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# Oscillatory contraction of insect fibrillar muscle after glycerol extraction

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[Plates 21 and 22]

Insect fibrillar muscle can sustain oscillations in a mechanically resonant load because length changes of the active muscle are followed by delayed changes of tension. It has not been known whether this is an intrinsic property of the contractile mechanism, or whether it depends on the excitation-contraction coupling system. The object of the present investigations has been to decide between these two possibilities by examining the behaviour of glycerol-extracted fibrillar muscle from giant water bugs (*Hydrocyrius* and *Lethocerus*, Order: Hemiptera). The purpose of the glycerol extraction was to isolate the contractile protein system by selective destruction of cellular components other than the myofibrils.

Preparations consisting of single muscle fibres or small bundles of fibres were immersed in buffered salt solutions containing adenosine triphosphate (*ATP*), magnesium and calcium. Activation of the fibres could be produced, and the level of activity controlled, by varying the  $\text{Ca}^{2+}$  concentration, which was stabilized by the presence of a calcium buffer (ethylene glycol bis-( $\beta$ -aminoethyl ether)-*N*, *N'*-tetraacetate). Activity was detected by tension development and by the fact that length changes were followed by delayed changes of tension: this was revealed by transient analysis, which showed that a sudden release or stretch was followed by a delayed change of tension; by sinusoidal analysis, which showed a phase lag of tension on length (driven oscillation experiments); and by the fact that the preparation could sustain oscillations in a resonant lever system by overcoming its external damping (free oscillation experiments).

Attempts to raise the level of activity or the power output of the preparation sometimes produced a change in the mechanical properties of the muscle, characterized by a rise of tension and an alteration in the oscillatory behaviour (the 'high tension' state). Inadequate diffusion of *ATP* into, or adenosine diphosphate (*ADP*) out of, the preparation was probably the cause of this phenomenon.

Under the conditions of the present experiments, the threshold  $\text{Ca}^{2+}$  concentration varied from  $2 \times 10^{-8} \text{ M}$  to  $12 \times 10^{-8} \text{ M}$ . However, the sensitivity of the preparation to  $\text{Ca}^{2+}$  depended on the concentration of  $\text{Mg}^{2+}$ , which had a weak antagonistic effect on the action of  $\text{Ca}^{2+}$ , and on the mechanical conditions. The effect of muscle length was of particular importance, as stretching the fibre seemed to be equivalent to raising the  $\text{Ca}^{2+}$  concentration. It is suggested that this effect might provide the control mechanism by which changes of muscle length lead to delayed changes of tension.

From studies of the effect of varying the chemical conditions it is concluded that fluctuations in the levels of  $\text{Ca}^{2+}$ ,  $\text{H}^+$ , *ATP*, *ADP*, and inorganic phosphate are not responsible for the oscillatory behaviour of the glycerinated preparation. The mechanical properties of the living and glycerinated muscle are compared; it is concluded that raising the  $\text{Ca}^{2+}$  concentration is equivalent to stimulation of the living muscle, and that the oscillatory contractions produced in the two cases are essentially the same. The glycerol-extraction procedure was not entirely successful in producing structural isolation of the contractile protein system, but possible participation of other cellular components in the oscillatory contraction is considered to have been excluded by the chemical conditions (in particular, by the presence of the calcium buffer). It is therefore concluded that the delay between change of length and change of tension is an intrinsic property of the contractile protein system.

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## INTRODUCTION

Studies made during tethered flight have shown that the flight muscles of certain insects undergo cyclical length changes at frequencies that are completely independent of the pattern of excitation (Pringle 1949; Roeder 1951). Muscles that give this *asynchronous* type of contractile response are usually described as *fibrillar*, because their fibres contain very large myofibrils (Tiegs 1955). The reviews by Pringle (1957, 1965) and Boettiger (1960) give full accounts of the structural, biochemical and physiological peculiarities of these muscles, and of their distribution in the Insecta.

Investigations of the mechanical properties of living insect fibrillar muscles have provided an explanation for the asynchronous nature of their contractile responses *in vivo*. It has been shown that the pattern of length and tension changes in the active muscle is determined almost entirely by the mechanical characteristics of the load. If the muscle is coupled to a mechanically resonant system of suitable natural period, then the length and tension changes are sinusoidal (Boettiger 1957a, b; Machin & Pringle 1959); on the other hand, if the load is a 'click' system, then the muscle undergoes a series of alternate abrupt changes of length and tension (Boettiger & Furshpan 1954; Pringle 1954). This unusual behaviour results from the fact that in muscles of this type, unlike vertebrate skeletal muscle, shortening is followed by a *delayed* fall of tension, and elongation is followed by a *delayed* rise of tension. Each cycle of length change results in a net transfer of energy from the muscle to the load, and the oscillation or 'click' sequence therefore continues as long as an active state is maintained in the muscle.

It has been suggested by Machin & Pringle (1960) that the delay between change of length and change of tension might involve the excitation-contraction coupling system, with some intracellular membrane system as the transducer element, or that it might be an intrinsic property of the contractile mechanism. In this paper we have tried to decide between these two possibilities by examining the properties of insect fibrillar muscle after glycerol extraction. A preliminary report of this work has already been given by Jewell, Pringle & Rüegg (1964).

## MATERIAL AND SOLUTIONS

## (a) Material

The fibrillar muscles used in the present experiments were the dorsal longitudinal muscles of the giant (tropical) water bugs, *Hydrocyrius colombiae*, *Lethocerus cordofanus*, and *L. maximus* (Order: Hemiptera). Each muscle contained approximately 6000 fibres, about 70 µm in diameter, and up to 1.5 cm in length. The muscles were glycerinated *in situ*, using the schedule given by Huxley (1963), and stored at -18°C for 1 to 20 weeks. Because insect muscles contain so little connective tissue, it was a simple matter to prepare single fibres or small bundles of fibres by teasing with fine steel needles in a drop of 50% glycerol. The preparation was then transferred to the mechanical apparatus, where it was fixed between glass or stainless steel hooks by means of a fast-setting glue (celluloid dissolved in acetone).

(b) *Solutions*

All solutions were made up in glass distilled water that had been passed through Amberlite 120-H ion-exchange resin. The inorganic reagents were of analytical grade; the *EGTA* (ethylene glycol bis-( $\beta$ -aminoethyl ether)-*N,N'*-tetraacetic acid) was obtained from the General Chemical Company, Sudbury, Middlesex; and the *ATP* (adenosine triphosphoric acid, disodium salt) and L-histidine hydrochloride came from the Sigma Company, London.

Stock solutions were made of KCl, MgCl<sub>2</sub>, *EGTA* and Ca*EGTA* (made by mixing exactly equimolar amounts of *EGTA* and CaCl<sub>2</sub>). Fresh solutions of histidine and *ATP* were made up as required. Three basic solutions, designated *A*, *B* and *C*, were used throughout; their compositions were as follows:

solution *A*: 40 mM K<sup>+</sup>, 5 mM Mg<sup>2+</sup>; 45 mM Cl<sup>-</sup>, 5 mM (histidine Cl)<sup>-</sup>; pH 7·0, ionic strength 0·055.

solutions *B* and *C*: 65 mM K<sup>+</sup>, 10 mM Na<sup>+</sup>; 51 mM Cl<sup>-</sup>, 10 mM (histidine Cl)<sup>-</sup>; 5 mM (Mg*ATP*)<sup>2-</sup>, and either 2 mM *EGTA*<sup>2-</sup>(solution *B*) or 2 mM (Ca*EGTA*)<sup>2-</sup>(solution *C*); pH 7·0, ionic strength 0·082.

The Ca<sup>2+</sup> concentration in solutions *B* and *C* was stabilized by the presence of the chelating agent, *EGTA*. Over the range 10<sup>-8</sup> to 10<sup>-5</sup> M Ca<sup>2+</sup> (pH 7·0), this substance behaves as a calcium buffer with a very low affinity for the other metal ions present, notably Mg<sup>2+</sup> (Weber & Winicur 1961; Portzehl, Caldwell & Rüegg 1964). The formulae given by Portzehl *et al.* (1964) were used to calculate apparent association constants for the chelation of Ca<sup>2+</sup> and Mg<sup>2+</sup> at pH 7·0 from the true association constants given by Bjerrum, Schwarzenbach & Sillén (1957) for ionic strength 0·1 and temperature 20 °C. The combined apparent association constant for chelation of Ca<sup>2+</sup> at zero Mg<sup>2+</sup> concentration was 4·83 × 10<sup>6</sup>, and the corresponding value for chelation of Mg<sup>2+</sup> at zero Ca<sup>2+</sup> concentration was 40·5. No significant errors arose from the use of these constants in calculating the Ca<sup>2+</sup> concentration in our solutions, in which the experimental conditions were slightly different (ionic strength 0·08, temperature 20 to 24 °C).

Solutions containing various Ca<sup>2+</sup> concentrations in the range 10<sup>-9</sup> to 10<sup>-5</sup> M could be obtained by mixing solutions *B* and *C* in different proportions. The following formulae were used to calculate the Ca<sup>2+</sup> concentration:

$$\frac{[\text{Ca } \textit{EGTA}]}{[\text{free } \textit{EGTA}]} \approx \frac{\text{volume of solution } C}{\text{volume of solution } B} \quad (\text{i})$$

$$[\text{Ca}^{2+}]_0 = \frac{[\text{Ca } \textit{EGTA}]}{[\text{free } \textit{EGTA}]} \times \frac{1}{4.83 \times 10^6} \quad (\text{ii})$$

$$[\text{Ca}^{2+}]_{\text{Mg}} = [\text{Ca}^{2+}]_0 (1 + 40[\text{Mg}^{2+}]) \quad (\text{iii})$$

The approximation given by equation (i) is very close provided that solution *C* contains exactly equimolar quantities of CaCl<sub>2</sub> and *EGTA*. By substitution of the ratio from equation (i) in equation (ii) the Ca<sup>2+</sup> concentration, [Ca<sup>2+</sup>]<sub>0</sub>, for zero Mg<sup>2+</sup> concentration is obtained. Finally, in equation (iii) the Ca<sup>2+</sup> concentration is corrected for the presence of Mg<sup>2+</sup>. In all our experiments except those in which the Mg<sup>2+</sup> concentration was varied, this correction could be neglected because the

$Mg^{2+}$  concentration was very low owing to chelation of  $Mg^{2+}$  by the *ATP*. (Solutions *B* and *C* contained equimolar (5 mm) concentrations of  $MgCl_2$  and *ATP*, and from the association constants given by Martell & Schwarzenbach (1956) it can be calculated that the  $Mg^{2+}$  concentration was only about  $10^{-5}M$ .)

Solution *B* contained a small amount of calcium due to impurities, especially in the *ATP*, which Seidel & Gergely (1963*a*) and Lorand, Demovsky, Meisler & Molnar (1963) have shown may have a calcium content of up to 0.02 %. However, because of the presence of *EGTA*, it is unlikely that the  $Ca^{2+}$  concentration of solution *B* exceeded  $10^{-9}M$ .

#### EXPERIMENTAL TECHNIQUE

The two techniques that have been used to investigate the mechanical properties of glycerinated muscle fibres were designed to detect the characteristic features of activity observed in the living fibrillar muscle, namely, tension development, and the property that changes of length result in delayed changes of tension. The first two parts of this section are concerned with the techniques used in (*a*) free oscillation experiments, and (*b*) driven oscillation experiments, and the last part (*c*) gives details of the methods of calculation used in these experiments.

##### (*a*) Free oscillation experiments

As in the experiments of Machin & Pringle (1959) on living fibrillar muscle, the principle of the free oscillation technique was to examine the behaviour of the muscle when coupled to a mechanically resonant load.

###### (i) Apparatus

The resonant load consisted of a light, vertically orientated, stainless steel lever, the upper end of which was fixed to a weak torsion band. The lever provided the inertia and the torsion band the restoring force necessary for mechanical oscillation. The preparation was horizontal in orientation; one end was glued to the lever and the other end was glued to a similar strip of stainless steel, which was rigidly mounted on a micrometer movement. The length of the preparation could be adjusted by means of the micrometer. Movements of the lever were detected by a small mirror which was mounted on the torsion band; this reflected a beam of light on to a ground glass screen placed about 120 cm from the mirror. An arrangement of lenses made the spot of light intense enough to be photographed by a 35 mm Cossor oscilloscope camera running at 1 in/s (2.54 cm/s).

###### (ii) Load parameters

The load parameters of the lever system were its stiffness, damping, and equivalent mass. The stiffness of the system had to be rather low ( $350 \text{ dyn cm}^{-1}$ ) in order to make it sensitive enough for use with single fibre preparations. The recording conditions were therefore *auxotonic*, so the system was not very suitable for accurate measurements of tension development. The damping of the system was due mainly to the viscous drag of the bathing solution on the tip of the lever,

and it varied with the depth of immersion. The damping constant could be calculated from the damping factor of the transient response; for example, in record A (i) of figure 3, plate 21, it was  $0.24 \text{ dyn s cm}^{-1}$ . The natural frequency of the lever system with a muscle fibre attached and immersed in the bathing solution depended on the interaction of the combined stiffness of the torsion band and the preparation with the total equivalent mass of the lever system, preparation, and the fluid displaced. It was always 40 c/s or less, and frequencies down to about 5 c/s could be obtained by the judicious placing of small pellets of Plasticine on the lever system to increase its equivalent mass. The present experiments differed from those of Machin & Pringle (1959) in that the equivalent mass was the only load parameter that could easily be varied.

#### (iii) Interpretation of results

The important feature of the free oscillation technique was that it provided a simple means of detecting a delay between change of length and change of tension, because this was equivalent to the presence of *negative damping* in the system (that is, to the muscle acting as a source of power). If the negative damping due to the muscle exceeded the positive damping due to the viscosity of the bathing solution, then a sustained oscillation was the result. The amplitude of oscillation depended on the negative damping, and it therefore provided an index of the delay between change of length and change of tension at that frequency. The power output of the muscle fibre could be calculated from the frequency and amplitude of oscillation, and the damping constant (see below).

#### (iv) Experimental details

The natural frequency of the lever was first adjusted to the required value, which was usually above 10 c/s because the apparatus was difficult to handle at low frequencies, and the stainless steel brackets were set exactly 0.5 cm apart. The fibre was then glued in place, and a solution bath (capacity 5 ml.) was raised around it by means of a movable platform. The solution was changed in one of two ways: in some experiments baths containing different solutions were raised around the fibre, but this produced artifacts due to surface tension effects during the solution change, and it sometimes resulted in breakage of the fibre; in other experiments (including those illustrated by figures 2 and 3, plate 21, and figures 6 and 7, plate 22), a single bath with inlet and outlet tubes was used as a flow-through system. This arrangement avoided the surface tension effects, but large volumes of solution were required to ensure adequate flushing of the system, and it was not possible to produce abrupt changes in the composition of the bathing solution.

##### (b) Driven oscillation experiments

The technique used in the present experiments was very similar to that developed by Machin & Pringle (1960) for studies of living fibrillar muscle. It was unfortunate that at the time the present experiments were made this technique had not been developed to the point where it was sensitive enough for use with single fibre

preparations. Bundles of 5 to 10 muscle fibres were used, and it was acknowledged at the outset that problems might arise due to inadequate diffusion of ATP into the core of the preparation during activity.

### (i) Apparatus

The preparation, which was horizontal in orientation, was fixed between two glass hooks. One was an extension of the anode deflector shaft of an RCA 5734 force transducer, which was carried on a microscope movement so that the length of the preparation could be adjusted. The other hook was mounted on the armature of a Goodmans V 47 electro-magnetic vibrator, which was operated as part of a servomechanism (see Machin 1959). The drive for the vibrator was provided by amplifiers designed and built by Cambridge Prototypes Limited, of Cambridge, and the movement of the vibrator armature was sensed by a variable inductance transducer system (Cambridge & Haines 1959). The output of this displacement transducer provided the negative feedback of the servo control system; it linearized the performance of the vibrator, increased its mechanical output impedance, and ensured that the vibrator movement followed any sinusoidal input with negligible phase error and amplitude distortion over the frequency range used in the present experiments (0 to 150 c/s).

When no external input was applied to the vibrator driver system, the recording conditions were essentially *isometric*, because the stiffness of the apparatus was about  $4 \times 10^5$  dyn cm<sup>-1</sup>, and *aperiodic*, because any resonances in the apparatus were at high frequencies compared with those at which the muscle could produce power. Accurate measurements could therefore be made of tension development by the preparation under these conditions. However, the apparatus was usually operated with an external input, and the tension was measured during the imposition of length changes on the muscle. In most of the present experiments the input was a sine wave (sinusoidal analysis), but some studies were also made with a square wave input (transient analysis).

### (ii) Sinusoidal analysis

The application of this technique to biological systems has been discussed by Machin & Pringle (1960) and by Machin (1964). When sinusoidal length changes were imposed on a preparation bearing tension (due to stretch or activity), the tension also showed sinusoidal variations, but the phase relationship between length and tension changes depended on the frequency of oscillation and the state of the preparation. The tension change could be resolved into two components: one in-phase with length, which measured the elastic properties of the preparation, and the other in quadrature to length (that is, either leading or lagging by 90°), which provided a measure of its viscous properties. The resolution of tension changes into these two components was done by feeding the output of the force transducer into a Solartron resolved components indicator (type VP. 253.3); this was used in conjunction with a matching oscillator (type OS.103.3), which provided the input to the vibrator driver system and the reference input for the resolved component indicator.

The resolved components of tension change were converted into elastic and viscous moduli, as described in § (c), and the results obtained at a number of frequencies were displayed as a vector modulus plot (see, for example, plot (c) of figure 4A). Each point refers to a particular frequency, and its coordinates give the viscous and elastic moduli of the muscle at that frequency. If a line is drawn from the point to the origin, its length gives the dynamic modulus of the muscle and the angle that it makes with the abscissal axis gives the phase difference between length and tension changes. If the line is above the abscissal axis, there is a phase *advance* of tension on length at that frequency, and the viscous modulus is *positive* (that is, work is being done by the apparatus on the muscle). On the other hand, if the line is below the axis, there is a phase *lag* of tension on length and the viscous modulus is *negative* (that is, work is being done on the apparatus by the muscle).

### (iii) *Transient analysis*

The principle of this technique is to examine the response of a system to a sudden change in its input (see Machin 1964). Transient analysis has been used extensively in studying the mechanical properties of various types of muscle, including living insect fibrillar muscle (Boettiger 1957 *a, b*). Some studies of the transient response of the glycerinated material have been made to see if it behaves in the same way as living muscle when subjected to rapid releases and stretches. Rapid movements of the type required were obtained by supplying the vibrator driver system with a square wave input from the Solartron oscillator; the resulting tension changes were displayed on a Tektronix 502 A oscilloscope and photographed with a Cossor 35 mm camera.

### (iv) *Experimental details*

The glass hooks were set 0.5 cm apart, and the preparation was glued between them. A solution bath was raised around the preparation, and its length was adjusted to give the required tension. In all the driven oscillation experiments the solution was changed by raising different baths of solution around the preparation. The amplitude of the imposed length change was usually restricted to about 2  $\mu\text{m}$ , r.m.s., which corresponded to just over 0.1 % (peak to peak) of the muscle length, in order to avoid errors due to the curvature of the elastic characteristics of the muscle (see Machin & Pringle 1960). Greater amplitudes were used in experiments where high power outputs were required. The frequencies used in making a sinusoidal analysis were as follows: 1, 2, 3, 4, 5, 7, 10, 20, 30, 40, 80 and 100 c/s, and it was usually possible to make a complete ascending or descending series of measurements in 4 to 5 min. For the remainder of the time, a continuous sinusoidal movement was imposed on the muscle, and the outputs of the length and force transducers were displayed on the X and Y axes of the oscilloscope. The elliptical tension-length plot obtained provided a useful monitor of the state of the preparation; the shape and inclination of the loop gave rough indications of the viscous and elastic properties of the muscle, the sense of rotation showed the direction of power transfer between the muscle and the apparatus, and the mean deflexion on

the *Y* axis gave the mean tension in the preparation. The mean tension obtained in this way was the same as the isometric tension developed in the absence of imposed length changes. The 'idling' frequency used for monitoring purposes was  $f_{V\max}$  (defined below), which was 5 c/s for preparations of *H. colombiae* and *L. cordofanu*s, and 2 c/s for *L. maximus*.

(c) *Methods of calculation*

(i) *Muscle fibre dimensions*

No attempt was made to determine the cross-sectional area of each fibre used because of the problems that arise due to irregularities of the transverse profile of muscle fibres (see Blinks 1965). Measurements of the diameter of glycerinated fibres suspended in solution *B* (a relaxing medium) indicated that the fibre population was fairly uniform, with a mean diameter of 70  $\mu\text{m}$ , so a standard cross-sectional area of  $3.85 \times 10^{-5} \text{ cm}^2$  was assumed for each. Similarly, the unstretched length of the preparation (nominally 0.5 cm) was assumed as the standard fibre length, and  $1.92 \times 10^{-5} \text{ g}$  was assumed as the standard fibre weight.

(ii) *Conversion to absolute units*

The standard dimensions were used to convert tensions from dynes per fibre ( $\text{dyn fibre}^{-1}$ ) into kilogrammes force per square centimetre ( $\text{Kg cm}^{-2}$ ); amplitudes of movement from microns (peak to peak) into percentages of the muscle length; and power outputs from output per fibre ( $\text{erg s}^{-1} \text{ fibre}^{-1}$ ) to output per gramme wet weight of muscle ( $\text{erg s}^{-1} (\text{g muscle})^{-1}$ ).

(iii) *Elastic and viscous moduli*

The expressions given by Machin & Pringle (1960) were used to convert the results of sinusoidal analysis into elastic and viscous moduli:

$$E_e = \frac{\Delta F_e \times L}{\Delta L \times A}, \quad E_v = \frac{\Delta F_v \times L}{\Delta L \times A},$$

where  $E_e$  and  $E_v$  ( $\text{Kg cm}^{-2}$ ) are the elastic and viscous moduli,  $L$  (cm) and  $A$  ( $\text{cm}^2$ ) are the standard fibre length and cross-sectional area, and the remaining quantities are root mean square amplitudes:  $\Delta L$  (cm) is the length change, and  $\Delta F_e$  and  $\Delta F_v$  ( $\text{dyn fibre}^{-1}$ ) are the in-phase and quadrature components of the tension change, respectively.

(iv) *Power outputs*

The oscillatory power output of the fibre could be calculated as follows:

$$\begin{aligned} \text{power output} &= \text{r.m.s. velocity} \times \text{r.m.s. force}, \\ P &= 2\pi f \cdot \Delta L \times \Delta F_v, \end{aligned}$$

where  $P$  ( $\text{erg s}^{-1} \text{ fibre}^{-1}$ ) is the power output, and  $f$  (c/s) is the frequency of oscillation. In the driven oscillation experiments,  $\Delta F_v$  was obtained directly from the

resolved component indicator, and in the free oscillation experiments it was calculated as follows:

$$\text{r.m.s. force} = \text{r.m.s. velocity} \times \text{damping constant},$$

$$\Delta F_v = 2\pi f_v \Delta L \times D,$$

where  $D$  (dyn s cm $^{-1}$ ) is the damping constant of the system.

#### (v) Frequency parameters

Three frequency parameters will be used in referring to the vector modulus plot:  $f_{V_{\max}}$ , the frequency at which the viscous modulus has its maximum negative value (5 c/s in figure 4);  $f_{V_0}$ , the frequency at which the viscous modulus changes from negative to positive (about 22 c/s, by interpolation, in figure 4); and  $f_{P_{\max}}$ , the frequency at which the power output is maximum (about 10 c/s in figure 4).

## RESULTS

### (a) Preliminary observations

Some preliminary experiments were made to establish the approximate conditions for activation of the glycerinated material. First, myofibrils (diameter 5  $\mu\text{m}$ ) were teased from muscle fibres of *L. cordofanus* and examined by phase-contrast microscopy. In buffered salt solution the sarcomere length was about 2.7  $\mu\text{m}$  and there were  $H$  zones, but no detectable  $I$  bands. Irrigation of the myofibrils with a solution containing 5 mM MgATP and Ca $^{2+}$  at impurity level ( $\sim 10^{-5}$  M) resulted in shortening of the sarcomeres. Length changes of a few per cent were associated with the appearance of  $C_z$  contraction bands (Hodge 1955; Hanson 1956), and shortening to sarcomere lengths less than about 2.4  $\mu\text{m}$  also resulted in  $C_m$  contraction bands.

Single glycerinated fibres were then set up in the free oscillation apparatus, and immersion in the MgATP solution resulted in the development of tensions up to 0.2 Kg cm $^{-2}$ . Relaxation was produced by adding EDTA (ethylene diamine tetra-acetate, 2 mM) to the solution, but the tension could be re-established either by adding CaCl $_2$  to this solution or by immersing the preparation in a fresh batch of the original MgATP solution. The cycle of contraction and relaxation could be repeated many times, but often after three or four cycles the tension development was less and the fibre began to oscillate at about the natural frequency of the lever system. On other occasions, oscillations were only detected when the tension was rising after immersion in the MgATP solution, or when it was falling after the addition of EDTA. These results suggested that the appearance of oscillations depended on the Ca $^{2+}$  concentration, so in all the subsequent experiments this was stabilized and controlled by the presence of a Ca $^{2+}$  buffer system (EGTA).

### (b) The 'resting' preparation

When the preparation was immersed in buffered salt solution (solution A) at the beginning of each experiment, it was very inextensible. Figure 1a shows the type of vector modulus plot that was obtained by sinusoidal analysis at a mean tension

of  $0.029 \text{ Kg cm}^{-2}$ . When the preparation was transferred to an *ATP* solution containing a low  $\text{Ca}^{2+}$  concentration (solution *B*), there was always a transient rise of tension followed by complete relaxation. When the tension was reset at its previous value by stretching the preparation, sinusoidal analysis showed that the elastic modulus had fallen to about a half and the viscous modulus to about a quarter

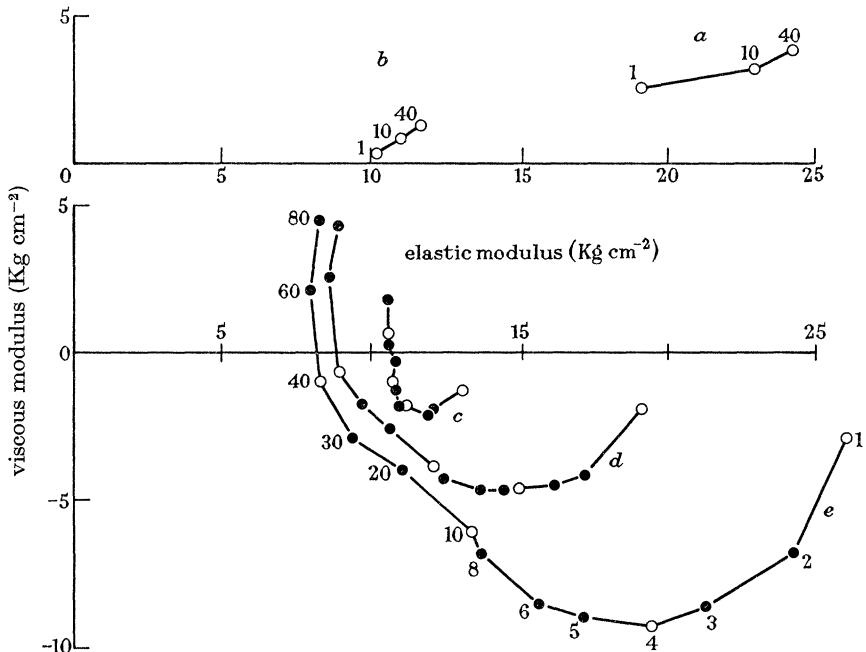


FIGURE 1. Driven oscillation experiment. *Upper graph*: vector modulus plots illustrating the plasticizing action of *ATP* at different frequencies (indicated alongside plots); (a) buffered salt solution (solution *A*), (b) low  $\text{Ca}^{2+}$ , *ATP* solution (solution *B*). *Lower graph*: vector modulus plots showing different levels of activity in mixtures of solutions *B* and *C*, giving the  $\text{Ca}^{2+}$  concentrations indicated in the following table:

plot	solution	$[\text{Ca}^{2+}]$	mean tension ( $\text{Kg cm}^{-2}$ )
a	<i>A</i>	—	0.029
b	<i>B</i>	$\sim 10^{-9} \text{ M}$	0.029
c	<i>C/B</i> (0.25)	$5.2 \times 10^{-8} \text{ M}$	0.041
d	<i>C/B</i> (0.67)	$1.4 \times 10^{-7} \text{ M}$	0.088
e	<i>C/B</i> (1.00)	$2.1 \times 10^{-7} \text{ M}$	0.152

Preparation: *L. cordofanus* (6 fibres, length 0.5 cm); temp. 22°C. Oscillation: amplitude 0.13%; frequencies (c/s) as indicated. The frequencies for plots *c* and *d* were the same as for plot *e*, and the points at 1, 4, 10 and 40 c/s are shown as open circles to facilitate their identification.

of the corresponding values in solution *A* (figure 1*b*). The elastic modulus could also be estimated from the change in natural frequency of the free oscillation apparatus produced by coupling it to the muscle fibre (figure 3, plate 21); measurements by either method gave values (5 to 10  $\text{Kg cm}^{-2}$ ) that corresponded well with those obtained by Machin & Pringle (1960) from living insect fibrillar muscle at comparable resting tensions. The shape of the vector modulus plot was also similar, so the glycerinated muscle in solution *B* was regarded as the equivalent of the

living muscle in the resting state. Before activation was produced by raising the  $\text{Ca}^{2+}$  concentration, the preparation was always stretched to give an initial tension of 1 to 4 dyn fibre $^{-1}$  (0.025 to 0.1 Kg cm $^{-2}$ ) in solution *B*.

### (c) Calcium activation

#### *Free oscillation experiments*

Figure 2, plate 21, shows the results of two experiments in which activation was produced by transferring the preparation from solution *B* to mixtures of solutions *B* and *C* giving different  $\text{Ca}^{2+}$  concentrations. In both experiments, raising the  $\text{Ca}^{2+}$  concentration to  $8.9 \times 10^{-8} \text{ M}$  resulted in oscillations but the mean tension remained unchanged. The effects produced by immersing the preparation in solutions containing higher  $\text{Ca}^{2+}$  concentrations differed in the two experiments: in one the mean tension increased (figure 2*d*, *e*, plate 21), and in the other it remained unchanged (figure 2*a* to *c*); but in both cases the amplitude of oscillation increased. The fibres varied a good deal in the extent to which the mean tension increased upon activation, but in all other respects they behaved identically. Returning to solution *B* resulted in a fall of tension and decay of the oscillation (figure 2*f*, *g*). The oscillation could be switched on and off repeatedly by raising and lowering the  $\text{Ca}^{2+}$  concentration, and once switched on it would last for many hours (the longest recorded period was 23 h). Although oscillation was possible at  $\text{Ca}^{2+}$  concentrations up to the limit of the range covered by the *EGTA* buffer ( $\sim 10^{-5} \text{ M}$   $\text{Ca}^{2+}$  at pH 7.0), the increase in amplitude produced by solutions containing more than  $5 \times 10^{-7} \text{ M}$   $\text{Ca}^{2+}$  was slight.

Figure 3, plate 21, shows that both the natural frequency of the lever and the frequency of the oscillatory contraction depended on the equivalent mass of the moving system (which could be varied by altering the amount of Plasticine attached to the lever). The photographic records of the lever motion have been arranged in three horizontal rows (*A*, *B*, *C*), each corresponding with a different equivalent mass, and in vertical groups to show the following:

(i) The transient response of the lever system alone with the tip of the lever immersed in the bathing solution. From the known stiffness of the system (350

#### DESCRIPTION OF PLATE 21

FIGURE 2. Free oscillation experiment. Records showing activation by raising the  $\text{Ca}^{2+}$  concentration. Two single fibre preparations, length 0.5 cm; temp. 20°C. Records (*a* to *c*)—*L. cordofanus*; (*a*)  $8.9 \times 10^{-8} \text{ M}$   $\text{Ca}^{2+}$ , 10 s interval between two parts of record; (*b*)  $2.1 \times 10^{-7} \text{ M}$   $\text{Ca}^{2+}$ ; (*c*)  $1.2 \times 10^{-6} \text{ M}$   $\text{Ca}^{2+}$ . Records (*d* to *g*)—*L. maximus*; (*d*)  $8.9 \times 10^{-8} \text{ M}$   $\text{Ca}^{2+}$ ; (*e*)  $1.9 \times 10^{-6} \text{ M}$   $\text{Ca}^{2+}$ ; (*f*, *g*) continuous record showing termination of oscillatory response on returning to solution *B*. Calibrations: between Records (*c*) and (*d*); zero tension corresponds with lower edge of record in each case.

FIGURE 3. Free oscillation experiment. Records showing the dependence of the frequency of oscillation on the natural frequency of the lever system and the mean tension in the fibre. The natural frequency was as follows: (*A*) 6.9 c/s, (*B*) 17 c/s, (*C*) 1.9 c/s. Records: (i) transient response of lever system alone, (ii) transient response of lever when attached to a resting fibre in solution *B*, (iii) active oscillation produced by raising the  $\text{Ca}^{2+}$  concentration to  $2.1 \times 10^{-7} \text{ M}$ . The tension was varied by stretching the fibre in solution *B*. Preparations: *L. cordofanus*, length 0.5 cm; temp. 21°C; Rows *A* and *B* single fibre, Row *C* two fibres. Calibrations: as for figure 2.

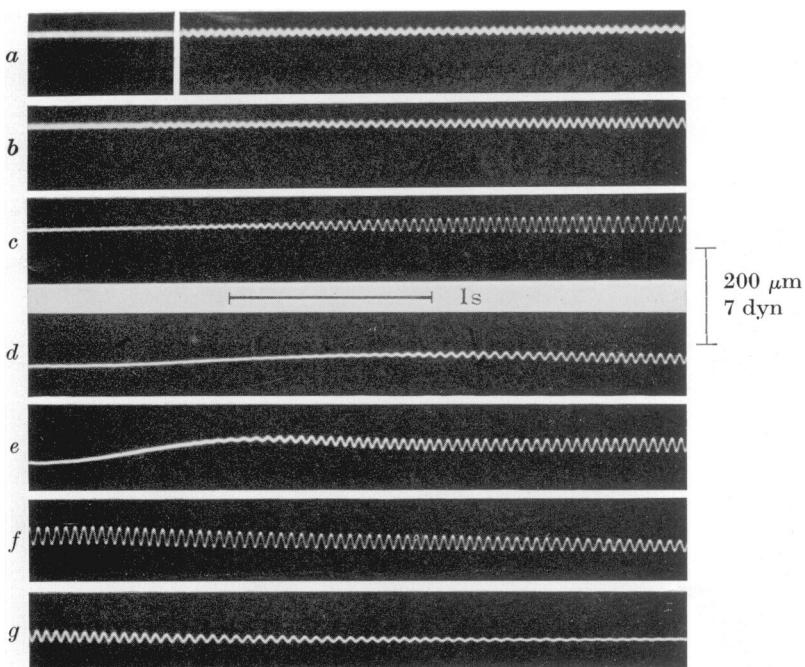


FIGURE 2

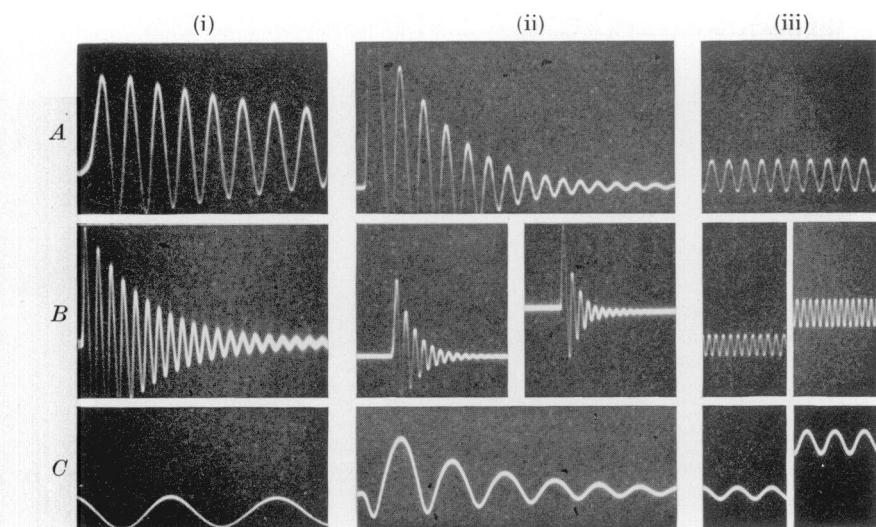


FIGURE 3

(Facing p. 438)

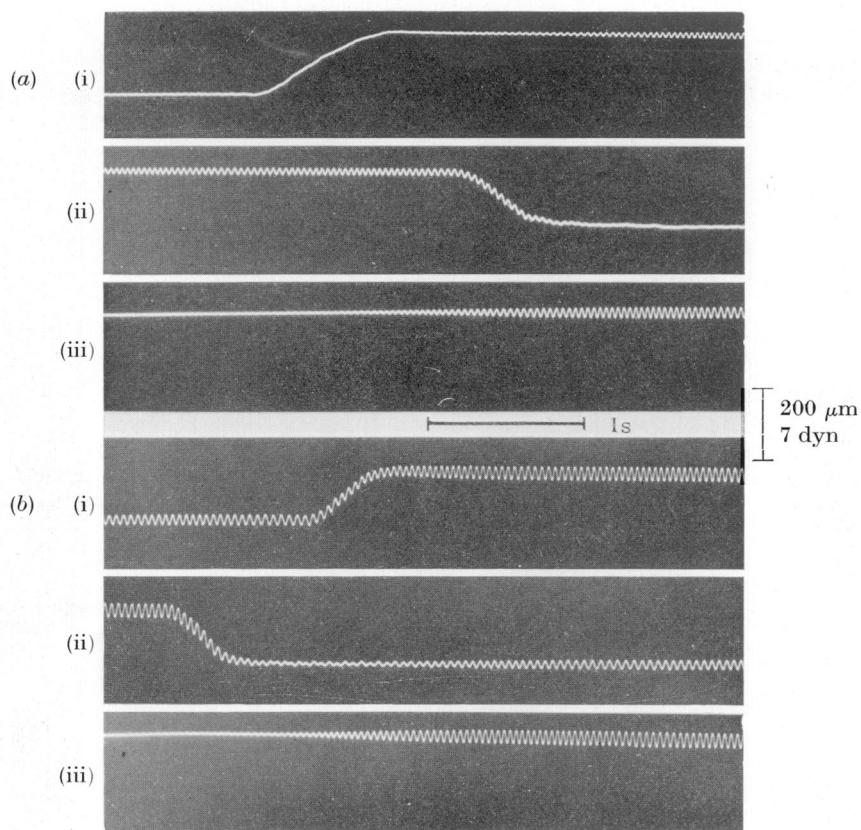


FIGURE 6

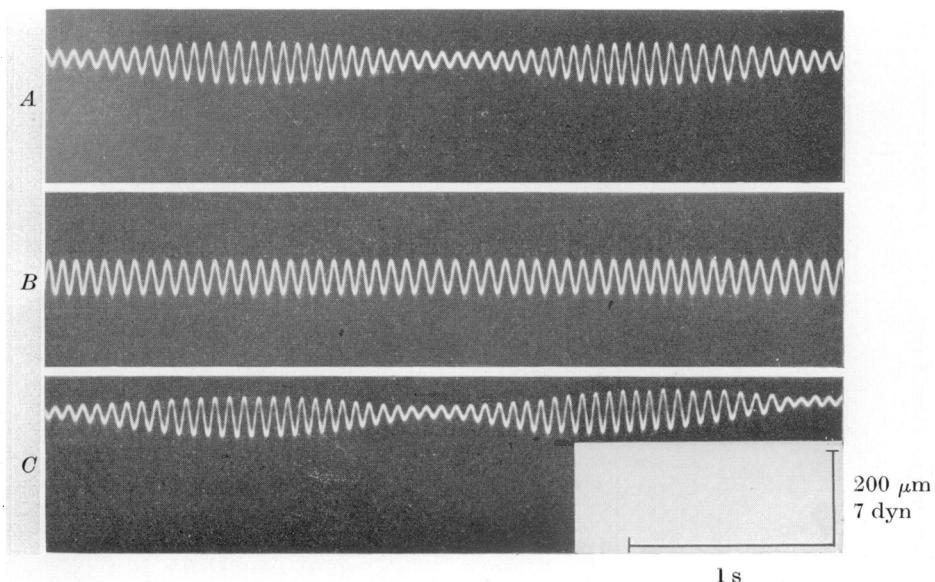


FIGURE 7

dyn cm<sup>-1</sup>), the frequency, and the damping factor, the equivalent mass and the damping constant could be calculated; for example, in record A (i) the frequency was 6.9 c/s, the equivalent mass 186 mg, and the damping constant 0.24 dyn s cm<sup>-1</sup>.

(ii) The transient response of the lever system when attached to a resting muscle fibre immersed in solution B. The damping factor was increased due to the positive viscous modulus of the resting muscle fibre, and the frequency was greater because of the increase in overall stiffness of the system. From the change in frequency, the stiffness of the fibre could be estimated if it was assumed that the equivalent mass was not altered by the presence of the muscle fibre: the value obtained from record A (ii) was 505 dyn cm<sup>-1</sup> which corresponds with an elastic modulus of 6.6 Kg cm<sup>-2</sup>.

(iii) The oscillation produced by activating the preparation. The frequency was greater than before because of the increase in elastic modulus produced by activation; this effect was most marked when the natural frequency was low, as in the records of series C. From the frequency and amplitude of oscillation, and the damping constant, the power output of the fibre could be calculated; for example, in record A (iii) the frequency was 11.1 c/s, the amplitude 64 µm (peak to peak), and the power output was  $6 \times 10^{-3}$  erg s<sup>-1</sup>, which corresponds to 310 erg s<sup>-1</sup> (g muscle)<sup>-1</sup>.

Experiments in which the equivalent mass was varied showed that oscillation was possible over a wide range of frequencies (about 5 c/s up to 45 c/s). It was apparent from the driven oscillation experiments that the power output was maximal at fairly low frequencies, but for technical reasons it was more convenient to use frequencies of 10 c/s or above in the free oscillation experiments.

#### Driven oscillation experiments

Experiments on the effect of raising the Ca<sup>2+</sup> concentration were carried out as follows: the resting tension was set at about 0.03 Kg cm<sup>-2</sup> in solution B and a vector modulus plot obtained; the Ca<sup>2+</sup> concentration was then raised by changing the solution for a mixture of solutions B and C, and the vector modulus plot was determined when the mean tension had reached a plateau; the preparation was

#### DESCRIPTION OF PLATE 22

FIGURE 6. Free oscillation experiment. Records showing the effect of stretch on the activity of a single fibre in solutions containing (a)  $8.9 \times 10^{-8}$  M Ca<sup>2+</sup>, (b)  $2.9 \times 10^{-7}$  M Ca<sup>2+</sup>. Records: (i) showing activity at low tension, followed by the effect of a stretch, (ii) continuation of same record, showing effect of release; (iii) showing activity at high tension, produced by immersing the fibre in Ca<sup>2+</sup> solution after stretching it in solution B. Preparation: *L. cordofanus*, length 0.5 cm; temp. 20°C. Calibrations: between Records (a) and (b); zero tension corresponds with the lower edge of the record in each case.

FIGURE 7. Free oscillation experiment. Records showing spontaneous amplitude modulation of the oscillatory response of a single fibre at high mean tensions. Records: (A) mean tension 10 dyn, (B) mean tension lowered to 7 dyn by releasing the fibre, (C) mean tension restored to 11 dyn by stretching. Preparation: *L. cordofanus*, length 0.5 cm, activated by a solution containing  $2.9 \times 10^{-7}$  M Ca<sup>2+</sup>; temp. 21°C. Calibrations: shown in inset on record (C); zero tension corresponds with the lower edge of the record in each case.

then returned to solution *B*, where the resting tension was reset (if necessary) and the vector modulus checked at the idling frequency; the preparation was then transferred to the next mixture of solutions *B* and *C*, and so on. The results of a typical experiment are shown in figure 1*c* to *e*, which gives vector modulus plots and the mean tensions at three different activating  $\text{Ca}^{2+}$  concentrations. The

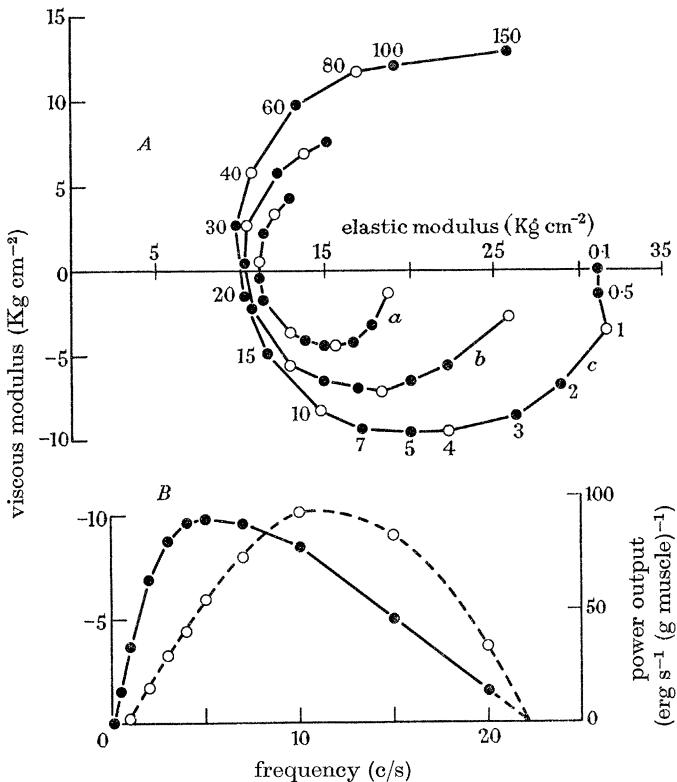


FIGURE 4. *A.* Driven oscillation experiment. Vector modulus plots showing a progressive increase in the level of activity during prolonged immersion in a solution containing  $4.8 \times 10^{-7} \text{ M Ca}^{2+}$ . (a) Obtained 5 min after immersion; the mean tension rose from  $0.078 \text{ Kg cm}^{-2}$  to  $0.082 \text{ Kg cm}^{-2}$  during the measurements; (b) 20 min after immersion, mean tension rising from  $0.155 \text{ Kg cm}^{-2}$  to  $0.201 \text{ Kg cm}^{-2}$ ; (c) 40 min after immersion, mean tension steady at  $0.247 \text{ Kg cm}^{-2}$ . Preparation: *H. colombiae* (8 fibres, length 0.5 cm); temp.  $23.5^\circ\text{C}$ . Oscillation: amplitude 0.12%; frequencies (c/s) as indicated alongside plot (c), which included additional observations at 0.1, 0.5, 15 and 150 c/s. Points obtained at 1, 4, 10 and 40 c/s are shown as open circles to facilitate their identification.

*B.* Same experiment as figure *A*. Variation of viscous modulus (solid circles, left ordinate) and power output (open circles, right ordinate) with oscillation frequency (abscissa). Constructed from data of vector modulus plot (c).

threshold for this preparation was  $2.3 \times 10^{-8} \text{ M Ca}^{2+}$ ; at this concentration, there was no increase in mean tension above the resting value ( $0.029 \text{ Kg cm}^{-2}$ ), but the viscous modulus was slightly negative over the frequency range 2 to 8 c/s (maximum value  $-0.6 \text{ Kg cm}^{-2}$  at 4 c/s). This vector modulus plot has not been included in figure 1 because it was a tight cluster of points located at precisely the same position as plot (c) on the elastic axis. Increases in the  $\text{Ca}^{2+}$  concentration above

the threshold value produced the following effects, which are regarded as indications of an increasing level of activity in the preparation: (1) an increase in mean tension, (2) an increase in the rate of rise of tension, (3) increases in the negative viscous modulus at frequencies below  $f_{V_0}$ . The frequency parameters of the vector modulus plot were independent of the  $\text{Ca}^{2+}$  concentration, except when this was just above the threshold value (figure 1c).

Experiments of this type were often impossible because of a tendency for the mean tension and negative viscous modulus at frequencies below  $f_{V_0}$  to increase with time, even though the  $\text{Ca}^{2+}$  concentration remained constant. Figure 4A shows an extreme example of this phenomenon; vector modulus plot (c) was the final steady state performance reached after almost an hour of immersion in the same solution, and plots (a) and (b) show intermediate levels of activity observed during that period. If a preparation in this state was relaxed by immersion in solution B for a few minutes, and then returned to the original solution, the build-up of activation was repeated starting at a low level as before. The experiment of figure 1 was selected to illustrate activation by raising the  $\text{Ca}^{2+}$  concentration because this tendency for the performance to improve at a given  $\text{Ca}^{2+}$  concentration was slight.

Sometimes a very gradual rise of tension and oscillatory activity of the type seen in figure 4A was followed by a dramatic change in the mechanical properties of the muscle; there was a rapid increase in mean tension and a reduction of the frequency parameters of the vector modulus plot to as little as one tenth of their previous values. This change from 'low tension' state to 'high tension' state could often be produced by raising the  $\text{Ca}^{2+}$  concentration above a critical level, which varied from one preparation to another and to some extent with time in a given preparation. Vector modulus plots obtained in such an experiment are shown in figure 5A. Immersing the preparation in a solution containing  $2.1 \times 10^{-7} \text{ M Ca}^{2+}$  produced the 'high tension' state; the mean tension was  $0.520 \text{ Kg cm}^{-2}$ ,  $f_{V_0}$  was 4 c/s and  $f_{V_{\max}}$  was 1 c/s or less. The preparation was relaxed by immersion in solution B, and then transferred to a solution containing  $1.4 \times 10^{-7} \text{ M Ca}^{2+}$ ; this produced the 'low tension' state, with mean tension of  $0.132 \text{ Kg cm}^{-2}$ ,  $f_{V_0}$  about 40 c/s and  $f_{V_{\max}}$  4 c/s. Further treatment with the solution containing  $2.1 \times 10^{-7} \text{ M Ca}^{2+}$  again gave the 'high tension' state.

The maximum negative viscous modulus was about the same in the 'high tension' and 'low tension' states, but the power output was less in the former state because of the reduction in frequency parameters. When the preparation was immersed in solutions containing high  $\text{Ca}^{2+}$  concentrations (up to  $10^{-5} \text{ M Ca}^{2+}$ ), there was little change in the maximum negative viscous modulus, but the frequency parameters fell further and the maximum power output was reduced accordingly.

#### (d) Effects of varying the chemical conditions

Experiments were made to examine the behaviour of the preparations in solutions that differed in various respects from those specified previously. The effects of the following variations were studied by either free or driven oscillation techniques.

## (i) Ionic strength

Solutions like *B* and *C* were prepared with different KCl contents to give ionic strengths of 0.05 and 0.18. Activation could be produced in both cases by raising the  $\text{Ca}^{2+}$  concentration.

## (ii) pH

Similar solutions were made in which the pH was adjusted to either 6.2 or 7.8, and oscillation still occurred on activation.

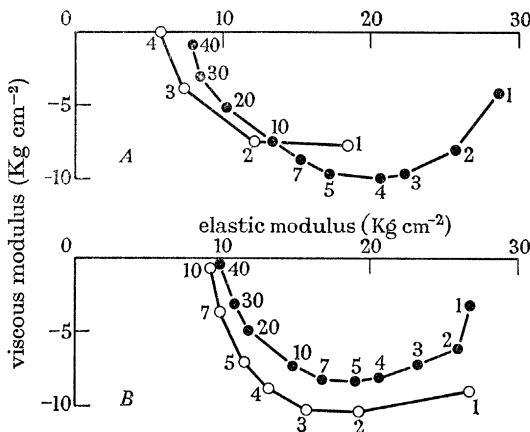


FIGURE 5. *A*. Driven oscillation experiment. Vector modulus plots showing the 'low tension' state (●) and the 'high tension' state (○) produced by different  $\text{Ca}^{2+}$  concentrations. ●,  $1.4 \times 10^{-7} \text{ M Ca}^{2+}$ , mean tension  $0.132 \text{ Kg cm}^{-2}$ ; ○,  $2.1 \times 10^{-7} \text{ M Ca}^{2+}$ , mean tension  $0.520 \text{ Kg cm}^{-2}$ . Preparation: *L. cordofanum* (6 fibres, length 0.5 cm); temp. 22°C. Oscillation: amplitude 0.13%; frequencies (c/s) as indicated.

*B*. Driven oscillation experiment. Vector modulus plots showing reversal of the 'high tension' state (○) by stopping oscillation. Preparation immersed in solution containing  $1.1 \times 10^{-7} \text{ M Ca}^{2+}$  throughout. ○, Plot obtained 25 min after immersion, mean tension  $0.218 \text{ Kg cm}^{-2}$ . The oscillatory drive was then switched off for 14 min. ●, Plot obtained after re-starting the oscillation, mean tension  $0.071 \text{ Kg cm}^{-2}$ . Preparation: *H. colombiae* (8 fibres, length 0.5 cm); temp. 24.5°C. Oscillation: amplitude 0.12%; frequencies (c/s) as indicated.

(iii) Reduced  $\text{Mg}^{2+}$  concentration

Experiments were made with the following solutions, which contained no added  $\text{Mg}^{2+}$  except at impurity levels:

solution *D*: KCl 40 mM, histidine 10 mM.

solution *E*: solution *D*, with EGTA 2 mM and EDTA 1 mM.

solution *F*: solution *E*, with ATP 5 mM.

The pH was adjusted to 7.0 in each case. Solutions *E* and *F* included EDTA, which chelates  $\text{Mg}^{2+}$ , in order to remove any  $\text{Mg}^{2+}$  introduced into the bathing solutions by the preparation or as impurities in the chemicals used, especially the ATP (see Offer 1964). Because of the presence of both EDTA and EGTA, these solutions therefore contained virtually no divalent cations.

In solution *D*, the tension was adjusted to  $0.026 \text{ Kg cm}^{-2}$  and sinusoidal analysis gave results that were indistinguishable from those obtained in solution *A*. The effect of transferring the preparation to solution *E* was rather like that obtained in going from solution *A* to *B* (see figure 1*a, b*). The tension dropped to zero immediately, and when the preparation was stretched to produce a tension of  $0.026 \text{ Kg cm}^{-2}$ , sinusoidal analysis showed that the viscous and elastic moduli had fallen to about half. However, during the next 10 min they crept back to their original values in solution *D*. Following subsequent immersion in solution *F*, the tension and both moduli rose for about 4 min and then returned slowly to their initial values during the next 10 min. After this it was impossible to activate the preparation by raising the  $\text{Ca}^{2+}$  concentration of either  $\text{Mg}^{2+}$  free solutions or the usual solutions *B* and *C*. It was also noted that treatment with  $\text{Mg}^{2+}$  free solutions made the fibres exceptionally fragile.

#### (iv) Raised $\text{Mg}^{2+}$ concentration

Two experiments were made on the effects of raising the  $\text{Mg}^{2+}$  concentration. In the first it was found that the activity produced by raising the  $\text{Ca}^{2+}$  concentration from about  $10^{-9} \text{ M}$  to  $1.1 \times 10^{-7} \text{ M}$  could be abolished by raising the  $\text{Mg}^{2+}$  concentration from about  $10^{-5} \text{ M}$  to  $2 \times 10^{-3} \text{ M}$ . The level of activity in the preparation could then be controlled by varying the  $\text{Mg}^{2+}$  concentration and keeping the  $\text{Ca}^{2+}$  concentration constant. In the second experiment, the level of activity produced by  $2.5 \times 10^{-7} \text{ M}$   $\text{Ca}^{2+}$  in the presence of  $1.3 \times 10^{-3} \text{ M}$   $\text{Mg}^{2+}$  was found to be the same as that produced by  $1.1 \times 10^{-7} \text{ M}$   $\text{Ca}^{2+}$  in the presence of about  $10^{-5} \text{ M}$   $\text{Mg}^{2+}$ . Although  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$  had antagonistic actions on the level of activity, it is clear that the sensitivity to  $\text{Ca}^{2+}$  was much greater than that to  $\text{Mg}^{2+}$ .

#### (v) $\text{MgATP}$ concentration

Oscillatory contractions could be obtained by raising the  $\text{Ca}^{2+}$  concentration of solutions containing either 3 or 8 mM  $\text{MgATP}$ . The amplitude of oscillation was reduced when the  $\text{MgATP}$  concentration was less than 3 mM, and at 0.5 mM activation produced no detectable oscillation, but the tension developed was greater. These experiments were made with the free oscillation technique and the frequency was not varied sufficiently to exclude the possibility that oscillation might have occurred at a very low frequency (as in the 'high tension' state, figure 5*A*).

#### (vi) ATP-regenerating system

Some free oscillation experiments were made in which the fibre was immersed in solutions containing an *ATP*-regenerating system. In one experiment, a single fibre was bisected (in order to improve access by diffusion) and immersed in a solution *B* containing 0.25 mg/ml. of phosphoenolpyruvate kinase. Activation was produced by raising the  $\text{Ca}^{2+}$  concentration and oscillation occurred at 14 c/s with an amplitude of 0.5 %. When substrate (phosphoenolpyruvate) was added to the solution to give a concentration of 10 mM, the amplitude increased to 0.8 % and the frequency of oscillation remained unchanged.

(vii) *Sodium azide*

Sodium azide was used to inhibit mitochondrial enzymes that might have survived the glycerol extraction procedure, especially the *ATPases* (Sacktor 1953) and the enzymes concerned with oxidative phosphorylation (Dawson, Elliott, Elliott & Jones 1959). The presence of sodium azide (10 mM) made no difference to the oscillations produced by activation.

(viii) *Mersalyl*

The organic mercurial, mersalyl ('Salyrgan', Bayer Products), was used in an attempt to produce selective inhibition of relaxing factor if present (Hasselbach & Makinose 1961). This substance inhibits both relaxing factor activity and the actomyosin *ATPase* but, whereas the former action is irreversible, the action on the *ATPase* is partially reversible by an excess of cysteine. Studies with the free oscillation technique showed that activation of the fibre was prevented by pre-treatment with mersalyl (0.1 mM in solution A) for 1 to 4 min, but oscillations at 30% of the expected amplitude were obtained after subsequent treatment with cysteine (10 mM). After more prolonged pre-treatment with mersalyl, the abolition of the contractile response was irreversible.

(ix) *Ca<sup>2+</sup> buffer*

Raising the Ca<sup>2+</sup> buffering capacity by a factor of 10, by increasing the *EGTA* concentration to 20 mM, was without effect on the oscillatory activity.

(x) *H<sup>+</sup> buffer*

The behaviour of the preparation was also unchanged by doubling the H<sup>+</sup> buffering capacity, and by replacing the histidine with phosphate or *Tris* buffers at pH 7.0.

(e) *Effect of varying the muscle length**Free oscillation experiments*

The effect of stretching the preparation was to increase both the frequency and the amplitude of the oscillations produced by activation (see records B(iii) and C(iii) of figure 3, plate 21). Analysis of record B(ii) of that figure shows that stretching the fibre increased the natural frequency of the lever system by about the same amount as it increased the frequency of the oscillatory contraction. The effect of stretch on frequency may therefore be attributed to the slight increase in dynamic stiffness that would be expected on stretching the fibre if its elastic characteristics at rest and during activity are non-linear.

In the experiment of figure 6, plate 22, the effect of stretch on the amplitude of oscillation was examined at two Ca<sup>2+</sup> concentrations: (a)  $8.9 \times 10^{-8}$  M, which was about the threshold value, and (b)  $2.9 \times 10^{-7}$  M, which was well above the threshold. The records show that the amplitude of oscillation could be increased by stretching the fibre either *before* or *after* immersion in the Ca<sup>2+</sup> solution (compare records (i) and (iii)); that the effect could be reversed by releasing the fibre (record (ii)); and that the effect was most marked when the Ca<sup>2+</sup> concentration was low (figure 6a).

In some experiments, especially those in which the amplitude was large even at low tensions, stretching the fibre produced amplitude modulation at a very low frequency (0.5 c/s in figure 7A, plate 22). This effect disappeared when the tension was lowered by releasing the fibre (figure 7B), but it reappeared when a further stretch was applied (figure 7C).

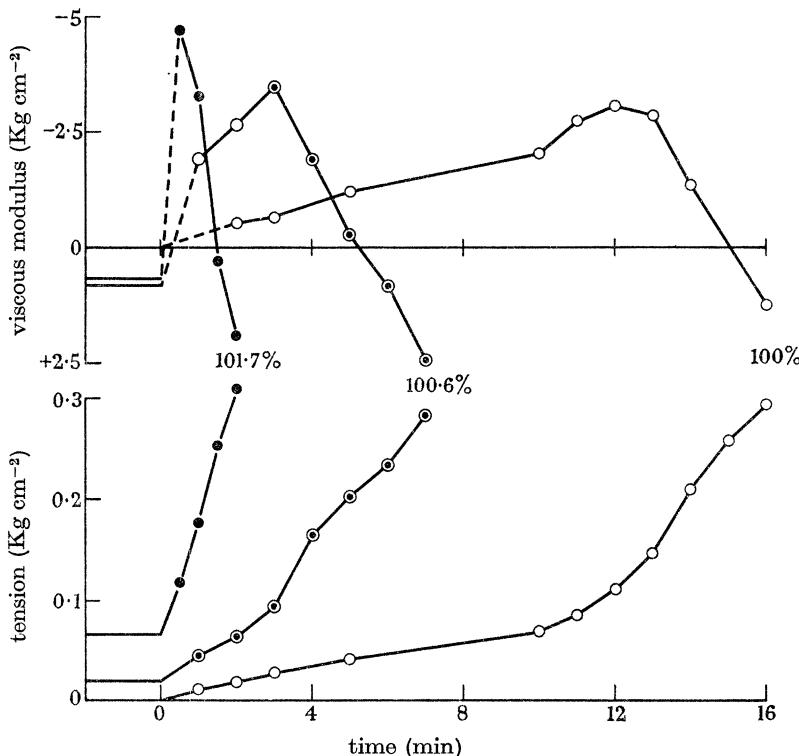


FIGURE 8. Driven oscillation experiment. Graphs showing the transition from 'low tension' state to 'high tension' state at three different muscle lengths. *Upper graph:* viscous modulus at 5 c/s. *Lower graph:* mean tension. The horizontal lines on the left of the figure show the initial conditions in solution B; at time zero activation was produced on each occasion by raising the  $\text{Ca}^{2+}$  concentration to  $6.9 \times 10^{-8} \text{ M}$ . Preparation: *L. cordofanus* (8 fibres, length as indicated, where 100% = 0.506 cm); temp. 24.5°C. Oscillation: amplitude 0.13%; frequency 5 c/s.

#### Driven oscillation experiments

Figure 8 shows the results of an experiment in which activation by raising the  $\text{Ca}^{2+}$  concentration to  $6.9 \times 10^{-8} \text{ M}$  always produced the 'high tension' state, but the time at which this appeared depended on the initial length (or tension) in solution B ( $< 10^{-9} \text{ M Ca}^{2+}$ ). When the muscle length was adjusted to give zero resting tension, activation produced a slow increase of both mean tension and negative viscous modulus at 5 c/s (as in the experiment of figure 4). This behaviour continued for 12 min, but then the mean tension rose more rapidly and the viscous modulus began to decrease, eventually becoming positive because  $f_{V0}$  had fallen to below 5 c/s (as in figure 5A). The normal resting state was then restored by

returning the preparation to solution *B*. When the same sequence of observations was made at a slightly longer muscle length (100.6%, giving a resting tension of 0.020 Kg cm<sup>-2</sup> in solution *B*), the transition from the 'low tension' state to the 'high tension' state was greatly accelerated, and when the muscle length was even longer (101.7%, giving a resting tension of 0.065 Kg cm<sup>-2</sup>) the preparation seemed to pass into the 'high tension' state as soon as the Ca<sup>2+</sup> concentration was raised.

#### (f) Power output

##### *Free oscillation experiments*

In the experiment of figure 7, plate 22, the fibre produced a steady amplitude of 64 µm peak to peak (1.28%) at a frequency of 13.8 c/s (figure 7*B*); this gives a calculated power output of  $9.4 \times 10^{-3}$  erg s<sup>-1</sup> fibre<sup>-1</sup> or 490 erg s<sup>-1</sup> (g muscle)<sup>-1</sup>. The peak amplitude during the amplitude modulation at high tension (figure 7*A* and *C*) was 1.60%, and the corresponding power output was 756 erg s<sup>-1</sup> (g muscle)<sup>-1</sup>. These were the highest values that were obtained in the free oscillation experiments, but no systematic efforts were made to adjust the mechanical conditions for optimal performance.

##### *Driven oscillation experiments*

Figure 4*B* shows that the maximum power output at a given level of activity and amplitude of oscillation occurred at about  $2 \times f_{V\max}$ , not at  $f_{V\max}$  as stated by Machin, Pringle & Tamasige (1962). When the amplitude of oscillation was restricted to the low value used in routine experiments (~0.1%), the maximum power output did not exceed about 100 erg s<sup>-1</sup> (g muscle)<sup>-1</sup>. Higher power outputs could be obtained by increasing the amplitude of movement, but this often resulted in the appearance of the 'high tension' state. In the experiment illustrated by figure 9, raising the amplitude first to double and then to 5 times the usual value resulted in proportional increases in the quadrature component of tension ( $\Delta F_v$ ); the power output was therefore increased by factors of 4 and 25, respectively, and the negative viscous modulus remained unchanged. When an attempt was made to increase the power by a factor of 100 (amplitude  $\times 10$ ),  $\Delta F_v$  first showed a more than proportional increase, but it then fell rapidly; at the same time, the mean tension rose and  $f_{V0}$  fell from about 50 to 7 c/s. The preparation was then in the 'high tension' state, but this was reversed almost completely by stopping the oscillation for 2 min. On restoring the oscillatory drive at high amplitude, the 'high tension' state re-appeared with the same characteristics as before. Finally, the amplitude was reduced to its original value (0.12%) and both the mean tension and  $\Delta F_v$  decreased, though at the time when the preparation was relaxed by immersion in solution *B* they were still slightly above their original values. The maximum *steady* power output in this experiment was obtained when the amplitude was 5 times the usual value; the calculated power output was  $18 \times 10^{-3}$  erg s<sup>-1</sup> fibre<sup>-1</sup> or 950 erg s<sup>-1</sup> (g muscle)<sup>-1</sup> at an amplitude of 0.6% and frequency 5 c/s.

The highest power output obtained in the driven oscillation experiments occurred in a similar experiment in which the amplitude was raised in steps over a period of nearly 4 h. After each increase, the preparation began to enter the 'high tension'

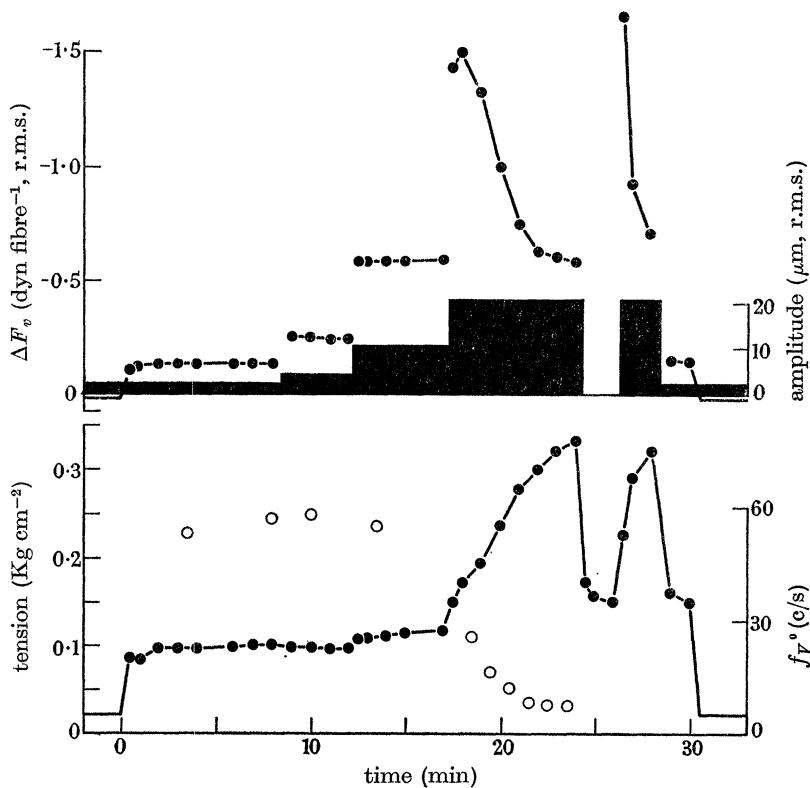


FIGURE 9. Driven oscillation experiment. Graphs showing reversible transitions from 'low tension' state to 'high tension' state produced by varying the amplitude of oscillation. *Upper graph:* solid areas, amplitude (right ordinate scale); ●,  $\Delta F_v$ , the quadrature component of tension at 5 c/s (left ordinate). *Lower graph:* ●, mean tension (left ordinate); ○,  $f_{v_0}$ , the frequency at which viscous modulus was zero (right ordinate). Preparation: *H. colombiae* (7 fibres, length 0.5 cm); temp. 24.5°C. At zero time activation was produced by transferring the preparation from solution B to a solution containing  $1.1 \times 10^{-7}$  M  $\text{Ca}^{2+}$ ; after 30 min it was returned to solution B.

TABLE 1. EFFECT OF AMPLITUDE OF OSCILLATION ON THE POWER OUTPUT

amplitude ( $\mu\text{m}$ , r.m.s.)	2.38	35.7
(%, pk-pk)	0.135	2.02
mean tension (dyn fibre <sup>-1</sup> )	12.9	12.1
( $\text{Kg cm}^{-2}$ )	0.336	0.314
quadrature tension (dyn fibre <sup>-1</sup> , r.m.s.)	-0.29	-4.17
viscous modulus ( $\text{Kg cm}^{-2}$ )	-15.6	-15.2
power output (erg s <sup>-1</sup> fibre <sup>-1</sup> )	0.00086	0.187
(erg s <sup>-1</sup> (g muscle) <sup>-1</sup> )	45	9700

*Experimental details.* Preparation: *L. maximus* (6 fibres, length 0.5 cm); activated by solution containing  $1.1 \times 10^{-7}$  M  $\text{Ca}^{2+}$ ; temp. 22.5°C. Driven oscillation: frequency 2 c/s, amplitude as indicated.

state but this reversed spontaneously, leaving the preparation in a reasonably steady state. Table 1 shows the results that were obtained 58 min after immersion, when the amplitude was 0·135 %, and 195 min after immersion, when the amplitude was 2·02 % ( $\times 15$  increase). The calculated power output was  $9700 \text{ erg s}^{-1}$  ( $\text{g muscle}^{-1}$ ) at the high amplitude, but this was probably a slight overestimate of the true power output because the method of calculation assumes linear behaviour of the preparation. In fact, the tension-length plot was not perfectly elliptical; it showed some collapse at the end corresponding with muscle shortening, but no correction has been made for this because unfortunately the loop was not photographed.

(g) *The 'high tension' state*

In the experiments that have been described so far, the 'high tension' state developed in three ways:

- (i) Immediately after the preparation was immersed in a solution containing a  $\text{Ca}^{2+}$  concentration that exceeded the critical value for that preparation (figure 5A).
- (ii) After a gradual increase in the level of activity produced by prolonged immersion in an activating solution (figure 8). Under these circumstances, the change-over from 'low tension' to 'high tension' behaviour could be accelerated by increasing the initial resting tension by stretching the preparation.
- (iii) Following attempts to raise the power output of the preparation by increasing the amplitude of movement (figure 9).

The 'high tension' state showed a good deal of variability in the precise conditions required for its appearance, and once established its subsequent course was also rather unpredictable. If the rise of mean tension and reduction of frequency parameters were not excessive, then the 'high tension' state often fluctuated in intensity or reversed spontaneously. However, if it was more extreme (e.g. figure 5A), with very high mean tension and low frequency parameters, then positive steps were required to reverse it. One or more of the following procedures could be used:

- (i) Lowering the  $\text{Ca}^{2+}$  concentration (figure 5A). Complete restoration of the normal resting state could always be produced by returning the preparation to solution B.
- (ii) Stopping the oscillatory drive, or reducing its amplitude. This was effective when the 'high tension' state had been precipitated by increasing the amplitude (figure 9), and it was often successful under other circumstances. For example, figure 5B (open circles) shows the vector modulus plot obtained after a gradual increase in the level of activity; the mean tension was  $0\cdot218 \text{ Kg cm}^{-2}$  and the  $f_{V_0}$  was about 10 c/s, so this represented an intermediate state which was less extreme than the 'high tension' state illustrated by figure 5A. It was reversed by switching off the oscillatory drive, and the vector modulus plot shown by solid circles in figure 5B was obtained shortly after restarting the oscillation, the mean tension then being  $0\cdot071 \text{ Kg cm}^{-2}$ .
- (iii) By driving the preparation at its  $f_{V_0}$ , or at a higher frequency. The former procedure involved continuous adjustment of the frequency to keep the quadrature component of tension at zero. Both procedures reduced the power output of the

preparation to zero, and when the frequency was greater than  $f_{v_0}$  work was being done by the apparatus on the muscle.

(iv) Agitation of the bathing solution. This produced repeatable reversals of the 'high tension' state, but the effect was only transient.

The last finding suggested that the 'high tension' state might be due to gross concentration gradients in the preparation and the solution, and some preliminary experiments were made to exclude the more obvious possibilities. Adding concentrated ATP solution to the bathing solution had only a transient reversing action, so this might have been simply due to agitation of the solution. Improving the H<sup>+</sup> buffering did not prevent the appearance of the 'high tension' state.

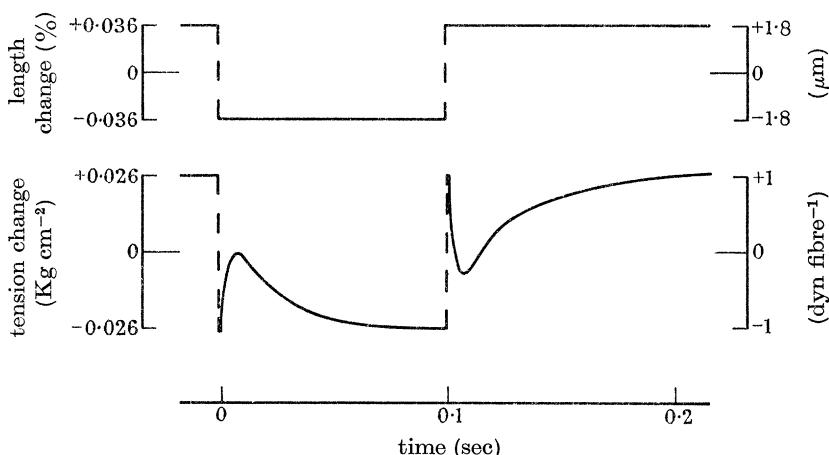


FIGURE 10. Graphs showing the transient response of the active preparation to rapid changes of length. *Upper graph*: part of a sequence of releases and stretches (amplitude 3.6  $\mu\text{m}$ , frequency 5/s). *Lower graph*: the corresponding tension changes, where zero indicates the tension recorded in the absence of any imposed length changes ( $0.320 \text{ Kg cm}^{-2}$ ). These graphs have been replotted from 35 mm photographic records which did not allow resolution of the very earliest events of the transient response (indicated by dashed lines). Preparation: *H. colombiae* (6 fibres, length 0.5 cm); temp. 23.2 °C. Activation was produced by a solution containing  $1.1 \times 10^{-7} \text{ M Ca}^{2+}$ .

#### (h) Results of transient analysis

Figure 10 shows the results of transient analysis of a glycerinated preparation. Activation was produced by a solution containing  $1.1 \times 10^{-7} \text{ M Ca}^{2+}$  and there was a gradual improvement in performance, which resulted in a final steady tension of  $0.320 \text{ Kg cm}^{-2}$  and negative viscous modulus of  $11 \text{ Kg cm}^{-2}$  at 5 c/s. The input to the vibrator was then changed from a sine wave to a square wave of the same periodicity (5/s), and the length and tension changes shown in figure 10 were recorded. As in vertebrate skeletal muscle, the immediate effect of an abrupt decrease in the muscle length (quick release) was a rapid drop in tension. There was then some redevelopment of tension at the shorter length, but this was quickly superseded by a delayed fall of tension even though the conditions remained isometric. When the muscle length was restored abruptly to its initial value (quick stretch), the tension rose immediately, as it does in vertebrate skeletal muscle

under comparable circumstances. At the longer length the tension began to fall again, but there was then a delayed rise of tension which persisted until the next cycle of length changes (not shown).

## DISCUSSION

### (a) *Properties of glycerol-extracted fibres*

#### (i) 'Resting' state

The experiments commenced with the fibres in a buffered salt solution, in which they showed the characteristic high stiffness and viscosity of a muscle in rigor (figure 1*a*). The reduction of elastic and viscous moduli that followed immersion in an *ATP* solution containing a low  $\text{Ca}^{2+}$  concentration (figure 1*b*) could be attributed to the known plasticizing action of *ATP* in the absence of actomyosin *ATPase* activity (Weber & Portzehl 1952).

#### (ii) *Features of activity*

The present experiments have shown that raising the  $\text{Ca}^{2+}$  concentration activates glycerinated fibres of insect fibrillar muscle, as it does isolated actomyosin preparations (Weber & Winicur 1961), myofibrils (Weber, Hertz & Reiss 1963), and glycerinated fibres from vertebrate striated muscles (Seidel & Gergely 1963*b*; Filo, Bohr & Rüegg 1965). Activity was detected by two changes in the mechanical properties of the preparation: first, tension development, which was greatest when no net shortening occurred (see caption, figure 1) and less or absent when conditions were auxotonic (figure 2, plate 21); secondly, by the fact that length changes in the preparation resulted in delayed changes of tension. This was revealed by transient analysis (figure 10), which showed that a sudden release or stretch is followed by a delayed change of tension; by sinusoidal analysis (figure 1), which showed a phase lag of tension on length; and by the fact that the preparation could sustain oscillations in a mechanically resonant load by overcoming the external damping of the system (figure 3, plate 21).

As in the other studies cited above, the level of activity was determined by the  $\text{Ca}^{2+}$  concentration. When this was raised there was greater tension development, a higher rate of rise of tension, and a more pronounced delayed effect of length on tension, which appeared in the free oscillation experiments as an increase in amplitude of movement (figure 2, plate 21), and in the driven oscillation experiments as a greater negative viscous modulus and phase lag at a given frequency (figure 1).

#### (iii) *Chemical conditions for activation*

In the present experiments activation was always produced by raising the  $\text{Ca}^{2+}$  concentration of a solution containing *MgATP* but very little  $\text{Mg}^{2+}$ . The sensitivity of the preparation to  $\text{Ca}^{2+}$  depended on the concentration of  $\text{Mg}^{2+}$ , as this ion had a weak antagonistic effect on  $\text{Ca}^{2+}$  activation. Antagonism between the actions of these two divalent cations has been observed in actomyosin preparations (Weber & Winicur 1961; Maruyama & Watanabe 1962) and in glycerinated fibres of

vertebrate striated muscle (see Hasselbach 1964). In solutions containing virtually no divalent cations (solution *F*), the preparation developed tension, but did not oscillate.

The composition of the bathing solution was varied in other respects. It was found that  $\text{Ca}^{2+}$  induced oscillations were insensitive to small variations of pH and ionic strength; and unaffected by increasing the  $\text{H}^+$  or  $\text{Ca}^{2+}$  buffering capacity, by the presence of an *ATP* regenerating system (phosphoenolpyruvate + kinase), and by including inorganic phosphate in the bathing solutions. It therefore seems unlikely that fluctuations in the levels of  $\text{Ca}^{2+}$ ,  $\text{H}^+$ , *ATP*, adenosine diphosphate (*ADP*), or inorganic phosphate could be responsible for the oscillations.

The chemical conditions for activation of the glycerinated muscle were therefore essentially the same as those required for contraction of glycerinated vertebrate striated muscle. There is evidence that myofibrils teased from living fibrillar muscle of *Calliphora* (Hanson 1956; Jewell & Rüegg, unpublished) and *Drosophila* (Aronson 1962) behave differently in that they remain relaxed when irrigated with solutions containing *ATP* and divalent cations, but these observations were not made under strictly comparable chemical conditions to those used here.

#### (iv) Mechanical conditions for activation

In order to detect oscillatory activity by either the driven or free oscillation technique, the frequency had to be adjusted to a value less than the  $f_{V_0}$  of the preparation. The greatest effect of activation on the viscous modulus occurred at about the  $f_{V_{\max}}$ , and the greatest effect on the elastic modulus was at very low frequencies (figure 1). An important mechanical condition determining the sensitivity of the preparation to  $\text{Ca}^{2+}$  was the muscle length (or tension). If the preparation was stretched, the level of activity produced by a given  $\text{Ca}^{2+}$  concentration was increased. This was shown in both free oscillation (figure 6, plate 22) and driven oscillation experiments (figure 8) by applying the stretch before activating the preparation. The effect of stretch could therefore be regarded as shifting the strength-response curve of the preparation towards lower  $\text{Ca}^{2+}$  concentrations, giving an imaginary set of parallel curves corresponding with the steady state condition at each muscle length. The question that arose from this concept was whether it is possible to move from one strength-response curve to another if the muscle is stretched or released during activity. The experiments of figure 3, plate 21, and figure 6, plate 22, showed that this was indeed possible; the amplitude of oscillation could be reversibly increased by stretching the preparation after activation. This observation was particularly significant because the slight increase of frequency produced by the stretch would be expected *per se* to decrease the amplitude of oscillation due to the shape of the vector modulus plot (see figure 4). Isolated observations made during driven oscillation experiments confirmed the existence of this stretch effect.

In view of these findings, it is pertinent to inquire to what extent the delay between length and tension changes could be due to a similar mechanism. In rabbit psoas myofibrils the degree of contraction is dependent on the amount of  $\text{Ca}^{2+}$  bound to myofibrils (Weber, Herz & Reiss 1964a): it therefore seems possible

that the effect of stretch might be to produce a delayed increase in the amount of bound  $\text{Ca}^{2+}$ . On the basis of the existing data it is impossible to decide between this and some other mechanism that is entirely independent of  $\text{Ca}^{2+}$ /stretch interaction.

(v) *Threshold  $\text{Ca}^{2+}$  concentration*

The values obtained for the threshold  $\text{Ca}^{2+}$  concentration depended on the chemical conditions, and the method of measurement. The important effects of the  $\text{Mg}^{2+}$  concentration and the degree of stretch on the sensitivity of the preparation to  $\text{Ca}^{2+}$  have just been considered. The driven oscillation technique was best for determining the threshold because the frequency was easily varied, and very low negative viscous moduli could be detected. Oscillation could not be detected by the free oscillation technique until the negative viscous modulus was sufficient to overcome the positive damping of the lever system, and the apparatus was difficult to handle at the very low frequencies required for studies of the threshold concentration at  $f_{V_{\max}}$ . For these reasons, the values obtained by the driven oscillation technique (2 to  $5 \times 10^{-8}$  M) were always lower than those obtained by the free oscillation technique (5 to  $12 \times 10^{-8}$  M) under comparable conditions. Both techniques gave slightly lower threshold  $\text{Ca}^{2+}$  concentrations for oscillation than for tension development.

There was a good deal of variation from one preparation to another, but the range was no greater than that observed in other  $\text{Ca}^{2+}$  activated actomyosin systems (Weber, Herz & Reiss 1964*b*). The variation may have been due in part to errors in the calculation of the  $\text{Ca}^{2+}$  concentrations, due to slight departures of the pH from 7.0 (for *EGTA*,  $\Delta p\text{Ca} = 2.67 \Delta \text{pH}$ ). The threshold  $\text{Ca}^{2+}$  concentrations observed in the present experiments were about an order of magnitude lower than in other actomyosin systems, including living crustacean muscle (Portezhl *et al.* 1964), but in view of the dependence on mechanical and chemical conditions it is possible that the 'physiological' threshold is quite different.

(vi) *'High tension' state*

The change from 'low tension' to 'high tension' state is illustrated most clearly in figure 9, where it was produced by increasing the amplitude of oscillation. Figure 5 shows vector modulus plots of the fully developed 'high tension' state (figure 5*A*) and of an intermediate state (figure 5*B*), in which the increase of mean tension and the reduction of frequency parameters were less marked. The intermediate state usually followed a gradual improvement in the performance of the preparation of the type shown in figures 4*A* and 8; in fact the vector modulus plots in figure 4*A* do show a reduction of  $f_{V_0}$  with increasing tension. Thus it is possible to trace a continuous transition from the 'low tension' state, with gradual improvement in performance, through an intermediate state to the fully developed 'high tension' state.

Clues to the nature of the 'high tension' state are provided by two observations: first, the fact that agitating the solution produced a reversal suggests that large concentration gradients existed in and around the preparation; secondly, the

common feature of all the circumstances in which the 'high tension' state appeared was that the power output of the preparation was increasing, and the common feature of all the means used to reverse the 'high tension' state was that they reduced the power output. If increases in power output are accompanied by increases in *ATPase* activity, then accentuation of any radial concentration gradients would be expected because of the diffusion-limited access of *ATP* to the preparation. In the centre of a fibre bundle, it is conceivable that there could be a large reduction in *ATP* concentration, with a corresponding increase in *ADP* and possibly a slight fall of pH, which would be accompanied by an increase of  $\text{Ca}^{2+}$  concentration. If regional differences in the chemical conditions are associated with corresponding differences in mechanical properties, then it seems possible that the 'high tension' state is the resultant behaviour of a non-uniform preparation.

The proof of this hypothesis depends on the identification of the critical chemical condition. Local changes of pH (and therefore of  $\text{Ca}^{2+}$  concentration) do not appear to be responsible because increasing the  $\text{H}^+$  buffering capacity did not affect the development of the 'high tension' state. *ATP* and *ADP* are the other possibilities, but no systematic efforts were made in the present experiments to distinguish between them. The fact that in free oscillation experiments, reducing the  $\text{MgATP}$  concentration below 0.5 mm resulted in large tension increases but no oscillation (see Barany & Jaisle 1960) suggest that reduction of *ATP* in the core of the preparation could be the critical condition.

The amplitude modulation that was sometimes produced in free oscillation experiments by stretching the fibre (figure 7, plate 22) also occurred under circumstances of raised power output, and it was abolished when the power output was reduced by releasing the fibre. This phenomenon might be related to the spontaneous fluctuations in the intensity of the 'high tension' state that often occurred in driven oscillation experiments. If the muscle fibre has the characteristics that (1) stretch leads to an increase in power output and *ATPase* activity, and (2) a reduction in *ATP* level is accompanied by a reduction in power output and *ATP* splitting at a given frequency ('high tension' state), then the system might be expected to show instability due to the time lag introduced by *ATP* diffusion. This would provide an explanation of the amplitude modulation and the fluctuation in 'high tension' state. It might conceivably also explain the low-frequency 'auto-oscillations' that were obtained in glycerinated rabbit psoas muscle fibres under special conditions by Lorand & Moos (1956) and Goodall (1956). It is our intention to make a study of the properties of glycerinated rabbit psoas muscle, using the present techniques, in order to see what relationship 'auto-oscillations' bear to the oscillatory contractions obtained from insect fibrillar muscle.

(b) *Identity of the oscillatory contractions of living and glycerinated insect fibrillar muscle*

Unfortunately a complete systematic study has not yet been made of the living dorsal longitudinal muscle of the water bug, but preliminary experiments made in this Department (Pringle, Machin & Jewell, unpublished) have shown that it has essentially the same characteristics as the basalar muscle of the lamellicorn

beetles, which has been the subject of detailed studies by Machin & Pringle (1959, 1960). There are certain quantitative differences, notably in the frequency parameters, which are lower in the water bug muscle as would be expected on dimensional grounds from the relative sizes of the two types of insect.

#### *Free oscillation experiments*

As no experiments of this type have been made on the living water bug muscle, the results of the present work have been compared with the findings of Machin & Pringle (1959) on another fibrillar muscle. They show some striking similarities:

- (i) Activation was characterized by the appearance of oscillations at about the natural frequency of the system, and in some fibres by an increase in tension which preceded the onset of oscillation (figure 2, plate 21).
- (ii) Oscillations continued as long as the active state was maintained (up to 23 h). When the activating influence was withdrawn, the tension decreased and the oscillations decayed with the same time course (figure 2*f, g*, plate 21).
- (iii) Gradation of activity affected the increase in mean tension and the amplitude of oscillation, but not the frequency (figure 2).
- (iv) The frequency of oscillation depended primarily on the mechanical characteristics of the load, and to a slight extent on the length (or tension) of the fibre (figure 3, plate 21).

By altering the damping of the load, Machin & Pringle (1959) were able to vary the amplitude of oscillation. With very light damping, values up to 10% of the muscle length were obtained, but the maximum power output occurred at about 6%. The maximum amplitude observed in our free oscillation experiments was only 1.5%, but this may have been due to excessive external damping. It is also possible that the mechanical and chemical conditions were not ideal, because the power outputs obtained in these experiments compared very unfavourably with those observed by Machin & Pringle (1959), and they were very much lower than the values obtained in the driven oscillation experiments (discussed below).

#### *Driven oscillation experiments*

The results of the driven oscillation experiments will be compared with the findings of Machin & Pringle (1960) on the living beetle muscle, and with the available unpublished information on the living water bug muscle.

- (i) *Features of activation.* Activation of the glycerinated preparation by raising the  $\text{Ca}^{2+}$  concentration produced an increase of tension and a change in the vector modulus plot. Although maximal activation was probably never achieved in the present experiments, the increases of mean tension that were observed (excluding the 'high tension' state) were generally greater than those produced by the living beetle muscle at the same initial tension (Machin & Pringle 1959). The vector modulus plots obtained from both the glycerinated material and the living muscles of water bugs and beetles during activity showed striking similarities in their general shape and in the magnitudes of the negative viscous modulus at  $f_{V\max}$ . In both cases, raising the level of activity resulted in greater negative viscous moduli at frequencies below  $f_{V0}$ , and there was little change in  $f_{V\max}$  except at low levels of

activity. The vector modulus plots differed in that raising the stimulus frequency (Machin & Pringle 1960, figure 10) increased the elastic modulus at all oscillation frequencies, whereas raising the  $\text{Ca}^{2+}$  concentration (figure 1) only increased it at low frequencies.

(ii) *Frequency parameters.* Vector modulus plots of the living and glycerinated water bug muscle at the same temperature have shown consistent differences in their frequency parameters. The  $f_{V\max}$  of *L. cordofanus* (figure 1) and *H. colombiae* (figure 4) was usually 5 c/s at low or moderate tensions, and it fell to 1 c/s or less (figure 5) when the preparation entered the 'high tension' state. Living muscles from these species have a  $f_{V\max}$  of 12 to 15 c/s at the same temperature. A similar discrepancy exists in the case of *L. maximus*, in which the corresponding figures were 2 c/s after glycerination and about 8 c/s in the living state.

In contrast to the low values of  $f_{V\max}$  after glycerination,  $f_{V0}$  was usually *higher*. However, this parameter was more dependent on the tension than  $f_{V\max}$ ; for example, in the experiment of figure 4A,  $f_{V0}$  fell from 35 to 23 c/s as the mean tension gradually increased, but  $f_{V\max}$  remained unchanged. The highest values of  $f_{V0}$  obtained (at low tensions) for *L. cordofanus* and *H. colombiae* were 45 to 55 c/s, and the figure for the living muscle is about 30 c/s. Similarly, in the case of *L. maximus*, the corresponding values for the glycerinated and living muscles were 30 to 40 c/s and about 19 c/s, respectively.

No explanation can be offered at present for these differences in the frequency parameters of the living and glycerinated muscles. The variability of the values obtained from the glycerinated material has not yet been fully explored, and it is possible that under other chemical and/or mechanical conditions they may be more closely related to the frequency parameters of the living muscle.

(iii) *Temperature coefficient.* Only one experiment was made on the temperature dependence of the frequency parameters. In a glycerinated preparation of *H. colombiae*, a reduction of temperature of 10°C led to a reversible halving of  $f_{V\max}$  and  $f_{V0}$ . This indicates a positive temperature coefficient of about 2, which agrees very well with the findings of Machin *et al.* (1962).

(iv) *Power output.* The maximum value obtained by Machin & Pringle (1959) from the living beetle muscle was  $3 \times 10^5$  erg s<sup>-1</sup> (g muscle)<sup>-1</sup>. No details are given about that particular experiment, but from others that were described it can be inferred that the frequency of oscillation was about 24 c/s and the amplitude about 6% (peak to peak). The highest value obtained from the glycerinated muscle (table 1) was about  $10^4$  erg s<sup>-1</sup> (g muscle)<sup>-1</sup>, which is about 30 times less than the value quoted above. However, the amplitude of oscillation was only 2% and the preparation was being driven at its  $f_{V\max}$ , not at  $f_{P\max}$  (see figure 6B). Although these factors are sufficient to account for the difference in the power outputs obtained from the living and glycerinated muscle, it is unlikely that the power output of our preparation could have been raised to the level seen in the living muscle because of the limit imposed by the appearance of the 'high tension' state.

(v) *Transient response.* As in the case of living fibrillar muscle (Boettiger 1957a, b), the dominant feature of the transient response to rapid changes of length was a delayed change of tension in the same direction as the length change (figure 10).

By the application of the Fourier theorem, it is theoretically possible to predict the transient response from the results of sinusoidal analysis (and vice versa), but in practice this is a complicated procedure for systems like muscle that contain non-linear elements. Although a complete Fourier synthesis of the transient response has not been attempted, it can be inferred that the response shown in figure 10 is qualitatively what would be expected from the vector modulus plot of the glycerinated preparation.

(vi) *Conclusion.* Activation of the glycerinated muscle by raising the  $\text{Ca}^{2+}$  concentration appears to produce an equivalent mechanical state to that produced by electrical stimulation of the living preparation. The free and driven oscillation experiments that have been described show many qualitative and some quantitative similarities between the behaviour of the glycerinated and living muscle. Certain quantitative differences, notably in the frequency parameters, require further study before their significance can be finally assessed, but it may be tentatively concluded that the oscillatory contractions obtained in the two cases are essentially the same.

#### *(c) Functional isolation of the contractile protein system*

The aim of our investigations has been to decide whether the delay between change of length and change of tension is an intrinsic property of the contractile protein system by examining the behaviour of glycerinated insect fibrillar muscle. It has been concluded that the oscillatory contraction produced by this preparation is essentially the same as that obtained in the living muscle, but the real significance of this conclusion depends upon our success in producing functional isolation of the contractile protein system.

Dr Sally Page of the Biophysics Department, University College London, kindly examined some of our glycerinated material in the electron microscope. Her micrographs showed areas of intact membrane, partially disrupted mitochondria, and large amounts of cellular debris; because of the latter it was not possible to exclude the presence of elements of the sarcoplasmic reticulum. Biochemical studies on the same material by Abbott & Chaplain (1965) have shown that many of the sarcoplasmic and mitochondrial enzymes retain some of their activity after glycerol extraction. Similar findings in glycerinated skeletal and cardiac muscle have been reported by Wilson, Elliot, Guthe & Shappiro (1959) and Naylor & Merrillees (1964), respectively.

Before it can be concluded that the delay between change of length and change of tension is a characteristic feature of the contractile protein system, the possibility that the oscillatory contraction of the glycerinated muscle might depend on the presence of non-myofibrillar elements that survived the process of glycerol extraction must be excluded. It could be argued that the delay between change of length and change of tension results from a delayed *indirect* effect of length on the contractile protein system, due to a transducer action in one of the non-myofibrillar elements present. The possibility that the cell membrane, the mitochondria, or the sarcoplasmic reticulum might be functioning as a length-sensitive transducer system will therefore be considered.

### (i) Surface membrane

Machin & Pringle (1960) concluded that the surface membrane does not behave as the length-sensing element on the grounds that during oscillatory contraction they were unable to detect any synchronous fluctuations of the membrane potential of the living muscle fibre. An intact surface membrane was certainly not necessary in our experiments, because bisected fibres showed identical behaviour to whole fibres. It may also be concluded that any areas of surface membrane that survived the process of glycerol extraction must either have been small, or have had altered permeability characteristics, because the mechanical studies gave no indication of any major hindrance to *ATP* access.

### (ii) Mitochondria

There are two ways in which the mitochondria might conceivably act as a transducer element: length changes could lead to (1) fluctuations in the  $\text{Ca}^{2+}$  level, due to an effect on their  $\text{Ca}^{2+}$  pumping activity (Slater & Cleland 1953; Lehninger 1964); or to (2) fluctuations in the *ATP* level, due to an effect on their *ATPase* activity or *ATP*-regenerating systems. Any significant variations in the  $\text{Ca}^{2+}$  concentration were probably excluded by the presence of the calcium buffer, *EGTA* (discussed in next section). Furthermore, mitochondria from other types of muscle do not appear to be capable of lowering the  $\text{Ca}^{2+}$  concentration to the levels required to control activity in this preparation (see Weber *et al.* 1964a). Fluctuations of the *ATP* concentration due to *ATPase* activity are probably of no importance because sodium azide, which inhibits the *ATPase* activity of mitochondria from insect fibrillar muscle (Sacktor 1953), had no effect on the oscillatory contraction. Fluctuations in the *ATP* concentration due to activity of the phosphorylating enzymes also seems improbable in the absence of added substrates, and on the grounds that these enzymes are also inhibited to some extent by sodium azide (Dawson *et al.* 1959). These conclusions are supported by the fact that Abbott & Chaplain (1965) have removed most of the sarcoplasmic and mitochondrial enzymic activity of the glycerinated material by detergent treatment without affecting the oscillatory activity of the fibres.

### (iii) Sarcoplasmic reticulum

It could be argued that length changes might have resulted in fluctuations in the  $\text{Ca}^{2+}$  concentration within the muscle fibre, because of an effect of length on the  $\text{Ca}^{2+}$  pumping activity of any elements of the sarcoplasmic reticulum that survived glycerol extraction. This possibility is regarded as unlikely for the following reasons: (1) increasing the  $\text{Ca}^{2+}$  buffering capacity of the bathing solution by raising the *EGTA* concentration from 2 to 20 mM had no effect on the oscillatory behaviour; (2) treatment with mersalyl followed by cysteine, which abolishes relaxing factor activity (Hasselbach & Makinose 1961), also had no effect; and (3) unpublished work in this laboratory by H. H. vom Brocke (personal communication) indicates that particulate fractions prepared from the glycerinated material have very little  $\text{Ca}^{2+}$  binding activity under the conditions of our experiments.

## (iv) Conclusion

Although glycerol extraction of insect fibrillar muscle does not produce structural isolation of the contractile protein system, we are of the opinion that our chemical conditions have ensured its functional isolation. It may therefore be concluded that in the glycerinated muscle the delay between change of length and change of tension, which is essential for oscillatory contraction, is an intrinsic property of the contractile protein system.

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