seen in mammalian and molluscan muscle, and the yield point appears at the same time in all muscle types so far investigated.

Second, the sartorius muscle of the frog *R. temporaria* displays a seasonal change. Its response to prior stimulation and the general level of stiffness alter with the seasons. A muscle tested in early spring will have a higher stiffness value than a muscle tested in late autumn. After stimulation, the muscles in early spring appear to remain in an active contracted state longer than summer muscles. There is no clear explanation for these seasonal changes, however a number of hypotheses have been put forward, the most likely trigger for the changes are probably hormone linked with consequent alterations in either the muscle myofibrillar material, receptor types or sensitivity, or electrical membrane properties.

Third, adrenaline (in un-physiological doses) does have pronounced effects on the stiffness of frog muscle and the rate of thixotropic recovery. It caused a lowering of stiffness and a speeding up of thixotropic stiffness

recovery.

In conclusion: relaxed muscle is not a static material, but is in a state of constant flux, both in the short and long term. Activation or movement can profoundly alter its state. Cross-bridge mechanics are the probable basis for all these changes.

Further work might extend the studies already made on actomyosin extracts (MacGinnis 1989). Muscle extract solutions allow the purification of both major muscle filaments, so that pure actin and myosin could be tested for intrafilament as well as interfilament bonding, without the complications of other fibrillar material. Seasonal changes may also be able to be mapped out with muscle extracts if there are myofibillar changes, resulting in differences in filament interaction depending on the time of the year.

Adrenaline, from the brief experiments reported here, would seem to have striking affects on muscle's stiffness. This decrease in stiffness, and consequent reduction in stability, is especially interesting in view of the potency of adrenaline as a tremorogen. Future work should address the role of muscle destabilisation in the production of tremor, with physiological doses of adrenaline, and in mammalian species.

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APPENDIX