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TWO RATES OF RELAXATION IN THE DORSAL LONGITUDINAL MUSCLE OF A LEECH

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INTRODUCTION

The somatic muscles of annelids are of the 'oblique striated' or 'helical smooth' muscle type. Those of leeches have been described in papers by Röhlich (1962) and by Pucci & Afzelius (1962), and those of earthworms by Ikemoto (1963), Lanzavecchia (1968*a*) and more recently by Mill & Knapp (1970).

Although some experiments on the mechanical properties of earthworm muscles have been carried out, very little work has been done on those of leech muscles. Investigations carried out on earthworm muscles (Tashiro & Yamamoto, 1971) have demonstrated that the contraction in the longitudinal muscle consists of a phasic and a tonic component. This is similar to the situation in the 'catch' muscles of molluscs, in which a.c. stimulation causes a phasic response and d.c. stimulation produces a tonic contraction which is sustained for a long period of time (Winton, 1937).

The following account forms part of an investigation into the mechanical properties of the dorsal longitudinal muscle of the horse leech, *Haemopis sanguisuga* (L.). The experiments to be reported here show that two types of relaxation can be obtained from this muscle, and serve to describe some of their mechanical and pharmacological characteristics.

METHODS

Specimens of the horse leech *Haemopis sanguisuga* were kept in aerated tanks of de-chlorinated tap-water at 10–12 °C. The preparation of the longitudinal muscle was made as follows. An incision was made along the whole length of the animal on each side. Then, with the animal pinned out dorsal side uppermost, the dorsal wall was carefully separated from the gut and connective tissue, so providing a strip of the dorsal wall approximately 5 cm long by 0·5 cm wide.

The strip was tied with cotton at each end and a small fish-hook attached to a length of fine chain was inserted through the tissue behind each piece of cotton. The chains were used to mount the muscle on a vertical Perspex electrode assembly so that the muscle lay against an array of alternately positive and negative silver wires with 4 mm spacing. One end of the muscle was fixed by attaching the chain to a hook set in the Perspex. The chain at the other end of the muscle was attached to a Devices Type 2STO2 strain gauge, in which two semi-conductor strain elements form two arms of a Wheatstone's bridge circuit, giving an output proportional to tension. The muscle was in a bath containing Ringer solution of the following composition (mM): NaCl 113·0; KCl 4·3; $CaCl_2$ 2·0; $NaHCO_3$ 1·6; Na_2HPO_4 1·0; the pH being adjusted to 7·4

with 10% HCl (modified from Pantin, 1946). Experiments were carried out through out the year at room temperature (20–24 °C).

The output from the strain gauge was amplified and displayed on a Devices M2 hot-wire pen-recorder. For some experiments the signal was differentiated using a Tektronix Type 3A8 operational amplifier mounted in a Type RM 565 dual-beam oscilloscope, in order to obtain a simultaneous recording of the rate of change of tension. A Grass S4 stimulator was used to produce either a single d.c. shock or a train of square-wave pulses for a controlled period of time. The stimulator output was fed through an emitter-follower circuit so that the presence of Ringer solution of low resistance on the electrode did not reduce the stimulus voltage.

THE PREPARATION

The preparation used for these experiments is part of the body wall of the leech and contains tissues other than the longitudinal muscles being studied. There is a thin outer layer of cuticle and epidermis, and under this a layer of circular muscle. A thin layer of muscle fibres running obliquely in both directions lies between the circular and longitudinal muscle. The latter is arranged in bundles of fibres surrounded by connective tissue, and is the major component of the body wall. The innermost layer is the spongy connective tissue known as botryoidal tissue. Fig. 1 (Pl. 1) shows part of a cross-section through the preparation. The muscle fibres themselves are long and spindle-shaped and have an outer cortex of contractile material and an inner core of sarcoplasm containing the nuclei, mitochondria, etc.

It is extremely difficult to dissect away the longitudinal muscle from all these other tissues. Consequently the complete preparation has been used and its complexity must be remembered in interpreting the results. The circular muscles, being orientated perpendicular to the direction of tension measurement, will have no effect on the tension recorded. The epidermis is extremely extensible – the leech when swimming may be at least six times its contracted length – and will have no effect on the tension when experiments are carried out at the normal unstretched length of the preparation. This also applies to the connective and botryoidal tissue which is soft and extensible. The oblique muscle fibres will probably contract when the preparation is stimulated and a component of the tension they develop will be in the longitudinal direction.

In order to estimate the proportion of each of the tissues present, cross-sections of the preparation were fixed in Bouin's fluid and stained with Cason's or Ehrlich's haematoxylin and eosin. They were photographed (Fig. 1A) and tracings were made (as in Fig. 1B) to delineate the tissue regions. The relative areas occupied by each tissue were measured by weighing cut-outs of the tracing paper. The figures obtained for one preparation are shown in Table 1.

From the table it can be seen that even along the length of one preparation the amount of botryoidal tissue, and to a lesser extent connective tissue, is extremely variable. Therefore the amount of muscle present, if expressed as a percentage of the total cross-sectional area, is also variable. It is more meaningful to consider the amount of each type of muscle as a percentage of the total muscle area. The dorso-ventral muscle, which contracts to flatten the body of the leech for swimming, is not found in all sections and there is very little present.

Table 1. *Areas occupied by various tissues in a cross-section through the preparation of the dorsal body wall of the leech*

	Area occupied by each tissue (% of total cross-sectional area)		Area occupied by each type of muscle (% of total muscle area)	
	A	B	A	B
Epidermis	10·1	7·2	—	—
Connective tissue	9·5	14·9	—	—
Botryoidal tissue	17·5	38·9	—	—
Longitudinal muscle	52·0	32·6	82·6	83·6
Circular muscle	6·6	4·6	10·5	11·8
Oblique muscle	4·3	1·5	6·9	3·9
Dorso-ventral muscle	—	0·3	—	0·7

Section A is from a part of the preparation which was fixed while stretched longitudinally. Section B is from an unstretched region.

Only a certain proportion of the tension developed by the oblique muscle will be in the longitudinal direction, and this component depends on the length of the preparation. All the experiments described here were carried out at constant length, and so for any one preparation the contribution of the oblique muscle is constant. The cross-sectional area of the oblique muscle is always less than 10% of that of the longitudinal muscle, and therefore it is to be expected that the maximum longitudinal tension produced by the oblique muscle should never exceed 10% of the maximum tension developed by the longitudinal muscle, and will usually be much less than this.

RESULTS

Depending on the conditions of electrical stimulation, two types of contraction are shown by the longitudinal muscles.

Responses to pulse trains and to a.c. stimulation

Pulsed stimuli were applied as trains of 4 or 5 msec square-wave pulses at 50/sec, usually for $\frac{1}{2}$ sec. A stimulus of this type produced a contraction phase lasting about as long as the period of stimulation, followed by a relaxation in which the maximum rate of change of tension was about half that during the contraction phase. An increase in the stimulus intensity produced an increase in the peak tension, maximum tension being reached at a stimulus intensity in the region of 3–5 times threshold (Fig. 2*a*). In a series of contractions obtained at different stimulus intensities there was an approximately linear relation between the peak tensions reached and the maximum rates of relaxation, and also between the peak tensions and the maximum rates of contraction (Fig. 2*b*).

The time course of the relaxation was approximately, though not strictly, exponential, and its initial part, measured over the first second of relaxation, had a time constant τ_p which remained constant over the whole range of stimulus intensity. In the particular preparation to which Fig. 2 refers, the value of τ_p was $0{\cdot}71 \pm 0{\cdot}12$ sec (mean $\pm$ S.D., $N = 14$). The latter part of the relaxation tends to be slightly slower than it would be in the case of a true exponential decay and this deviation from the

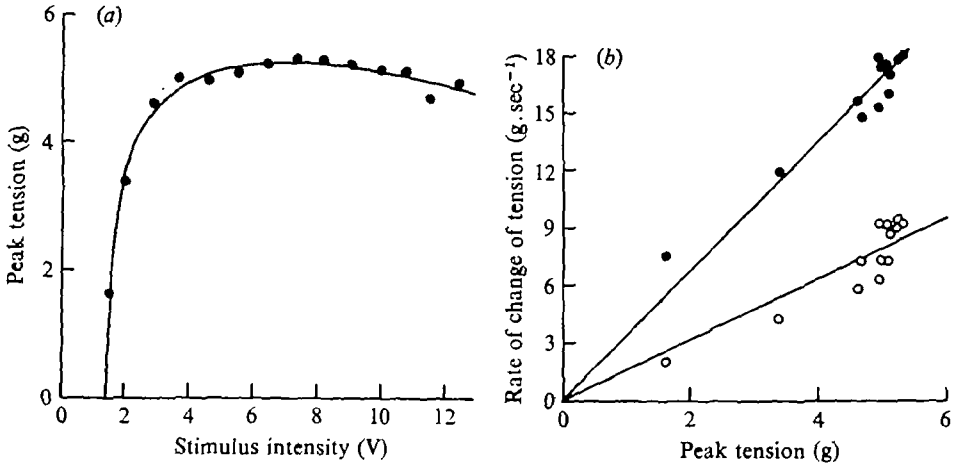

Fig. 2. Analysis of contractions produced by pulsed stimulation (5 msec pulses at 50/sec for 500 msec). (*a*) Relation between peak tension and stimulus intensity. (*b*) Relations between maximum rates of relaxation (○) and contraction (●), and peak tension.

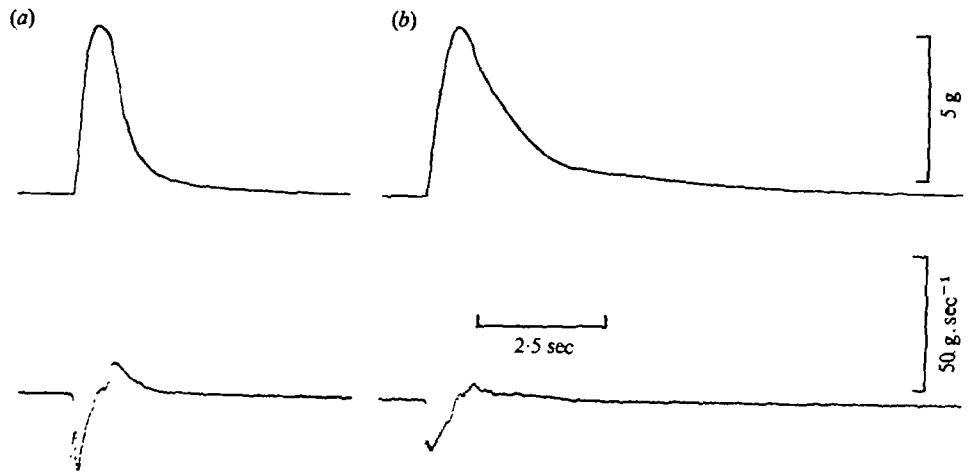

Fig. 3. Contractions at relatively low stimulus intensities. (*a*) Contraction produced by pulsed stimulation (4 msec pulses at 50/sec for 500 msec). (*b*) Contraction produced by 500 msec d.c. stimulation. Stimulation intensities were adjusted to produce contractions of equal magnitude. The upper traces show tension, and the lower traces a simultaneous recording of tension differentiated with respect to time.

exponential relaxation becomes greater at higher stimulus intensities, so that at very high voltages there appears to be a 'tail' on the tension record, as can be seen in Fig. 4(*a*).

A few experiments were performed using a 50 Hz a.c. stimulus. The resulting contractions appeared to be comparable in every way with those obtained with pulsed stimuli.

Responses to d.c. stimulation

A d.c. shock consisting of a single square-wave pulse of the same duration as the train of pulses making up a pulsed stimulus produced a contraction which differed in certain respects from that produced by the pulsed stimulus. At low stimulus

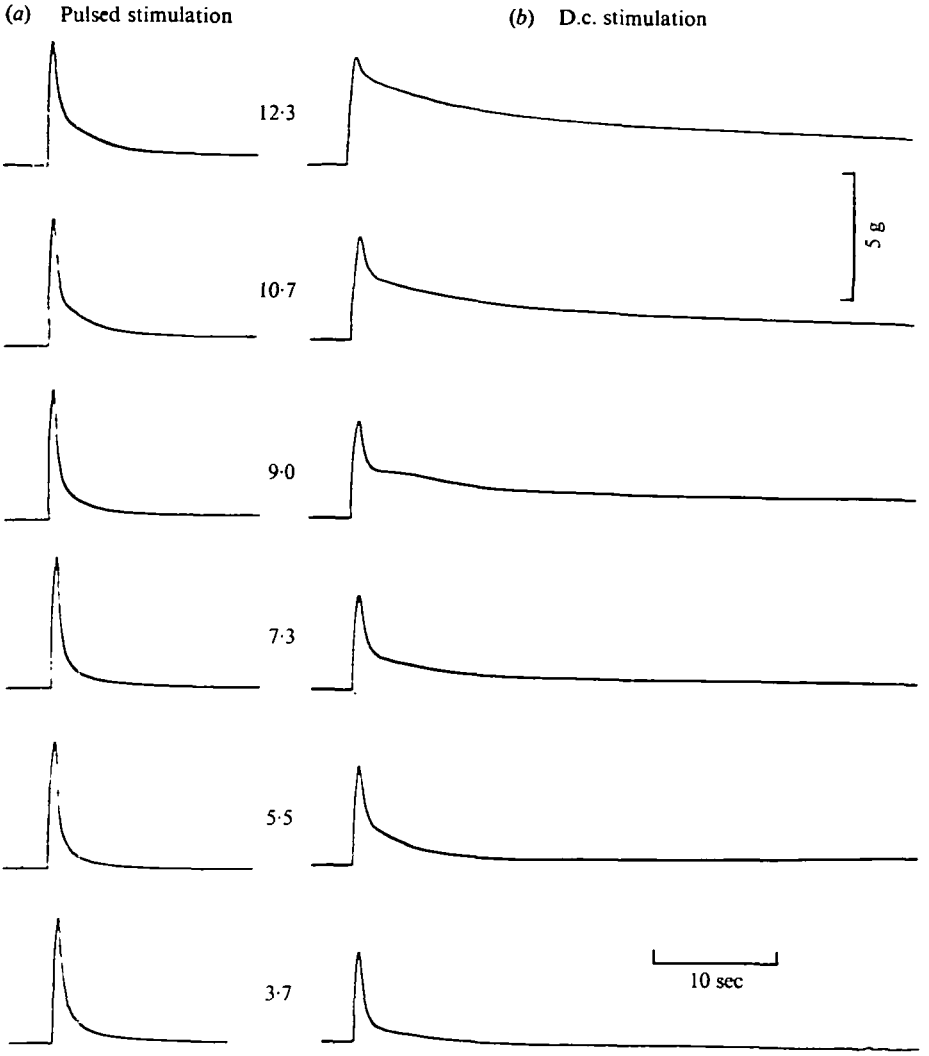

Fig. 4. Contractions produced by (*a*) pulsed stimulation and (*b*) d.c. stimulation of different intensities. All contractions are from the same specimen. Stimulation is by 5 msec pulses at 50/sec for 500 msec in (*a*), and by a 500 msec d.c. pulse in (*b*). Figures opposite each record show the stimulus voltage.

intensities (up to about 6 V or about 3–5 times threshold) there were linear relations between peak tension and the maximum rates of contraction and relaxation. However, the contraction rate was slightly slower and the relaxation rate was much slower than those of the pulsed response.

At higher stimulus intensities (up to 15 V or about 10 times threshold) the difference in relaxation from the two types of response became more marked. In the d.c. response, as the voltage was increased, the peak tension reached a maximum at about 5 V. The relaxation from the peak tension consisted of two components, both exponential, the second being slower than the first. The contraction therefore appeared to have a

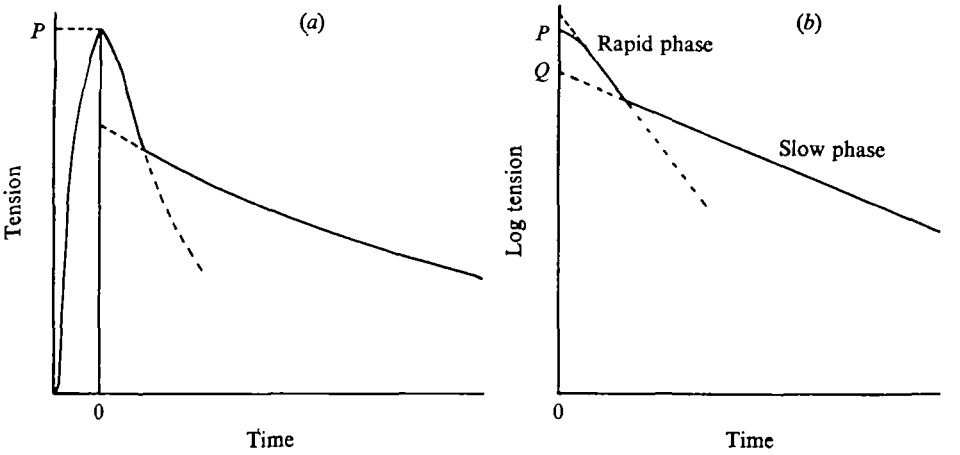

Fig. 5. Method of analysis of contractions produced by high-intensity d.c. stimuli. (*a*) Diagram of a typical tension trace. (*b*) Relaxation from the contraction in (*a*) replotted with tension on a logarithmic scale to measure the parameters used in Fig. 5. P is the peak tension of the contraction, and Q is the intercept on the tension axis of the slow phase, at time $t = 0$. τ_1 and τ_2 are the time constants of the rapid and slow phases respectively.

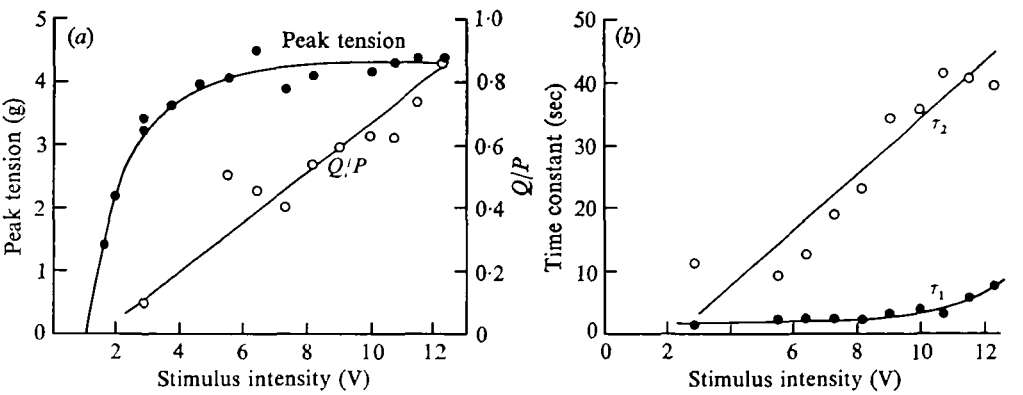

Fig. 6. Analysis of the d.c. contractions shown in Fig. 4(*b*), according to the method in Fig. 5. (*a*) The relation between peak tension and stimulus intensity, and between the ratio Q/P and stimulus intensity. (*b*) The relation between the time constants, τ_1 and τ_2, and stimulus intensity.

proportion of residual or 'catch' tension. The amount of this residual tension was greater at higher stimulus intensities; this can be seen in Fig. 4(*b*), which shows a series of contractions caused by d.c. stimulation of increasing intensity.

In order to obtain a quantitative description of the response to a high-intensity d.c. stimulus we have applied a simple and empirical method of graphical analysis. The relaxation phases of the tension records were plotted on semi-logarithmic graph paper. The two exponential components could then be seen as two straight-line regions on the graph, as is shown in Fig. 5. The second component, with the greater time constant, was extrapolated back to the start of the relaxation phase (time $t = 0$) to find its intercept on the tension axis at the point Q. The ratio Q/P then provides a measure of the relative magnitude of the residual tension in any one contraction. The rates of

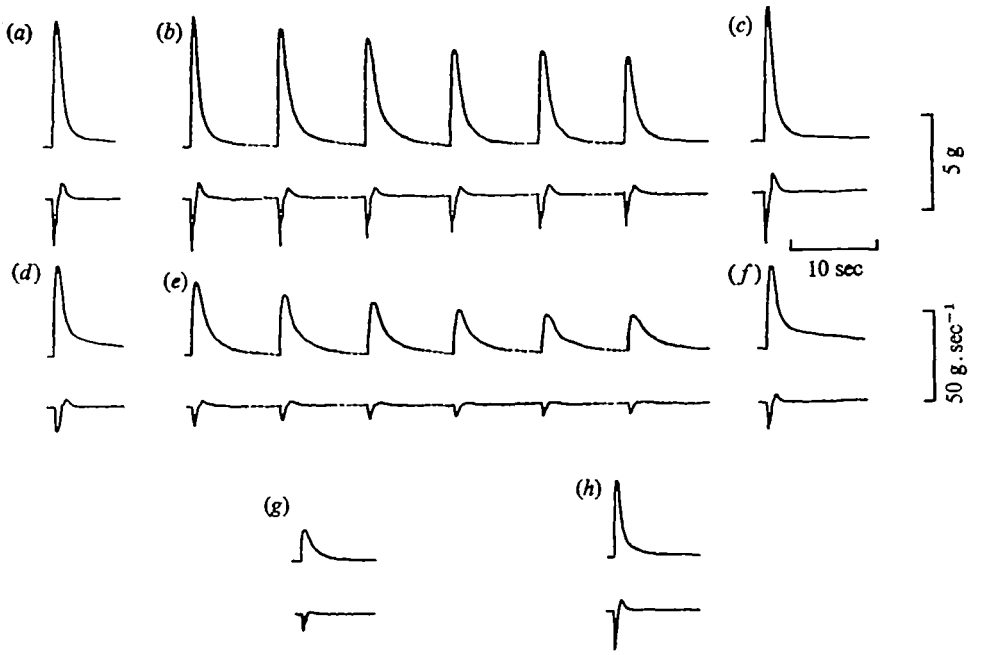

Fig. 7. Tracings of the records obtained in experiments on fatigue. (*a*) Normal contraction caused by pulsed stimulation. (*b*) Every fifth contraction in a series of thirty pulsed stimuli. (*c*) Single pulsed stimulus after one minute's rest. (*d*), (*e*), (*f*) Similar sequence with d.c. stimulation. (*g*) Response to a single d.c. stimulus after a series of thirty pulsed stimuli. (*h*) Response to a single pulsed stimulus after a series of 30 d.c. stimuli. Upper traces show the tension record and lower traces a simultaneous record of the rate of change of tension.

relaxation in the first and second phases were described by their time constants τ_1 and τ_2 respectively.

Fig. 6 shows the results of applying this analysis to the series of tension records shown in Fig. 4(*b*). The time constant of the initial rapid phase, τ_1, increased slightly with increasing stimulus intensity from 0·65 sec at 2·85 V to 3·3 sec at 12·3 V. (This compares with a value of $0{\cdot}71 \pm 0{\cdot}21$ sec for τ_p in this particular preparation.) The time constant of the slow phase, τ_2, also increased with increasing stimulus intensity, from 4·9 sec at 2·85 V to 17·4 sec at 12·3 V. This means that the muscle was taking over a minute to relax after stimulation at high intensities. The ratio Q/P increased from 0·1 at 2·85 V to 0·85 at 12·3 V.

Different preparations showed some variation in the magnitudes of these various parameters, but they always changed with stimulus intensity in the same way as in Fig. 6. Maximum values of the ratio Q/P varied between 0·30 and 0·85. The time constants varied slightly, and the transition between the two phases was more or less marked. In some cases it was very gradual, while in others there was a plateau of maintained tension before the second phase of relaxation began. Similarly there was sometimes a very small plateau just before a final relaxation to zero tension, and the proportion of this also increased with stimulus intensity.

Fatigue experiments

The muscle shows some fatigue on repetitive stimulation. The question arose as to whether fatigue of the response to pulsed stimulation is accompanied by fatigue of the response to d.c. stimulation, and vice versa. Fig. 7 shows the results of an experiment on this problem.

The muscle was subjected to a train of 30 standard stimuli (5 msec pulses at 50/sec for 500 msec for the pulsed stimuli, or 500 msec for the d.c. stimuli, both 3·7 V) at 10 sec intervals. For both the pulsed and the d.c. stimuli the peak tension and the maximum rates of contraction and relaxation declined gradually, but after a pause of 1 min all these parameters recovered. When the muscle was fatigued with 30 pulsed stimuli and 10 sec later a d.c. stimulus was given, the response resembled the last of a series of d.c. stimuli, i.e. it showed fatigue from which it recovered after a rest. Similarly a single pulsed stimulus at the end of a series of d.c. stimuli showed fatigue from which it could recover.

Pharmacological experiments on relaxation rates

In all these experiments the method used was as follows. The muscle preparation was made in the usual way and mounted on the electrode using standard Ringer solution in the muscle-bath. A series of pulsed stimuli followed by d.c. stimuli followed by pulsed stimuli was given, approximately five in each group, 2 min apart and of varying intensities up to 5 V. The preparation was then bathed for 20 min in the test solution and then the same sequence of stimuli was given. The muscle was washed in fresh aerated Ringer for 2 h, the medium being changed several times, and the sequence was repeated again. Several experiments were carried out for each substance tested, and it was found that each drug gave consistent results. Histograms showing the mean and standard deviation for both half-time of relaxation and maximum rate of relaxation divided by peak tension are shown in Fig. 8 for one experiment with each substance. The test substances were made up in Ringer solution in the concentrations stated.

(*a*) *Gamma-amino butyric acid* (10^{-5} *g/ml*)

For both the pulsed and the d.c. responses the half-time of relaxation increased by about 25% and the relaxation rate decreased by about 20%, so that there was no separation of the two responses. The contraction rate was not significantly affected.

(*b*) *High concentration of magnesium ion* (20 mM)

The 20 mM-$MgCl_2$ was substituted for sodium chloride in the Ringer solution. Again there was no difference between the pulsed and d.c. responses. In both, the half-time of relaxation was increased by 40–50%. The total tension was slightly reduced, though recovery was almost complete after replacement with standard Ringer solution. The rate of contraction was reduced, though not as markedly as the relaxation rate, which was reduced by 30–40%.

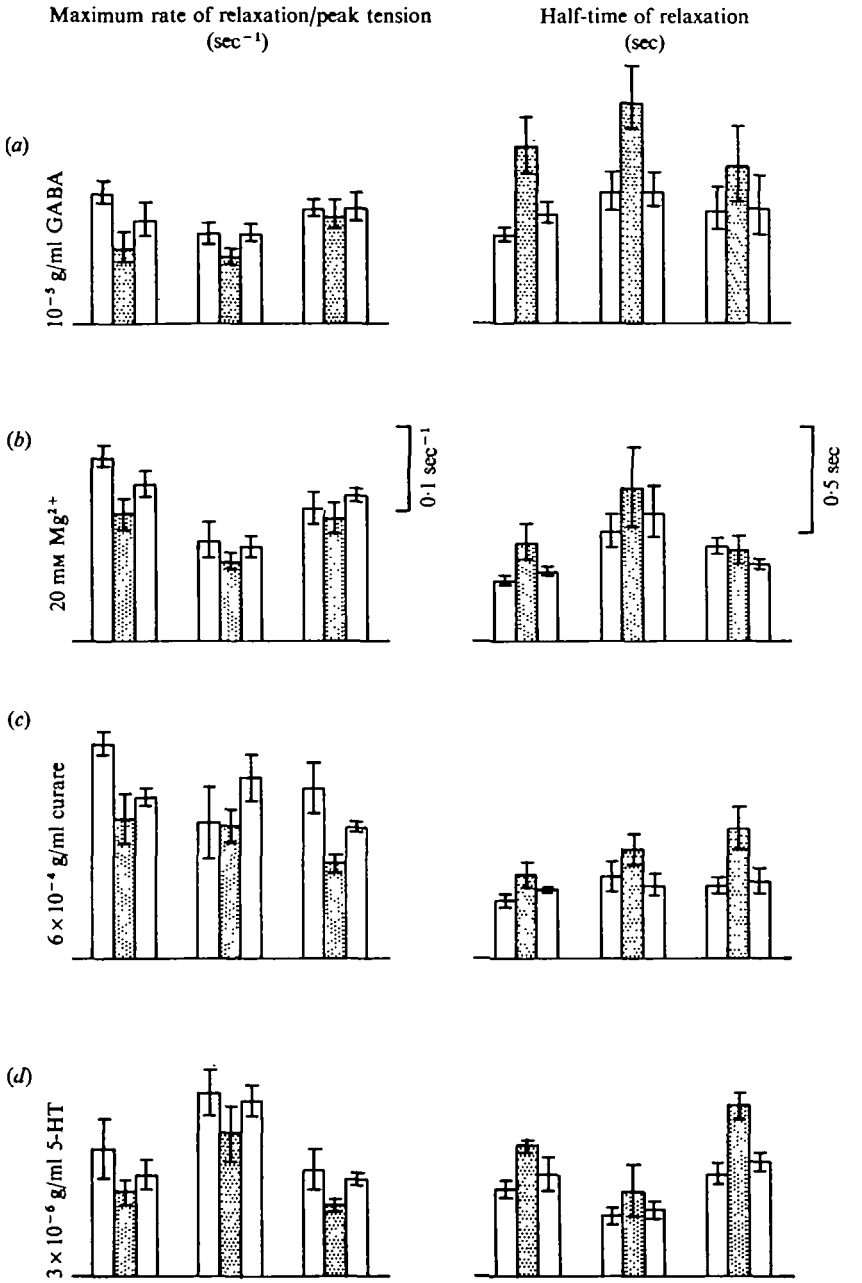

Fig. 8. The effect of various drugs on relaxation in the dorsal longitudinal muscle. The histograms show the mean and standard deviation of the maximum rate of relaxation divided by peak tension, on the left, and of the half-time of relaxation, on the right. Shaded columns indicate d.c. stimulation and the blank columns pulsed stimulation. There are three groups of stimulations shown in sequential order from left to right: in Ringer solution, in the test solution, and in Ringer solution again.

(*c*) *Curare* (6×10^{-4} *g/ml*)

A concentration of D-tubocurarine was used, sufficient to abolish an acetylcholine contracture comparable in size to the contractions due to electrical stimulation. The total tension was reduced slightly as with the high magnesium concentration, and it did not recover fully. The values of half-time and relaxation rate became more variable, though the mean values were not much affected.

(*d*) 5-*Hydroxytryptamine* (3×10^{-6} *g/ml*)

There was no separation of the two responses. Both showed an increase in the relaxation rate and a decrease in the half-time of relaxation by approximately 50%. There was a smaller increase in the contraction rate, so that a higher peak tension was reached during the 500 msec stimulation.

The 5-HT did not abolish the residual tension produced by higher intensity d.c. stimuli at a concentration which produced a significant effect on the rates of contraction and relaxation as measured at lower stimulus intensities. The contraction rate, and the relaxation rate of both components, appeared to be increased a little, but the proportion of residual tension was unaffected, and the transition between the two phases was still clear; it was not possible to selectively affect one component. Neither did 5-HT at a higher concentration abolish the residual tension.

DISCUSSION

The experiments described here show clearly that the rate of relaxation of electrically stimulated leech muscle depends upon the nature of the stimulus. Contractions produced by stimulation with d.c. always relax more slowly than do those produced by pulsed stimulation, and may show two distinct phases in relaxation, so that some appreciable 'residual tension' persists for up to 1 min or more after the contraction phase ends. We now have to consider possible mechanisms whereby these phenomena may be brought about.

One possibility is that the slow relaxation rate and residual tension could be caused by activity of nervous elements in the muscle continuing after the end of the period of stimulation. However, the experiments with curare seem to rule this out; the presence of curare in a concentration sufficient to block acetylcholine contractures of the muscle (we assume that such a concentration would be sufficient to block excitatory neuromuscular transmission) had no significant effect on the nature of either the contraction or relaxation of the muscle. The experiments with high concentration of magnesium ion are in agreement with this view, since the solutions would be expected to prevent the release of neuromuscular transmitter. The effects of high concentrations of magnesium ion on the rates of contraction and relaxation may perhaps be due to changes in the ionic concentrations inside the muscle fibres.

Hidaka, Ito, Kuriyama & Tashiro (1969) have shown that earthworm longitudinal muscles receive an inhibitory nerve supply, and that the action of gamma-amino butyric acid resembles the action of the inhibitory transmitter, and Stuart (1970) has shown that at least some longitudinal muscle fibres in the leech, *Hirudo*, receive an inhibitory innervation. Hence it is possible that the more rapid relaxation after a period of pulsed

timulation could be caused by stimulation of inhibitory nerve endings. However, if this were so we would expect the responses to pulsed and d.c. stimulation to become more alike in the presence of γ-amino butyric acid. More conclusive evidence against this possibility is provided by the results of high-intensity d.c. stimulation; the fact that the degree of residual tension rises as the stimulus intensity increases suggests that d.c. stimulation excites some extra mechanism in producing the residual tension, rather than failing to excite an inhibitory mechanism.

Another possibility is that there could be two types of muscle fibre in the preparation one of them with a much slower rate of relaxation than the other. The oblique muscle fibres could not be one of these types since they are not present in sufficient amount to account for the tensions involved; but it is possible that there could be two types of longitudinal muscle fibre. However, this would imply that the peak tensions reached during contraction should be greater at higher intensities of d.c. stimulation (and they are not) unless the onset of the slowly relaxing contraction were much slower than that of the rapidly relaxing one, being delayed until the end of the stimulus period. We do not consider that the records of the response to d.c. stimulation (as in Fig. 4(*b*)) are in accord with this explanation, but the possibility cannot be completely ruled out. In the earthworm longitudinal muscle, Tashiro (1971) and Tashiro & Yamamoto (1971) have observed biphasic responses in which a short high-intensity stimulus is followed by two successive contraction–relaxation cycles, termed phasic and tonic, in which the tonic phase is clearly very much slower than the phasic phase. It does seem possible that these two phases could be brought about by two separate types of muscle fibre.

A third possibility is that the muscle fibres are largely homogeneous in their physiological properties and that there is some feature of their membrane responses, coupling process or contractile system which can be modified or brought into action by d.c. stimulation, especially at high intensities. It has been suggested that in oblique-striated muscles there may be two contraction mechanisms: (*a*) the normal sliding filament mechanism in which sliding occurs between thick and thin filaments, and (*b*) a 'shearing' between adjacent thick filaments which results in a change in the angle of the striation (Rosenbluth, 1967; Knapp & Mill, 1971). However, there are strong geometrical arguments that the sliding and 'shearing' cannot be separated in this way (Lanzavecchia, 1968*b*). Furthermore, it is difficult to see how a long d.c. stimulus could affect the contractile mechanism directly to produce such 'shearing'. We therefore think it most likely that the d.c. stimulus acts by affecting the coupling process in some way, either directly or via some action on the membrane responses. For example, it is conceivable that a prolonged steady current could change the ionic distribution within the fibre so as to alter the kinetics of calcium accumulation by the sarcoplasmic reticulum during relaxation.

It is well known that a 'catch' system occurs in the anterior byssus retractor muscle of *Mytilus* and in other lamellibranch muscles. The body-wall muscles of annelids are similar to these molluscan 'catch' muscles in that their thick filaments are much larger in diameter than those of striated muscles, and it has been suggested that they contain paramyosin (Hanson & Lowy, 1957; Mill & Knapp, 1970). The 'catch' system in molluscan muscles is clearly of considerable use to the living animal in such prolonged contractions as are involved in keeping the shell closed, holding the animal

down on to its byssus, and so on. It is not so evident what use the residual tension which we have described for *Haemopis* could be, or, indeed, whether they occur in the intact animal. However, animals do frequently hang from the lid of an aquarium tank for long periods of time in the same position, supported only by the anterior sucker and with the body remaining at a length considerably less than the maximum. Some terrestrial leeches remain for long periods in an upright position attached only by the posterior sucker (Shipley, 1915). Behaviour of this type would be facilitated by the possession of a muscle system which under certain circumstances can have a very slow rate of relaxation.

SUMMARY

1. Experiments were performed to measure the mechanical properties of a preparation of the dorsal body wall of the leech *Haemopis sanguisuga*.

2. A quantitative histological description of this preparation is provided. It is concluded that the fibres of the dorsal longitudinal muscle are almost entirely responsible for the contractile properties of the preparation, as measured along its longitudinal axis.

3. The preparation was subjected to two types of electrical stimulation. Pulsed stimulation produced a fairly rapid contraction and relaxation. Contraction after a d.c. stimulus was slightly slower, and the relaxation consisted of an initial rapid phase followed by a much slower phase. The extent of the residual tension in this second phase was greater at higher stimulus intensities.

4. The muscle showed some degree of fatigue when subjected to a series of stimuli. Each type of electrical stimulus caused fatigue of both responses.

5. Curare had no effect on relaxation rates. Both phases of relaxation were somewhat slower in the presence of a high concentration of magnesium ion or γ-amino butyric acid, and faster in the presence of 5-hydroxytryptamine. It was thus not possible to separate the two types of response by pharmacological means.

6. The mechanism whereby two rates of relaxation are produced is discussed. It is suggested that d.c. stimulation affects excitation–contraction coupling in some way.

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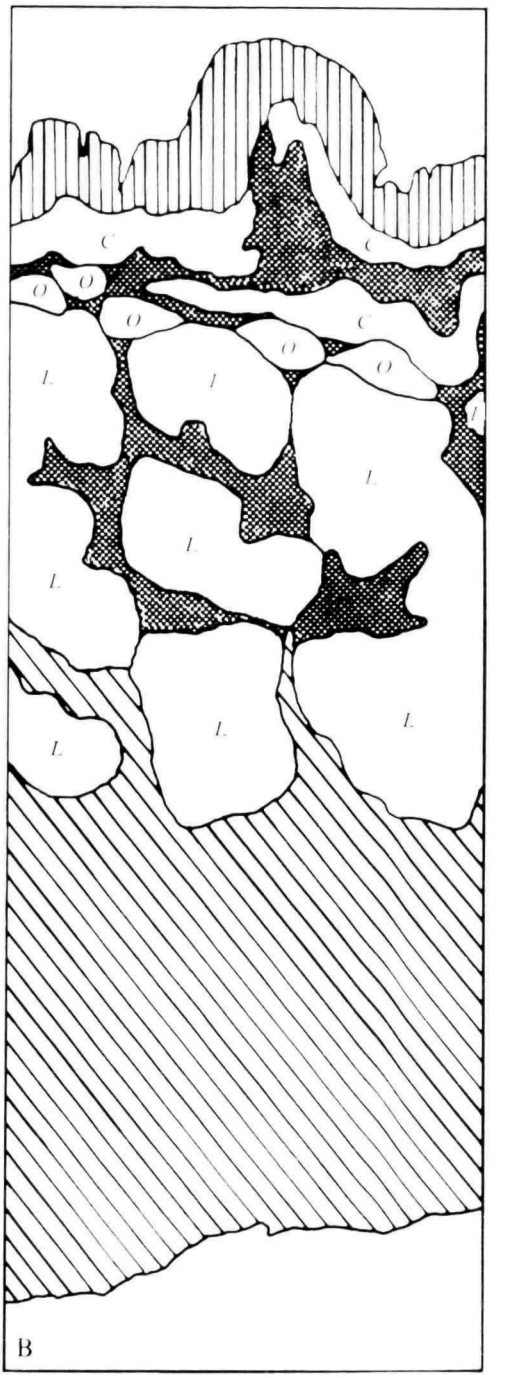

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EXPLANATION OF PLATE

Fig. 1 (A). Part of a cross-section through an unstretched preparation of the dorsal body wall, stained in Cason's stain. (B) Tracing of the photograph in A, to show the tissue regions. The vertical hatching shows the epidermis and the diagonal hatching the botryoidal tissue. The stippled area indicates connective tissue, and the blank areas are muscle, marked as follows: *L*, longitudinal muscle; *C*, circular muscle; *O*, oblique muscle.