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# CALCIUM TRANSIENTS IN ISOLATED AMPHIBIAN SKELETAL MUSCLE FIBRES: DETECTION WITH AEQUORIN

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SUMMARY

1. Single twitch muscle fibres isolated from frogs and toads were microinjected with the $Ca^{2+}$-sensitive bioluminescent protein aequorin. The fibres contracted normally and emitted flashes of light (aequorin responses) in response to stimulation for many hours thereafter.

2. No luminescence was detected from healthy fibres at rest.

3. The aequorin diffused from the site of injection at a rate consistent with a diffusion coefficient of $5 \times 10^{-8}$ $cm^2/sec$.

4. During trains of isometric contractions there was a progressive reduction in both the amplitude and the rate of decline of the aequorin response, an observation consistent with the theory that Ca is redistributed from sites of release to sites of sequestration under such circumstances.

5. In isometric tetani light emission continued to rise long after the plateau of force had been achieved. This and the fact that the amplitude of the tetanic aequorin response increased steeply with increasing stimulus frequency suggest that in tetani the sarcoplasmic $[Ca^{2+}]$ may normally be above the level required to saturate the contractile apparatus.

6. Both in twitches and in tetani the amplitude of the aequorin response increased slightly and then decreased substantially as the fibre was stretched progressively beyond slack length.

7. In potassium contractures the luminescent and mechanical responses first became detectable at about the same $[K^+]$, but for equivalent force luminescence was less intense than in twitches. The aequorin response was biphasic in solutions of high $[K^+]$.

8. Exposure of the fibre to $Ca^{2+}$-free solutions had no influence on either the mechanical or the luminescent responses in twitches. In $Ca^{2+}$-free solutions tetanic aequorin responses tended not to be maintained as well as normally, suggesting that intracellular Ca stores do become somewhat depleted.

9. In twitches the amplitude of the aequorin response probably reflects the amount of $Ca^{2+}$ liberated into the cytoplasm rather than a $[Ca^{2+}]$ in equilibrium with the myofilaments. Changes in the rate of decay of the aequorin response may reflect changes in the rate of Ca sequestration by the sarcoplasmic reticulum.

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10. In $K^+$-contractures and during the plateaus of tetani the aequorin signal changes slowly enough so that it seems unlikely that substantial gradients of $[Ca^{2+}]$ exist at the sarcomere level. Under such circumstances the amplitude of the aequorin response probably does reflect the $[Ca^{2+}]$ in equilibrium with the myofilaments.

## INTRODUCTION

The key role of Ca ions in excitation-contraction coupling in muscle is now generally accepted (for recent reviews see Fuchs, 1974; Ebashi, 1976). Much is known about the way in which the interactions of the contractile proteins are regulated by $Ca^{2+}$ (Weber & Murray, 1973). The function of the sarcoplasmic reticulum in maintaining a low intracellular Ca ion concentration ($[Ca^{2+}]$) in the resting muscle is well established (Inesi, 1972; MacLennan & Holland, 1975; Endo, 1977). The excitatory process at the surface membrane is well understood, and a good deal has been learned about the mode of spread of excitation into the internal membrane system (Costantin, 1975). Of all the factors involved in the control of muscular activity, least is known about the mechanisms responsible for the moment-to-moment control of the intracellular $[Ca^{2+}]$ during the normal contractions of living muscle. The development of knowledge about these mechanisms and about the relation of changes in intracellular $[Ca^{2+}]$ to contractile function has been impeded until recently by the lack of suitable methods for monitoring those changes.

One of the most promising approaches to the measurement of changes in cytoplasmic $[Ca^{2+}]$ has involved the intracellular injection of the $Ca^{2+}$-sensitive bioluminescent protein aequorin. This substance, first isolated by Shimomura, Johnson & Saiga (1962) from the luminescent jellyfish *Aequorea*, emits blue light in the presence of even very low $[Ca^{2+}]$, is relatively specific for $Ca^{2+}$, and requires no other cofactors (for review see Blinks, Prendergast & Allen, 1976). It was first used as an intracellular Ca indicator in the giant barnacle muscle fibre by Ashley & Ridgway (1970), and has subsequently found application in a variety of other cells (for references see Blinks, 1978). Known difficulties associated with the method are that aequorin is consumed in the luminescent reaction, that the relation between $[Ca^{2+}]$ and light intensity is not linear, that the sensitivity of the indicator to $[Ca^{2+}]$ is influenced by the composition of the cytoplasm, and that the kinetics of the luminescent reaction limit the ability of aequorin to track very rapid changes in $[Ca^{2+}]$ (see Discussion). Nevertheless, a large amount of useful information can be obtained from aequorin-injected cells despite these limitations.

Although the studies of Ashley & Ridgway (1970) yielded valuable insights into the regulation of contraction, their results with the giant barnacle muscle fibre cannot be related directly to the body of information that has been accumulated over the years on the mechanism of contraction of vertebrate striated muscle, particularly amphibian twitch muscles. The development of techniques for preparing, handling, and injecting concentrated solutions of aequorin through very small pipettes has now progressed to the point where it is possible to record luminescent responses from single amphibian muscle fibres with a fair degree of regularity. This paper represents a general description of the characteristics of those signals and the ways in which they may be modified by some important physiological variables. Preliminary reports

of some of our results have already been presented (Rüdel & Taylor, 1973; Rüdel, Taylor, Blinks & Mattingly, 1973; Taylor, Rüdel & Blinks, 1975; Rüdel, Blinks & Taylor, 1976).

## METHODS

### *Preparation, mounting, and stimulation of muscle fibres*

Single twitch muscle fibres were dissected from the tibialis anterior and semitendinosus muscles of the frogs *Rana temporaria* and *R. pipiens*, and from the tibialis anterior and iliofibularis muscles of the toad *Xenopus laevis*. *Rana temporaria* were obtained from the Frog Farm, County Meath, Ireland; the other species were purchased from Mogul-Ed, Oshkosh, Wisconsin, U.S.A. Dissections were carried out in a physiological salt solution (composition given below) containing $1{\cdot}5 \times 10^{-5}$ M-(+)-tubocurarine. The fibres were prepared with short tabs of tendon in which pinholes were made for the insertion of hooks. For injection, microscopic inspection, and most experiments, the fibres were suspended horizontally in a shallow uncovered glass-bottomed bath mounted on a temperature-controlled stage. For studies in which the bathing solution had to be changed rapidly, the fibres were transferred after injection to a small covered flush-through chamber similar in principle to that described by Hodgkin & Horowicz (1959). All results presented in this paper were obtained with an isometric recording system. The fibres were suspended between two fine stainless-steel hooks, one of which was fixed to the bath, and the other attached to a force transducer. An RCA 5734 mechanoelectronic transducer tube was used in many of the experiments; this was subsequently replaced by an AME Model AE strain gauge transducer (Aksjeselskapet Mikroelectronikk, Horten, Norway). The transducer was mounted on a micrometer slide which was used to adjust the fibre length. The fibres were stimulated with square-wave pulses of 0·5 msec duration and about twice threshold strength which were derived from a digital programmer and pulse generator and delivered through a Hewlett Packard 6824A DC amplifier. In the open bath, the stimulating electrodes were a pair of bright platinum plates 10 mm apart, extending the depth of the chamber and more than the length of the fibre. In the flush-through chamber, they were platinum wires 3 mm apart running parallel to the fibre along opposite sides of the chamber.

Before aequorin was injected, the contractile responses of the fibre to single pulses and to tetanic stimulation were recorded. The dimensions of the fibre were measured and sarcomere spacing in the region to be injected was estimated for at least one setting of fibre length by counting striations with a microscope (Zeiss-Oberkochen 40× water immersion objective, N.A. 0·75, or Nikon 20× water immersion objective, N.A. 0·33). In a few fibres the contractions were filmed at 20–50 frames/sec before and after the injection of aequorin. Methods for cinemicrography were essentially the same as those reported previously by Costantin & Taylor (1973).

### *Solutions*

The standard physiological salt solution contained (mM) NaCl 115, KCl 2·5, $CaCl_2$ 1·8, $Na_2HPO_4$ 2·15, $NaH_2PO_4$ 0·85; the pH was 7·2.

$Ca^{2+}$-free solution was identical to the standard solution except that $CaCl_2$ was omitted and 3 mM-$MgCl_2$ and 3 mM-EGTA (ethyleneglycol-bis($\beta$-aminoethylether)-*N*,*N*′-tetraacetic acid) were included instead (Stefani & Chiarandini, 1973).

High $Ca^{2+}$ solution contained only 83 mM-$CaCl_2$ and 2·5 mM-KCl (DeMello, 1973).

High $K^+$ solutions were made with methanesulphonate as the impermeant anion to maintain a constant $[K^+] \times [Cl^-]$ product, 2 mM-MOPS buffer (3-(*N*-morpholino)propanesulphonic acid) to maintain the pH at 7·2, 1 $\mu$g tetrodotoxin/ml. to prevent sodium-dependent propagated responses, sucrose to maintain a constant osmotic strength, and 1·8 mM-Ca acetate.

Inorganic salts were Fisher certified reagent grade except for NaCl which was biological grade. EGTA and methanesulphonic acid were obtained from Eastman Organic Chemicals; MOPS and tetrodotoxin from Sigma Chemical Co.; (+)-tubocurarine from E. R. Squibb & Sons; caffeine from Eli Lilly & Co.

### *Preparation of aequorin*

Aequorin, extracted from specimens of *Aequorea* (collected at Friday Harbor, Washington), was purified in 10 mM-EDTA (ethylenediaminetetraacetic acid) by a five-step sequence.

1. Differential ammonium sulphate precipitation (25–75 % of saturation cut).

2. Gel filtration on Sephadex G-50 (fine).

3. Ion-exchange chromatography on QAE Sephadex (A-50) with pH step and [NaCl] gradient elution.

4. Gel filtration on Sephadex G-50 (fine).

5. Ion-exchange chromatography on DEAE Sephadex (A-50) with [NaCl] gradient elution.

The product was shown by isoelectric focusing to be a mixture of luminescent proteins with isoelectric points between pH 4·4 and 4·7. Polyacrylamide gel electrophoresis revealed no bands detectable with Coomassie blue staining that had not emitted light when the gels were immersed in $CaCl_2$. Densitometric scanning of gels after sodium dodecyl sulphate gel electrophoresis indicated that about 97 % of the protein present ran as a single band. The specific photon yield of the product was $1{\cdot}6 \times 10^{16}$ photons/mg, calculated from measurements of light yield at 22 °C calibrated against the radioactive light standard of Hastings & Weber (1963) and on the basis of an extinction coefficient ($E^{1\%}_{1\,cm}$) at 280 nm of 27 (Shimomura & Johnson, 1969).

The purified aequorin was freed of NaCl and EDTA by passage through a 100 cm column of Sephadex G-25 (medium), equilibrated and eluted with 10 mM-ammonium acetate that had been decalcified by passage through a column of Chelex 100 (Bio-Rad Laboratories) in the ammonium form. The aequorin was then lyophilized in the presence of Chelex 100 beads. Since ammonium acetate sublimes during lyophilization, the product was virtually salt-free. For intracellular injection, the lyophilized aequorin was dissolved in 140 mM-KCl which had been freed of Ca by passage through a column of Chelex 100 in the potassium form. (KCl was used rather than distilled water so that conductivity would be high enough to allow membrane potentials to be detected with the injection pipette.) This solution, which had a concentration of active aequorin (determined from light yield) between 1 and 3 mg/ml., was filtered just before use by passing it through a small disk of 0·1 μm Millipore filter in a miniature holder made for the purpose. For details of these procedures see Blinks *et al.* (1978).

*Injection of aequorin*

Pipettes for microinjection were pulled from acid-washed glass with a standard microelectrode puller (Industrial Science type M-1); the puller was adjusted to produce abruptly tapered pipettes with tips small enough so that cells could be impaled without producing local contractures, but large enough so that nanoliter volumes of fluid could be expressed in a few seconds by the application of gas pressure to solutions inside. Satisfactory pipettes had resistances of 3–5 MΩ when filled with 3 M-KCl. The tips were too small to be resolved under green light with the light microscope (oil immersion objective, N.A. 1·30).

The pipettes were filled from the butt; a small amount (2–5 μl.) of freshly filtered aequorin solution was deposited at the shoulder of the pipette with a finely drawn-out piece of polyethylene tubing attached to a 5 μl. Eppendorf pipette. Air bubbles were dislodged from the tip of the pipette with a carefully washed, tapered glass filament. The pipette was then mounted in a holder similar in principle to that described by Grundfest, Kao & Altamirano (1954) which enabled us to inject and monitor the membrane potential through the same pipette. A washed platinum wire (silver rapidly inactivates aequorin) made contact with the aequorin droplet and nitrogen pressure could be applied to the inside of the pipette.

For injection, the isolated muscle fibre was stretched until it was somewhat taut (sarcomere spacing 2·6 μm or more). To stabilize the fibre a small fire-polished glass rod was sometimes brought up against its mid-section with one micromanipulator. The injection pipette was positioned near the fibre with a second micromanipulator, and its patency verified by ejecting aequorin into the $Ca^{2+}$-containing physiological salt solution while light emission was recorded. The fibre was then impaled under visual control. The potential difference between the platinum wires in the bathing solution and in the injection pipette was monitored as an indicator of membrane penetration. When penetration was verified by a sudden change in potential, pressure was applied to the pipette (usually intermittently) and gradually increased (from less than one to 5 or more atm) until the fibre swelled visibly around the site of impalement. When the pressure was released, the swelling rapidly subsided. The injection pipette was withdrawn from the cell and removed from the vicinity of the muscle bath (because the relatively large amount of aequorin remaining in the pipette emits a substantial amount of light no matter how carefully the tubing has been cleaned). The muscle fibre was then tested for its ability to contract normally and to emit light on stimulation. If the amount of light was judged insufficient for satisfactory recording, one or more additional injections were attempted.

On one occasion immediately after a successful injection, as well as at the end of many of our usual experiments, the muscle fibre was transferred to the cuvette of a calibrated integrating photometer for the purpose of determining the amount of active aequorin in the fibre. The fibre was placed in the cuvette in approximately 0·5 ml. of the high-$Ca^{2+}$ solution. The aequorin was then discharged by injecting 0·5 ml. 50 mM-caffeine into the cuvette while the integrated light output was recorded.

Muscle fibres were sometimes damaged during attempts to inject them, as was evidenced by the development of a local contracture around the injection site. If the break in the membrane was not too severe, it could often be resealed without discharging the injected aequorin by exposing the fibre to the high-$Ca^{2+}$ solution (DeMello, 1973) for a few minutes. When returned to the normal physiological salt solution, fibres that had been treated in this manner appeared normal, contracted normally, and showed aequorin responses that were indistinguishable from those of fibres that had been injected without any apparent damage. Nevertheless, the results that we consider most reliable were obtained from fibres that had suffered no apparent trauma, and survived for many hours giving stable mechanical and luminescent responses to stimulation. All Figures in this paper were made from fibres of this type.

### *Light recording*

The muscle bath was mounted in a totally darkened room adjacent to the laboratory containing most of the electronic equipment. The only potential source of background light in the dark room was the RCA 5734 transducer tube, and that was coated with a dense black paint. The pipette used for injecting aequorin was removed from the room, and no fluorescent lamps were present.

Light from the muscle fibre was detected with photomultiplier tubes mounted in several ways. For most purposes, an EMI 9635 B photomultiplier was mounted directly under the bath with the 45 mm photocathode as close (35 mm) to the muscle fibre as the dimensions of the housing and shutter would permit. The solid angle subtended by the photocathode was thus 1 steradian, or 8 % of the total solid angle about the light source. Light from the muscle fibre passed through a clear acrylic plastic window in addition to the glass bottom of the muscle chamber and the window of the photomultiplier tube. At other times the photomultiplier was mounted above the preparation with either a 25 mm diameter polished acrylic plastic rod, or a $10 \times 15$ mm rectangular glass fibre-optic light guide extending from the surface of the muscle chamber to the shutter of the photomultiplier. When the distribution of the injected aequorin was to be studied, two photomultipliers were used simultaneously (the EMI 9635 B mounted directly below the muscle chamber and an EMI 9502 B connected to a thin (0·5 mm) fibre-optic probe that was used to scan the length of the muscle fibre).

The photomultipliers were operated at room temperature with the anode at virtual earth potential. The overall voltage applied to the dynode chain was 1100 V in the case of the EMI 9635 B; 1300 V for the 9502 B. Light signals were recorded either with Zeltex ZA 801M1 operational amplifiers wired to convert anode current to voltage signals, or with an SSR Model 1120-11-5 photon counting system. The latter system includes an amplifier-discriminator which converts separate electron cascades from the photomultiplier into pulses of uniform amplitude and duration. These are both counted digitally and converted to an analogue signal by feeding them, after fourfold prescaling, into an operational amplifier wired as a 'leaky integrator'. The time constant of the analogue light recording system was $< 1$ msec except in the experiments on potassium contractures when it was 10 msec. The photon-counting system was used in an effort to measure the light emitted by muscle fibres at rest. The analogue output of the photon-counting system had no particular advantage over a simple record of anode current for the measurement of rapid Ca transients, but was often used for that purpose so that attempts to detect a resting glow could be made conveniently from time to time during an experiment.

The light and force signals, stimuli, and oscilloscope triggering pulses were recorded simultaneously with a chart recorder and with an Ampex SP-300 FM tape recorder (signal down 2 db at 625 Hz at tape speed used). The records shown in the Figures of this paper were all made from the tape recordings; signals were either photographed directly from an oscilloscope screen (white on black) or read out at low speed on an $X$-$Y$ plotter (black on white) after temporary storage in the digital memory of a Nicolet 1074 instrument computer. In some cases (indicated in the Figure legends) repetitive signals were averaged with the Nicolet instrument.

## RESULTS

Most of the experiments reported here were carried out on muscle fibres from *Rana temporaria*. Results from the other species studied were qualitatively similar; the origin of the muscle fibre used is indicated for each Figure.

### *Influence of the aequorin injection*

On several occasions the rather rapid injection process was filmed at 20–50 frames/sec through a compound microscope. When the films were projected in slow motion, it appeared that the injected solution lifted the surface membrane slightly from the underlying material and distributed itself in a patch some 100 $\mu$m in diameter around the tip of the pipette (see Pl. 1). The swelling of the fibre at the injection site subsided within a few seconds after the pressure was removed. When the pipette was first removed there was sometimes a small pimple in the surface membrane, but in fibres that survived the injection this soon disappeared, leaving no visible sign at the site of injection.

Assays carried out on a number of fibres at the end of the experiment and also on one freshly injected fibre indicated that the injection of about 1 nl. of a solution containing aequorin in a concentration of 1–3 mg/ml. was sufficient to give luminescent responses for twitches in which the peak photomultiplier signal was at least 30 times the dark current or count. The amount of aequorin usually injected is estimated to have been of the order of 1–5 ng.

Neither the injection nor the presence of aequorin appeared to influence the mechanical function of the muscle fibres as judged by any of the criteria available to us. The fibres responded with large force and light signals after local stimulation at one end, as well as after the transverse field stimulation normally applied. The tension records made in isometric twitches and tetani after the injection did not differ discernibly from those made before. During isometric tetani, the injected region of the fibre did not appear different or behave differently from the rest of the cell, either when it was viewed directly through the microscope, or when cine-films of contractions were projected in slow motion. Therefore, the action potential presumably propagated throughout the fibre and excitation–contraction coupling proceeded in an essentially normal manner after the injection. Aequorin-injected muscle fibres continued to behave normally throughout experiments lasting eight hours or more.

### *Diffusion of aequorin within the muscle fibre*

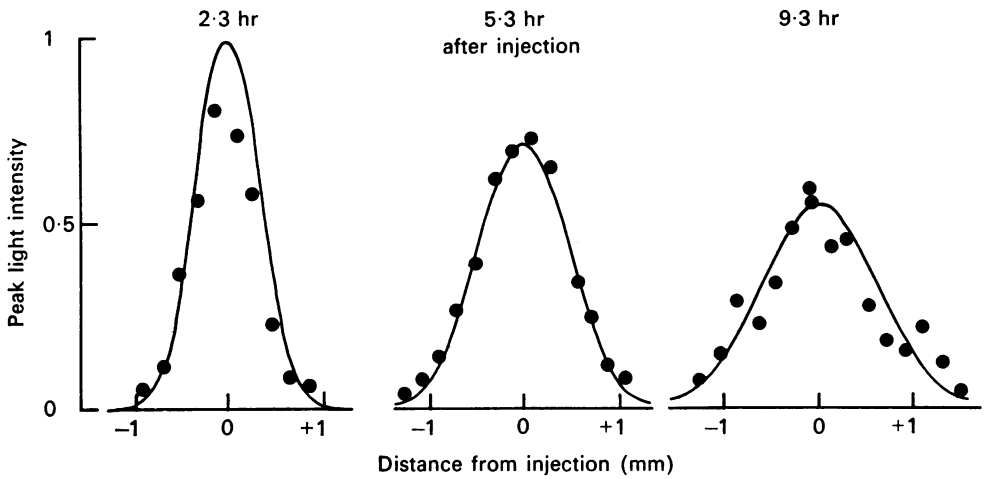
To estimate how rapidly the aequorin spread from the site of injection, we used a thin (0·5 mm diameter) fibre-optic probe to scan the light emission along the length of the muscle fibre. The probe was mounted in a micromanipulator, and was held about 0·5 mm above the fibre. It was then moved in 0·2 mm steps along the length of the fibre. At each step the fibre was stimulated to give a brief tetanus, and the light was recorded simultaneously through the probe and with the large-aperture photomultiplier mounted beneath the muscle chamber. The light measured with the probe was expressed as a percentage of that recorded through the large-aperture photomultiplier in order to correct for any depletion of the aequorin during the course of the experiment. The scan was repeated at intervals of several hours to determine how far the aequorin had spread from the site of injection. Text-fig. 1 shows results from the longest of three such experiments. The points represent the peaks of the aequorin responses recorded with the scanning probe, normalized to the peak of the aequorin responses recorded with the large photomultiplier

at that time. The fibre was scanned 2·3, 5·3, and 9·3 hr after the injection and a spreading of the aequorin during this 7 hr period is quite obvious from these three sets of measurements.

The curves drawn in Text-fig. 1 are theoretical ones calculated from the equation for diffusion in one dimension (Crank, 1956, p. 10)

$$\frac{C}{M} = \frac{\exp(-x^2/4Dt)}{2\sqrt{\pi Dt}}$$

where $C$ is the concentration in a plane at distance $x$ from the site of injection, $M$ is the amount of substance injected at $t = 0$ and $x = 0$, $t$ is the time after injection, and $D$ is the diffusion coefficient. Strictly, the equation should take the initial shape of the injected bolus into consideration, but after the early period of redistribution the results should not be greatly influenced by our assumption of an initial planar distribution. The distributions obtained with this equation were convolved with an empirically determined acceptance function that took into account the width of the fibre-optic probe and the fact that it accepted light from a conical space, not simply from the point immediately beneath its axis. The curves shown in Text-fig. 1 were calculated with a diffusion coefficient $D = 5 \times 10^{-8}$ cm²/sec. Curves calculated with $D = 3 \times 10^{-8}$ and $1 \times 10^{-7}$ cm²/sec gave substantially worse fits to the experimental points.

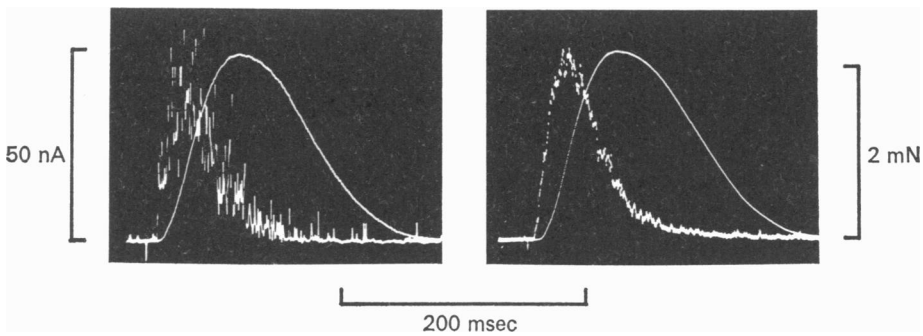



Text-fig. 1. Diffusion of aequorin from the site of injection. Single fibre from semitendinosus muscle of *Rana temporaria*. Striation spacing 2·3 μm; temperature 15 °C. The fibre was injected with aequorin at a single point near its middle. Light was recorded with a 45 mm photomultiplier mounted directly under the muscle chamber, and also through a thin (0·5 mm diameter) fibre-optic probe held in a micromanipulator. The probe was held so that it almost touched the fibre, and was moved in 0·2 mm steps to scan the length of the fibre. At each position, the muscle was stimulated with a brief (0·1 sec) 77 Hz tetanus. Successive scans were made 2·3, 5·3, and 9·3 hr after the injection. The points show the light intensity measured through the fibre-optic probe normalized with respect to the light intensity recorded (with the large-aperture photomultiplier) from the whole fibre. The curves show the distribution of light intensities (corrected for light acceptance by the probe) that would be expected if aequorin spread from the site of injection with a diffusion coefficient of $5 \times 10^{-8}$ cm²/sec.

### *Luminescence in resting fibres*

Although there is no level of [$Ca^{2+}$] below which aequorin luminescence does not occur (see Allen, Blinks & Prendergast, 1977), we were unable to detect luminescence from healthy aequorin-injected muscle fibres at rest. In an effort to measure the level of luminescence at rest, we used the photon counter in conjunction with an

EMI 9635 B photomultiplier tube that had a dark count of about 80/sec at 20 °C. The shutter between the muscle bath and the photomultiplier was alternately opened and closed for 1–5 min periods while the photon count was accumulated digitally. Although counts were made for aggregate periods of up to 30 min at various temperatures between 5 and 15 °C, we never found in healthy fibres that there was a significant difference between counts during the times that the shutter was open and closed. This was true whether the fibre was at slack length or stretched to a striation spacing of 3·6 $\mu$m or more. A few fibres that had been visibly damaged did have a resting glow shortly before they lost the ability to respond to stimulation.



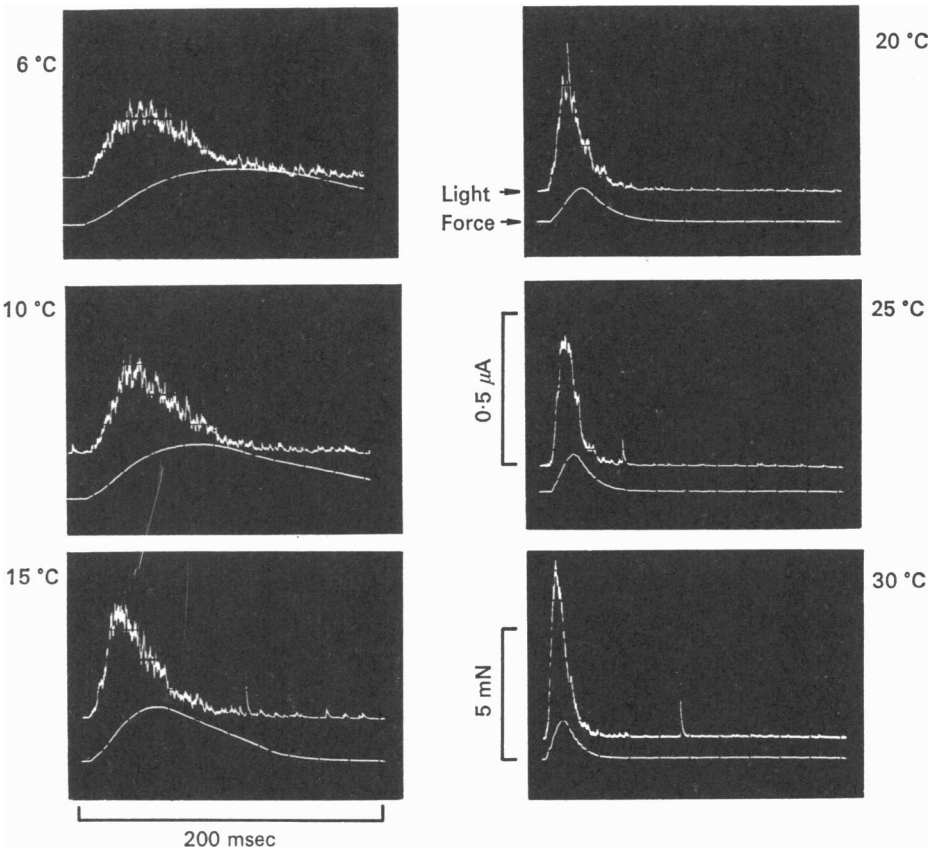

Text-fig. 2. Luminescent and mechanical responses in isometric twitches. Single fibre from tibialis anterior muscle of *Rana temporaria*. Striation spacing 2·3 $\mu$m; temperature 10 °C. Left panel shows records of light (noisy trace) and force from a single rested-state contraction. In the right panel seven such twitches have been averaged to reduce photomultiplier shot noise. The time of the stimulus is indicated by the vertical mark below the baseline.

### *The aequorin response to a single stimulus*

Aequorin-injected muscle fibres emit readily detectable flashes of light ('aequorin responses') in response to individual electrical stimuli. The intensity of these flashes varies with the amount of aequorin that has been injected, but the time course does not. The left-hand panel of Text-fig. 2 illustrates the characteristics of the aequorin response and associated force development in a single rested-state twitch. (Rested-state contractions are defined as those preceded by a period of rest long enough so that the influence of previous activity is no longer detectable (Blinks & Koch-Weser, 1961).) In the right-hand panel, seven such signals have been averaged in order to reduce photomultiplier shot noise. The light and force signals usually begin to rise nearly simultaneously, though the change in force may lag a few milliseconds behind the light. The luminescent response reaches its peak well before the mechanical, usually at about the time that the rate of force development is maximal. This correspondence is not precise, however; the relation varies somewhat from fibre to fibre, and is influenced by temperature and fibre length. The light intensity decays approximately exponentially, and usually has declined to less than 50 % of its peak level by the time peak force is achieved.

Our experimental procedure was such that we normally started recording light signals within a few minutes after the injection, and the rested-state aequorin

response was always essentially constant thereafter unless the experimental conditions were changed or the fibre was subjected to frequent tetanic stimulation (in which case the aequorin was sometimes depleted significantly). On several occasions we recorded aequorin responses from muscles stimulated to twitch regularly at intervals of 20–300 sec for periods of 2–4 hr. There was no regular tendency of the luminescent response to change with time during these periods. After a successful injection a muscle fibre could usually be used in an experiment for at least eight hours; during




Text-fig. 3. Influence of temperature on luminescent and mechanical responses in rested-state contractions. Isometric contractions of a single fibre from semitendinosus muscle of *Rana temporaria*; striation spacing 2·3 $\mu$m; temperatures as indicated. Calibrations are the same for all panels.

this time it could be stimulated to give thousands of twitches or dozens of tetani without a major reduction of the aequorin response. On several occasions we have recorded aequorin responses for more than 24 hr after a single injection. Experiments were usually terminated by exhaustion of the fibre or the experimenters, rather than by that of the injected aequorin.

Variations in temperature alter the amplitudes of the luminescent and mechanical responses in opposite directions. Text-fig. 3 shows a series of six rested-state contractions recorded from the same fibre at various temperatures. In this experiment

the peak intensity of the aequorin response increased 2·6-fold as temperature was raised from 6 to 30 °C, while there was a 40 % decrease in the peak tension developed. Both the luminescent and the mechanical responses become briefer as the temperature is raised. Although the time courses of the two responses are influenced in qualitatively similar ways by changes in temperature, the temporal relations between the two are modified somewhat. The tendency for the aequorin response to lead the mechanical one is more pronounced at low than at high temperatures: although the aequorin response always reaches its peak before the tension response does, the temporal discrepancy is relatively less pronounced at higher temperatures. Similarly, the tendency for the luminescent response to return to its baseline before the completion of relaxation is much more pronounced (in both absolute and relative terms) at low temperatures than at high.

*Aequorin responses during repetitive stimulation*

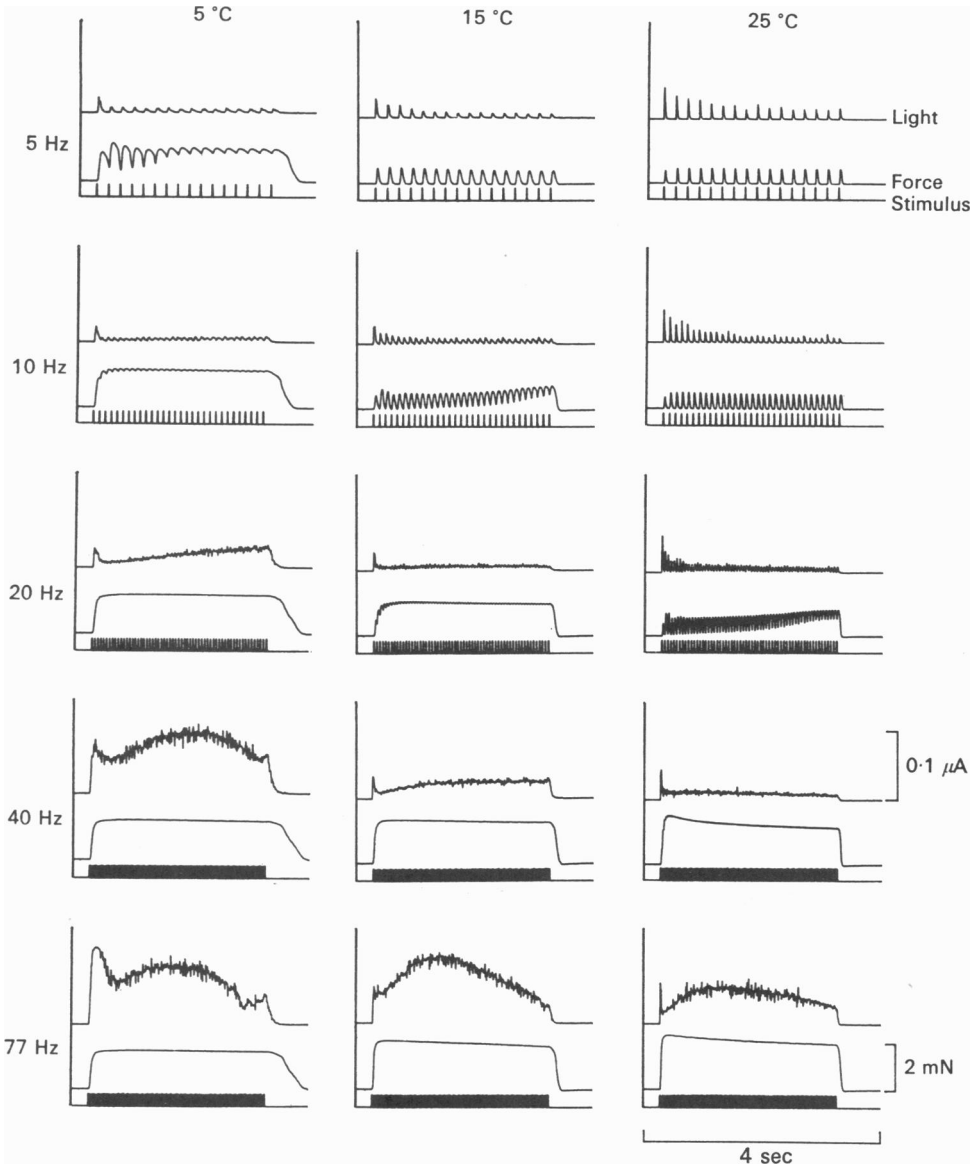
When trains of stimuli are applied to an aequorin-injected muscle fibre, the luminescent responses undergo progressive changes that suggest the operation of at least two progressive or cumulative processes. The patterns of these changes vary considerably with the temperature and stimulus frequency (Text-fig. 4), with the result that the individual processes are most evident under different experimental circumstances. The two processes are manifest as (1) a descending staircase of peak light intensity, and (2) a progressive slowing of the decay of the aequorin response.

*The descending staircase.* When a train of low-frequency stimuli is applied to a muscle fibre after a long period of rest, the first stimulus elicits a rested-state contraction. Subsequent responses form a characteristic descending staircase of peak light intensity that is obvious at all frequencies up to those producing a high degree of fusion of successive aequorin responses. The descending staircase is relatively brief, and at 15 °C is essentially complete within ten to fifteen contractions (Text-figs. 4 and 5). Stimulation can then be continued indefinitely without further change in the amplitude of the aequorin response. The approach to the steady state is faster at low temperatures than at high (Text-fig. 4). It also becomes faster with increasing stimulus frequency in such a way that the number of stimuli required to reach the steady state is roughly constant at any given temperature (Text-fig. 4). The extent to which the light intensity falls during the descending staircase increases with increasing frequency up to the point that the fall is distorted or cut short by the summation of successive responses. When a high degree of summation occurs, the initial descending staircase may be obscured altogether (Text-figs. 8 and 11).

The pattern of mechanical responses during the early part of a train of twitches is considerably more variable than that of the aequorin responses. In our experiments peak force usually rose somewhat over the first two or three twitches, and then fell slightly during the time that there was a descending staircase of aequorin responses (Text-fig. 4: 5 Hz and 10 Hz; Text-fig. 5). Sometimes these changes were followed by a long gradual rise in peak force. The relative prominence of the three mechanical changes was variable, and all were small in comparison to the descending staircase occurring simultaneously in the aequorin response.

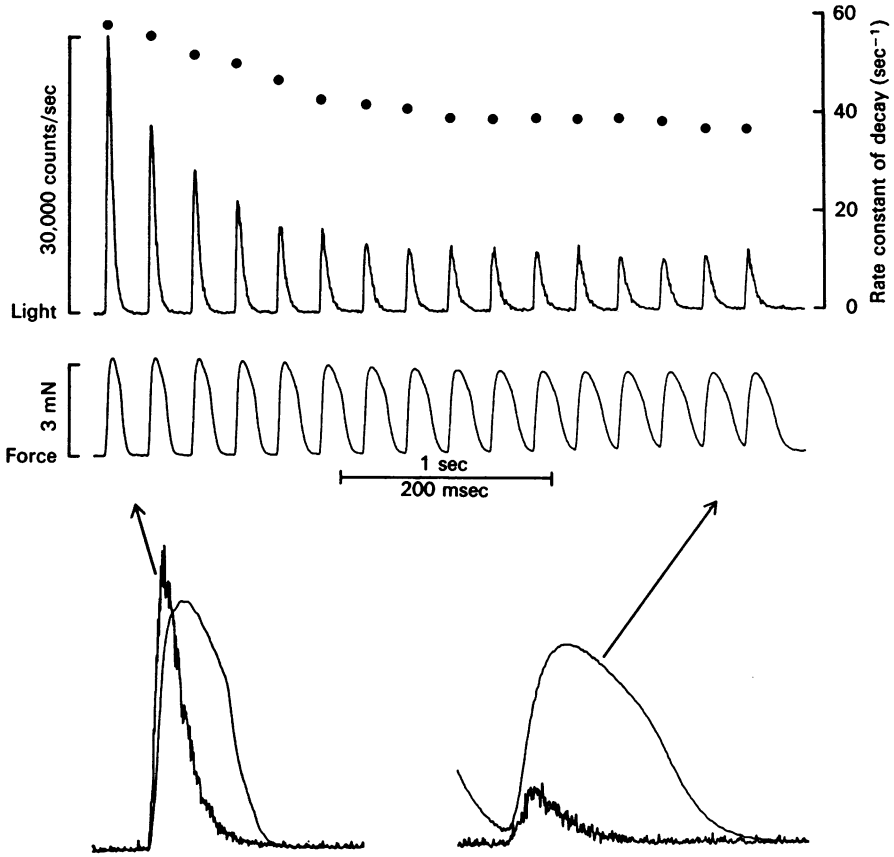
*Slowing of decay.* Although it is relatively inconspicuous in trains of unfused twitches, there is always a progressive increase in the half-time with which the

aequorin response declines in the successive contractions of a train following a period of rest. At very low frequencies of stimulation the time course can be followed by measuring the rate constants of decay of the individual aequorin responses in the train as in Text-fig. 5, but this seldom can be done satisfactorily without signal averaging. When the frequency of stimulation is increased to the point that




Text-fig. 4. Influence of temperature and stimulus frequency on fusion and summation. Isometric contractions of a single fibre from semitendinosus muscle of *Rana pipiens*. Striation spacing 2·3 $\mu$m; temperatures and stimulus frequencies as indicated. All records are from the same fibre; 3 sec tetani were separated by rest periods of at least 5 min, so the fibre was in the rested state at the start of each train of stimuli. Calibrations are the same in all panels.

successive aequorin responses are slightly fused, or become so during the course of the train, the slowing of decay becomes evident as a progressive rise in the level to which light intensity declines between stimuli (e.g., Text-fig. 4: 5°, 10 Hz). At low frequencies of contraction the progression of this change is very similar to that of the descending staircase (Text-fig. 5). At higher frequencies the descending staircase appears to run its course considerably more rapidly than the slowing of decay.

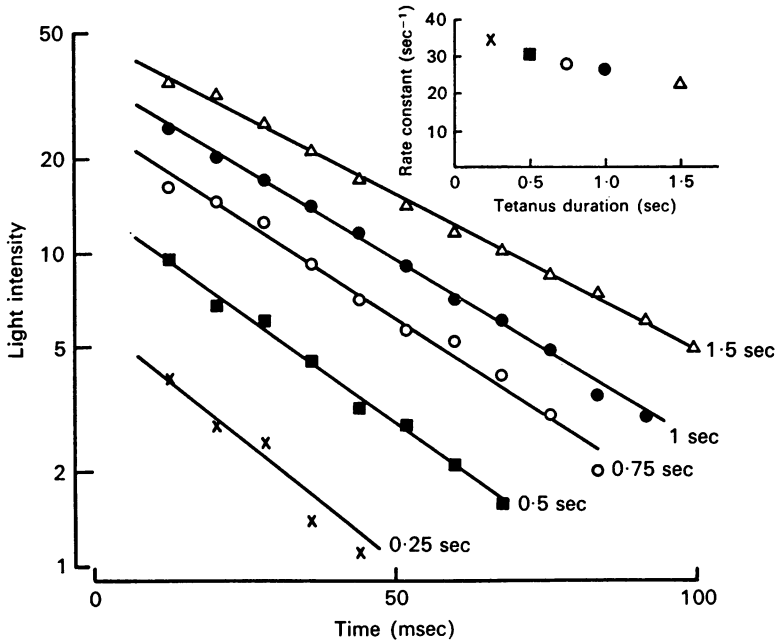



Text-fig. 5. Staircase phenomenon in successive isometric twitches. Single fibre from tibialis anterior muscle of *Xenopus laevis*. Striation spacing 2·1 μm; temperature 15 °C. Computer averaged records of ten trains of sixteen twitches at 5 Hz. Lower panels show high-speed tracings of the first and last contractions in the series. Points above light trace indicate rate constants for decay of luminescence in successive twitches.

The progression of the slowing of decay of the aequorin response in a series of twitches is also very much like that of the prolongation of mechanical response. The development of both these changes is most easily observed under conditions of stimulus frequency and temperature in which the responses to successive stimuli are partially fused. However, because at any given temperature the luminescent and mechanical responses begin to fuse at different frequencies (Text-fig. 4), it is not possible to use partially fused responses to compare the development of the two changes under identical conditions.

*Luminescent patterns in tetanic contractions*

Text-fig. 4 includes records of tetani made under a range of conditions of temperature and stimulus frequency. The aequorin responses show multiphasic patterns which can be interpreted as resulting from the summation of signals that undergo the two changes already described (the descending staircase and progressive slowing of decay) plus a third change that is obvious only at very high frequencies of stimulation (fade). At frequencies up to those at which fusion of successive aequorin responses begins to occur (approximately the point at which the mechanical responses become smoothly fused), the descending staircase is the dominant feature of the




Text-fig. 6. Influence of tetanus duration on the decay of the aequorin response. Single fibre from tibialis anterior muscle of *Rana temporaria*. Temperature 15 °C; striation spacing 2·3 $\mu$m, stimulus frequency 25 Hz. Semilogarithmic plots of the decay of light intensity after tetani of varying duration. Inset shows influence of tetanus duration on the rate constant for decay of light intensity.

pattern of luminescence during tetani. At higher frequencies of stimulation the initial fall in light intensity is followed by a rise that may carry the intensity well above the initial peak. This rise occurs only when successive aequorin responses are at least partially fused (i.e., do not return to baseline between stimuli), and presumably reflects progressive slowing of one or more of the processes responsible for terminating the aequorin response. At very high frequencies of stimulation, the amplitude of the tetanic aequorin response often tends to fade after one or two seconds of stimulation, but this is a much less regular feature than those described just previously. After the end of tetanic stimulation the aequorin response always declines much faster than force. As in the aequorin response associated with a twitch,

the decline of the tetanic aequorin response is fairly well described by a single exponential, the time constant of which varies with the conditions of the experiment.

As a check on the presumption that the gradual rise in luminescence during a tetanus was due to progressive slowing of a process responsible for terminating the aequorin response, we did the experiment illustrated in Text-fig. 6, in which the time course of the decline of light intensity was examined after tetani of various durations. As the tetanus was prolonged, there was a progressive decrease in the rate at which light intensity declined after the termination of stimulation. The inset of Text-fig. 6 shows that when the duration of the tetanus was increased from 0·25 to 1·5 sec, the rate constant of the decline dropped from 35 to about 25/sec. This type of analysis was carried out on fibres of both *R. temporaria* and *X. laevis* with similar results. Relaxation also became progressively slower as the duration of the tetanus was increased.

Over a wide range of frequencies the initial peak of light intensity in a tetanus following a long period of rest is equal to the aequorin response of a single rested-state contraction. In some fibres this level is not exceeded even during the plateau of a well-fused tetanus. However, when the frequency of stimulation is high enough to cause a large degree of summation even at the start of the train, the initial peak of light intensity may exceed that of the rested-state twitch (e.g., Text-fig. 4: 40 Hz, 5 °C). At very high frequencies the summation of successive responses may result in an initial peak of light intensity that is several times that high. This is more likely to occur at low temperatures than at high (cf. Text-fig. 4, 40 Hz at various temperatures). In this circumstance the decline from the initial peak is often more gradual than might be expected if it reflected only the influence of the process responsible for the descending staircase. Perhaps this reflects the simultaneous influence of the descending staircase and cumulation of responses.

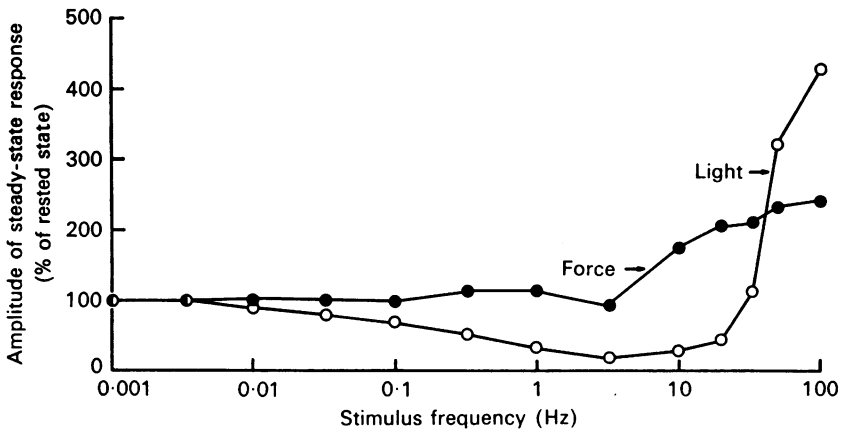
In comparing the luminescent and mechanical responses during tetanic contractions one is struck by the fact that the large fluctuations in light intensity just described are for the most part not accompanied by detectable changes in force. Mechanical tetani do tend to fade sometimes, and this tendency is most pronounced at high temperatures (Text-fig. 4). However, there is no obvious correlation between mechanical fade and luminescent fade or any other aspect of the luminescent response. Fibres sometimes failed to respond to some of the stimuli late in a train, and this was easily recognized by a jagged interruption in the record of luminescence (Text-fig. 4: 5°, 77 Hz). There was no corresponding break in the mechanical record unless the light intensity fell to very low levels.

### *Frequency–response relation*

The pattern of evolution of mechanical and luminescent responses after the initiation of stimulation has been illustrated for several stimulus frequencies in Text-figs. 4 and 5. The relation of the stable level of response eventually achieved to the frequency of stimulation is shown for a wide range of frequencies in Text-fig. 7. This graph covers a range of frequencies from those producing rested-state twitches at one extreme to the highest frequency the muscle fibre would follow at the other. (The aequorin responses in high-frequency tetani did not reach true steady states but tended to fade, as in the 77-Hz tetani of Text-fig. 4. In this situation the highest

light level attained after the initial descending staircase was plotted in lieu of a steady-state response.)

Under the conditions of the experiment shown in Text-fig. 7, contractions at frequencies below about 0·03 Hz were rested-state contractions. As the frequency of stimulation was increased above that point the descending staircase became progressively more pronounced and the eventual stable amplitude of the discrete aequorin responses decreased correspondingly. When the frequency became high enough that successive aequorin responses began to fuse (approximately 10 Hz in




Text-fig. 7. Influence of frequency of stimulation on amplitude of aequorin response and force developed in isometric contractions. Single fibre from semitendinosus muscle of *Rana temporaria*. Striation spacing 2·4 $\mu$m; temperature 15 °C. Open circles indicate amplitude of the aequorin response; filled circles the force generated when stable responses had been attained at each of the stimulus frequencies indicated. At the highest frequencies the aequorin response does not reach a true steady-state, so the peak light intensity is plotted. (Same fibre as in Text-figs. 12 and 13.)

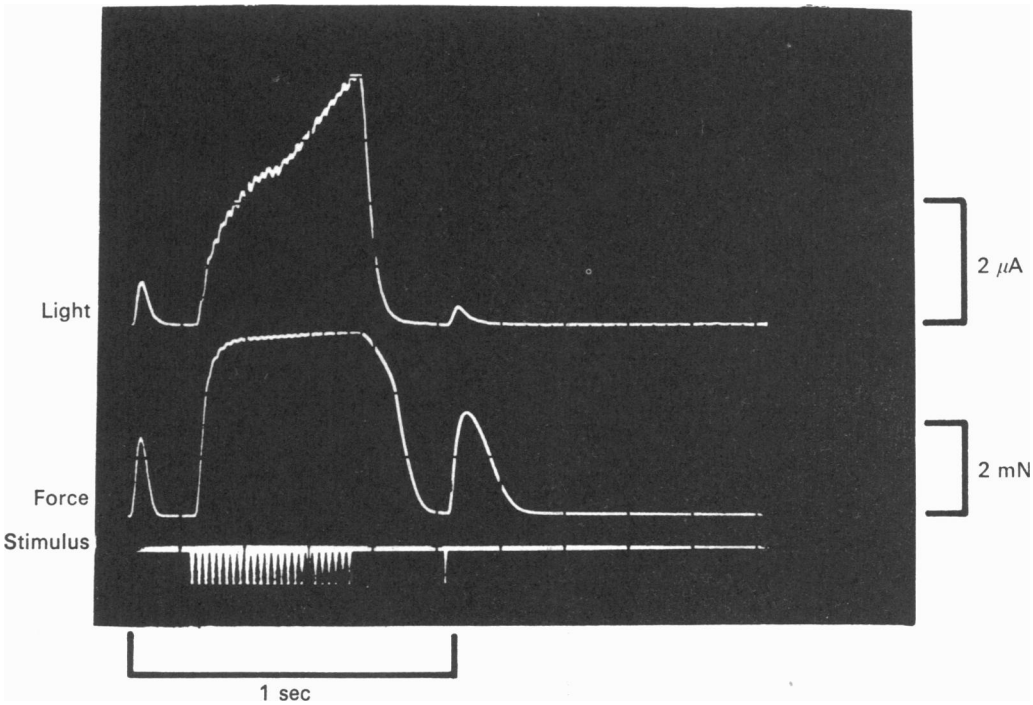
this fibre), summation occurred and the final level of light intensity began to rise again. From that point on it rose steeply with increasing frequency until the muscle fibre was no longer able to follow all of the stimuli. The curve shown in Text-fig. 7 was determined at 15 °C. At lower temperatures (see Text-fig. 4) the curves are shifted to the left and there is a reduction in the maximum frequency the muscle fibre will follow.

As one would expect from results already presented, Text-fig. 7 shows a poor correlation between peak light intensity and force developed. Force is well maintained as the peak light intensity of discrete twitches declines with increasing frequency, though in this experiment it did dip slightly at 3 Hz, where light intensity was lowest. In the range where summation of aequorin responses occurs, light intensity continues to rise steeply after force approaches a tetanic plateau.

### *Post-tetanic responses*

As may be seen from Text-fig. 7, more than 100 sec may be required after a single twitch to reestablish rested-state conditions as judged by the amplitude of the aequorin response. Similarly, it takes a considerable time after a tetanus for the

rate of decline of the aequorin response to return to the level seen in twitches. The decline of the aequorin response to a single stimulus delivered soon after the end of a tetanus is nearly as slow as that for the tetanus itself. Text-fig. 8 illustrates this with a record from a fibre that was stimulated to give in rapid succession a rested-state twitch, a short 50 Hz tetanus, and a post-tetanic twitch. In the post-tetanic twitch the aequorin response is much smaller in amplitude and decays with a lower rate constant than in the rested-state twitch. On the other hand, the mechanical response in the post-tetanic twitch is increased in both amplitude and duration (post-tetanic potentiation).

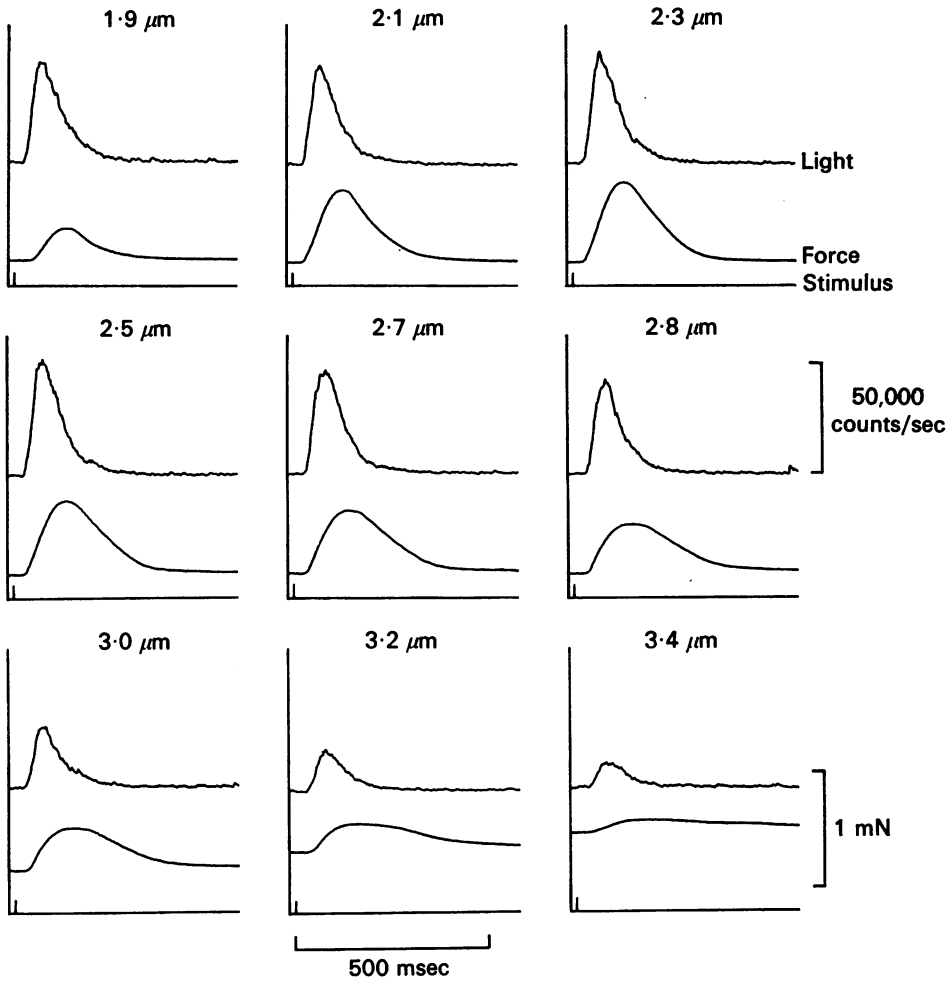



Text-fig. 8. Post-tetanic responses. Isometric contractions of a single fibre from iliofibularis muscle of *Xenopus laevis*. Striation spacing 2·5 μm; temperature 15 °C. First twitch is a rested-state contraction; this was followed by a 50-Hz tetanus, and a post-tetanic twitch.

If a short tetanus is followed by a second tetanus instead of a twitch, the form of the aequorin response in the second tetanus depends on the duration of the first tetanus and the time interval between the two. If the interval between tetani is short, the second luminescent response follows a time course much like that of the corresponding part of a long tetanus in which stimulation was not interrupted. There is no initial spike of luminescence, and the rise in light intensity is slower in the second tetanus than in the first, presumably because the rise reflects the summation of individual aequorin responses that are smaller (as a result of the process responsible for the descending staircase).

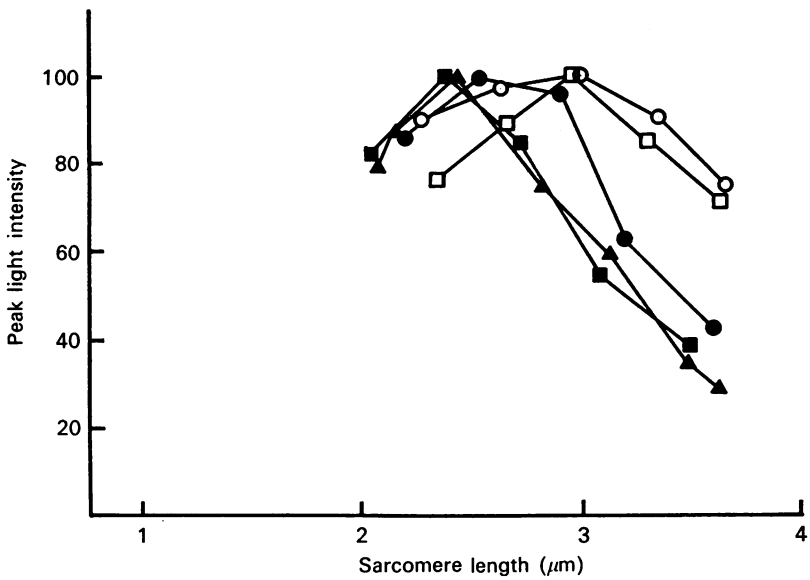
*Influence of fibre length*

All results described so far were obtained from fibres contracting isometrically at lengths (striation spacings from 2·1 to 2·5 $\mu$m) slightly greater than slack. Text-fig. 9 shows examples of luminescent and mechanical responses obtained from one fibre in twitches at various lengths. Each panel is an averaged record of four successive rested-state twitches at the estimated striation spacing indicated. Contractions at lengths of 2·1 $\mu$m or above were 'isometric', whereas at lower lengths the fibre was




Text-fig. 9. Aequorin responses and force developed in twitches at various fibre lengths. Single fibre from tibialis anterior muscle of *Rana temporaria*. Temperature 5 °C; sarcomere lengths as indicated. Averaged records of four successive rested-state twitches at each fibre length. Contractions at sarcomere lengths of 2·1 $\mu$m and above were isometric. At 1·9 $\mu$m the fibre was slack and shortened before developing tension. Striation spacing was measured in the region of the aequorin injection (middle of fibre) when the fibre was slightly stretched. All other sarcomere lengths are estimates calculated from changes of fibre length. At the three greatest lengths the upward displacement of the force baseline reflects the resting tension.

slack at rest and had to shorten before developing force. This particular fibre was studied at 5 °C; similar experiments were carried out on five other fibres at 15 °C. Although there was substantial quantitative variation in the responses of the six fibres to stretch, all showed the qualitative pattern of changes illustrated in Text-fig. 9. As the fibre was stretched progressively from slack length the amplitude of the force and light signals always increased somewhat at first, then decreased progressively with further stretch. Although the mechanical response underwent the characteristic prolongation with stretch originally described by Hartree & Hill (1921) (see also Huxley & Niedergerke, 1958), the duration of the aequorin response was not influenced appreciably by fibre length. In the fibre of Text-fig. 9 the greatest

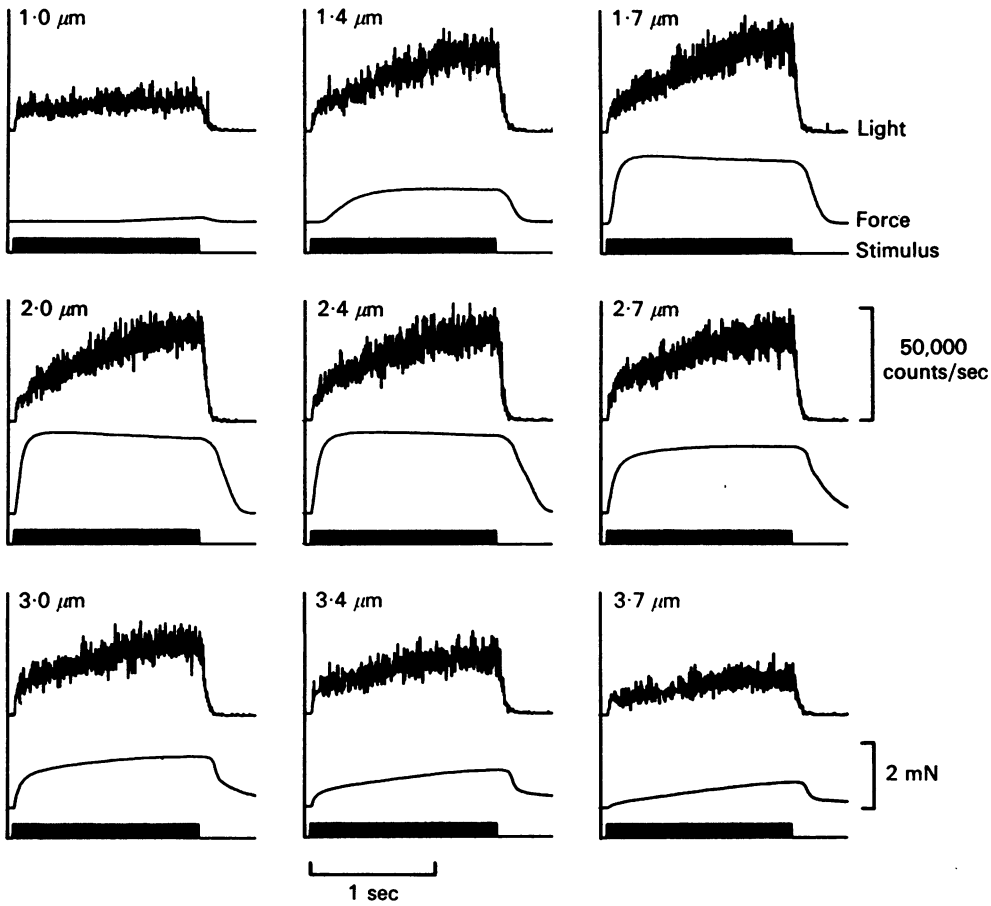



Text-fig. 10. Relation of peak light intensity to sarcomere length. Results from five single twitch fibres dissected from tibialis anterior muscles of *Rana temporaria* (separate frogs) and studied at 15 °C. Points show peak light intensity in rested-state twitch contractions as percentages of the maximum attained at any fibre length.

force and light responses were generated when the striation spacings were 2·3 and 2·5 μm respectively. There was considerable fibre-to-fibre variation in the sarcomere length at which each of these maxima was attained, and no obvious correlation between the lengths for the two maxima in individual fibres. The variation in the relation of peak light intensity to fibre length is illustrated in Text-fig. 10, which shows the results from the five fibres studied at 15 °C. The amplitude of the aequorin response was maximal between 2·4 and 3·0 μm, and decreased by as much as 70 % with stretch to 3·6 μm. At slack length (2·1 μm) the aequorin response was usually about 80 % of the maximum. At lengths calculated to be lower there was little further decrease in light intensity; the results are not shown in Text-fig. 10 because the contractions started from slack length and the aequorin response was probably nearly over before the fibres reached the calculated striation spacing if that length was reached at all. (Fibres do not shorten enough to develop tension in twitches at

calculated sarcomere lengths below about 1·8 $\mu$m.) The effects of stretch were of rapid, if not immediate, onset and were reversible, but the depression of the aequorin response produced by extreme stretch usually persisted for some minutes after the fibre was returned to slack length.

Text-fig. 11 shows a set of records obtained under conditions similar to those described for Text-fig. 9, except that each stimulus was a 50-Hz train lasting 1·5 sec and no signal averaging was applied. The peak amplitude of the light response was

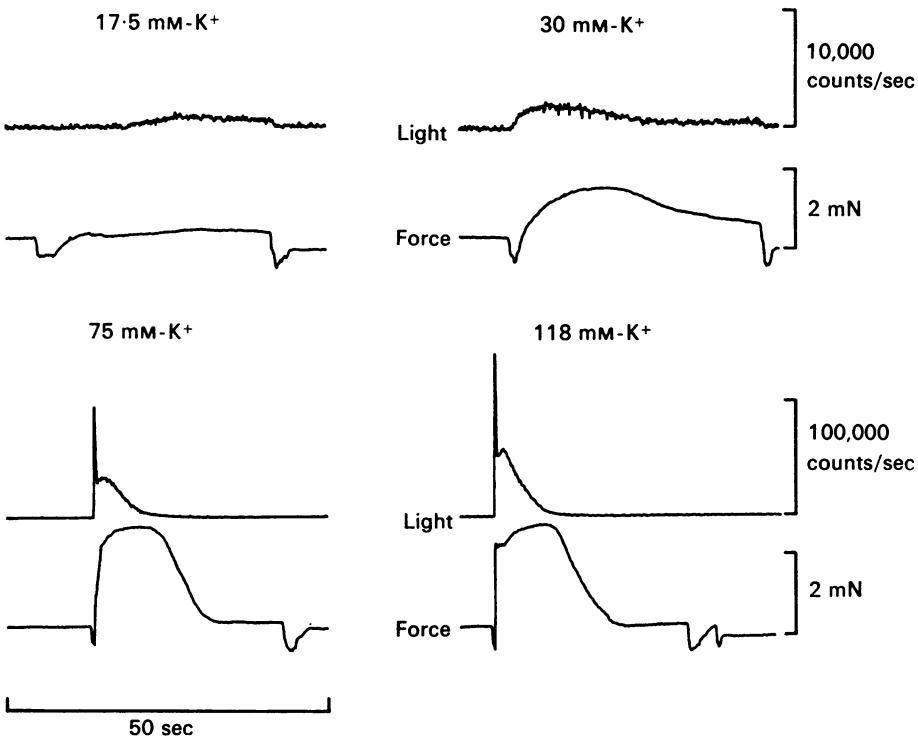



Text-fig. 11. Aequorin responses and force developed in tetani at various fibre lengths. Single fibre from tibialis anterior muscle of *Rana temporaria*. Temperature 15 °C, stimulus frequency 50 Hz, sarcomere lengths as indicated. Lengths were estimated as in Text-fig. 9; contractions at sarcomere lengths above 2·0 $\mu$m were isometric. Baselines of force records have been adjusted to the same level regardless of resting tension.

maximal at about 2·4 $\mu$m, and with stretch it decreased considerably. The upper left panel shows that when the fibre was allowed to shorten greatly, the aequorin signal rose for a much shorter time than in isometric contractions, and then stayed at a low level. In some fibres there was a tendency of the aequorin response to fade at such short sarcomere lengths even though it did not do so at greater lengths.

*The aequorin response to $K^+$-depolarization*

With electrical stimulation the action potential is normally all-or-nothing, producing a large and very brief depolarization of the surface membrane and T system. To examine the relation between luminescence and the generation of force at low sarcoplasmic [$Ca^{2+}$] and under conditions in which [$Ca^{2+}$] does not change so rapidly, we exposed aequorin-injected fibres to a series of bathing solutions of various [$K^+$] in which the regenerative spread of depolarization (longitudinally or into the T system) was prevented by tetrodotoxin. Each solution was flushed into the muscle

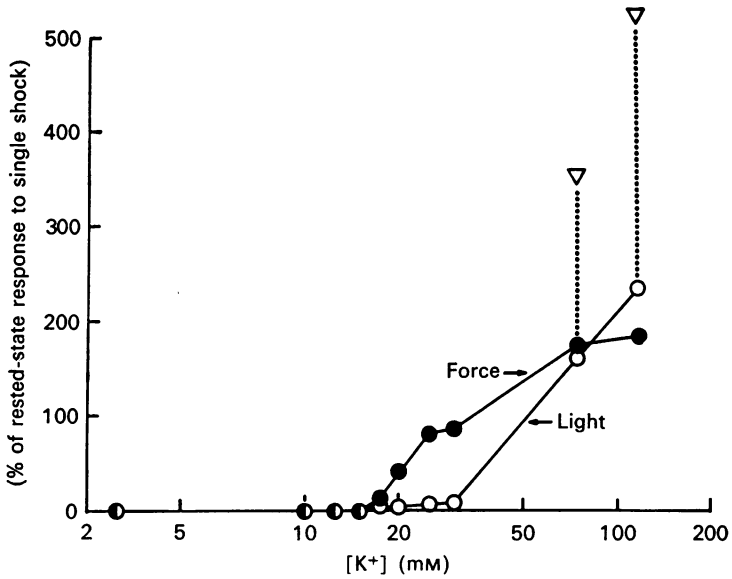



Text-fig. 12. Luminescent and mechanical responses in potassium contractures. Single fibre from semitendinosus muscle of *Rana temporaria*. Sarcomere length approximately 2·7 $\mu$m, temperature 15 °C. Downward deflexions on force record are flushing artifacts, and indicate the times at which the bathing medium was exchanged. In each panel, the first artifact marks the transition from 2·5 mM-$K^+$ to the concentration indicated, and the second artifact indicates replacement of the original solution. Tetrodotoxin, 1 $\mu$g/ml., was present throughout. The sensitivity of the light recording system is 10 times greater in the upper tracings than in the lower. Recording time constant for light was 10 msec. (Same fibre as in Text-figs. 7 and 13.)

chamber after a period of at least 5 min during which the fibre was exposed to the normal physiological salt solution (with tetrodotoxin). The range of [$K^+$] between 10 and 20 mM was covered in 2·5 mM steps because we were particularly interested in the relation between light and force near the point at which responses first became detectable. Our ability to detect small changes in force and light was limited by the

flushing artifact in the force record and by the fact that the resting glow was below our limit of detection. With these limitations we were not able to detect either mechanical or luminescent responses at 15 °C until [K+] was raised to 17·5 mM.

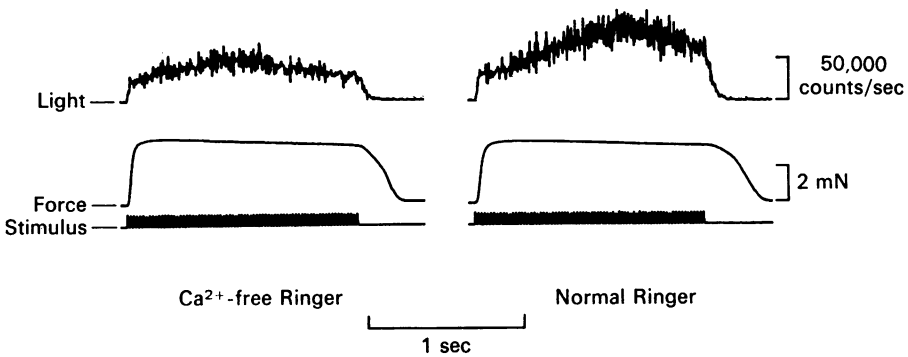
Text-fig. 12 shows a record made at 17·5 mM-K+, together with a series obtained from the same fibre at higher [K+]. At 17·5 mM-K+ both the aequorin and mechanical responses became detectable only after a substantial delay. At 30 mM-[K+] both responses were larger and considerably faster in onset. At the two highest concentrations, 75 and 118 mM, the aequorin responses were qualitatively different




Text-fig. 13. Relation of luminescent and mechanical responses to [K+]. Single fibre from semitendinosus muscle of *Rana temporaria*. Sarcomere length approximately 2·4 μm; temperature 15 °C. Open symbols indicate maximum light intensity, filled symbols peak force generated. All responses are expressed in terms of the force and light generated in rested-state twitch contractions of the same fibre. At the two highest [K+], the triangles indicate the light intensity in the initial flash; the circles indicate the peak of the slow phase of light emission (see Text-fig. 12). (Same fibre as in Text-figs. 7 and 12.)

from those at lower concentrations. They started with a spike which surged to some 40–70 times the amplitude observed in 30 mM-[K+]. The light intensity then dropped to about 40 % of the initial peak, at which point a more gradual secondary rise in luminescence occurred. By 10 sec light emission had largely subsided, though it remained above control levels for the duration of the exposure to high [K+]. Responses of this sort could be obtained repetitively in the same fibre, indicating that aequorin consumption was not responsible for terminating the luminescent response. The mechanical responses associated with the biphasic aequorin responses were characterized by an early rapid phase of onset that was clearly distinct from the more gradual final approach of force toward a plateau. At 15 °C maximum force was reached about 10 sec after the initial luminescent spike: after another 10 sec force had returned to the baseline while the fibre was still in high [K+] solution.

In Text-fig. 13 the maxima of luminescent and mechanical responses are plotted as a function of the [$K^+$] of the test solutions. Both measurements are scaled to the rested-state responses in ordinary twitches, and since these results were obtained from the same fibre used to make Text-fig. 7, a comparison of results from potassium contractures and electrical stimulation is possible. From Text-fig. 13 it is evident that force nearly equivalent to that of a rested-state twitch can be developed slowly in K contractures with a peak light intensity only about 10% as great as in the much briefer twitch. In solutions of high [$K^+$], force plateaued at a level very close to the tetanic force of the fibre, and the peak light intensity (in the initial flash) was comparable to that achieved in a 100-Hz tetanus.




Text-fig. 14. Influence of $Ca^{2+}$-free solution on the tetanic aequorin response. Single fibre from semitendinosus muscle of *Rana temporaria*. Sarcomere length approximately 2·4 $\mu$m; temperature 15 °C; stimulus frequency 50 Hz. The left-hand record was made after the fibre had been bathed in $Ca^{2+}$-free solution for 30 min during which time it was stimulated to give five rested-state twitches and one other 1·5 sec 50 Hz tetanus. The right-hand record was made 4·5 min after return to normal physiological salt solution.

### *Aequorin responses in $Ca^{2+}$-free solution*

It has long been clear that although small amounts of Ca enter the cell as a result of excitation, most of the Ca directly involved in activating the contractile proteins of vertebrate skeletal muscle must be released from intracellular stores (Sandow, 1965). The finding that isolated muscle fibres can function in a $Ca^{2+}$-free medium for a considerable time (Armstrong, Bezanilla & Horowicz, 1972) indicates that the influx of extracellular $Ca^{2+}$ does not play a key role in triggering intracellular Ca release. However, it does not necessarily follow from this that the aequorin response would not be significantly influenced by the influx of $Ca^{+2}$. Accordingly, we determined the influence of removing the extracellular $Ca^{2+}$ on the aequorin response. Aequorin-injected muscle fibres were bathed for periods of up to an hour in the $Ca^{2+}$-free solution containing 3 mM-$Mg^{2+}$ and 3 mM-EGTA. Twitches elicited at 5 min intervals during this time showed no significant changes in either the mechanical or the luminescent responses. The aequorin responses during tetani were distinctly more subject to fade in the $Ca^{2+}$-free solution (Text-fig. 14), though the mechanical responses faded relatively little.

## DISCUSSION

### *Distribution, consumption, and possible effects of injected aequorin*

The results shown in Text-fig. 1 make it clear that the longitudinal distribution of aequorin in the fibres that we have studied is neither uniform nor constant. Longitudinal spread is an order of magnitude slower than would be predicted from the diffusion coefficient ($8{\cdot}7 \times 10^{-7}$ cm$^2$/sec) measured by Shimomura & Johnson (1969) for aequorin in free solution, but only slightly slower than that reported by Ashley, Moisescu & Rose (1974) for the radial diffusion of aequorin in barnacle myofibrils. The retardation of spread is probably not due to the exclusion of aequorin from the myofilament lattice, since aequorin has a Stokes radius of 1·9 nm (Prendergast & Mann, 1978), which is an order of magnitude smaller than the interfilament distances of muscle. When aequorin is injected at one or a few points along a fibre, it probably distributes itself fairly uniformly across the fibre within a few minutes, but nothing approaching a longitudinally uniform distribution of the protein is achieved during the course of even a long experiment. Fortunately, the light intensity from a solution of given [$Ca^{2+}$] is directly proportional to the concentration of active aequorin. Thus, as long as the total amount of active photoprotein is constant, the redistribution of aequorin within a cell should not influence the amount of light emitted overall unless the calcium transients differ in regions of the cell that have different aequorin concentrations.

Two questions frequently arise in the context of the use of aequorin in muscle fibres: (1) how is it possible to record so many aequorin responses without discharging all of the aequorin, and (2) doesn't the aequorin interfere with contraction by competing with the contractile proteins for calcium ions? Both questions are best answered with some rough calculations.

After a typical injection some 1000 photon counts are registered during the aequorin response for a twitch. The optical geometry of our apparatus was such that about 8 % of the emitted light reached the photocathode, which has a quantum efficiency at 469 nm of about 20 %. Thus 1000 recorded counts correspond to about 62,500 photons emitted from the fibre. If the quantum yield of the luminescent reaction is about 0·3 (Shimomura & Johnson, 1970), this means that some 200,000 molecules of aequorin were consumed per twitch. A typical injection of aequorin contains more than 100,000 times this number.

In order to deal with the question of how much the presence of aequorin might alter free Ca concentrations, we shall assume that each aequorin molecule has three Ca binding sites, all of which must be filled for luminescence to occur (Allen *et al.* 1977), that all three binding sites have equal affinities for Ca, and that light intensity is proportional to the fraction of aequorin molecules having all three sites occupied. If $A$ is the number of aequorin molecules and $p$ is the probability of occupancy of a single Ca binding site, then the total number of sites occupied is $3pA$, and the number of molecules of aequorin with all three sites occupied is $p^3A$. The best available estimate of $p^3$ during a tetanus is $2 \times 10^{-3}$ (D. G. Allen and J. R. Blinks, unpublished; see p. 318 below), and thus $p$ is about 0·13. In the case of a typical (2 ng) injection $A$ was $10^{-13}$ mole, and the amount of Ca bound to aequorin ($3pA$) during a tetanus will have been $3{\cdot}9 \times 10^{-14}$ mole. If the aequorin was distributed in a 1 mm length of a muscle fibre 100 $\mu$m in diameter, the volume of distribution would have been $7{\cdot}9 \times 10^{-9}$ l., and the average concentration of Ca bound to aequorin about $5 \times 10^{-6}$ M. This is more than an order of magnitude lower than the concentration of Ca bound to the proteins of fully activated myofibrils ($2 \times 10^{-4}$ M) (Weber & Herz, 1963), and thus it seems unlikely that under the conditions of our experiments the injected aequorin would have bound enough Ca to interfere significantly with contraction. This conclusion is in accord with our observations that the force developed by isolated muscle fibres is unchanged by the injection of aequorin, and that by cinemicrography we could detect no abnormality of contraction at the site of injection.

*Factors influencing the interpretation of the luminescent signals*

*Obstacles to quantitative interpretation.* The interpretation of luminescent signals from aequorin-injected cells has been discussed extensively in a recent review (Blinks *et al.* 1976); only a few aspects will be considered in detail here. In short, it seems virtually certain that in muscle cells the aequorin response reflects a transient increase in the intracellular $Ca^{2+}$ concentration and not a reduction in the concentration of $Mg^{2+}$ or some other inhibitor of the luminescent reaction. However, a number of factors limit our ability to interpret the aequorin signals precisely in terms of $[Ca^{2+}]$, including (1) lack of sufficiently detailed information about the influence of environmental factors on the aequorin reaction, compounded by (2) lack of information about the intracellular environment, (3) the lack of a suitable method for uniformly controlling the intracellular $[Ca^{2+}]$, (4) the virtual certainty that there is spatial inhomogeneity of the intracellular $[Ca^{2+}]$ during activity, coupled with (5) the fact that the intensity of aequorin luminescence varies non-linearly with $[Ca^{2+}]$, and (6) kinetic limitations on the ability of the aequorin reaction to follow rapid changes of $[Ca^{2+}]$. An additional complication that has been discovered since the review was written is that at very low free Ca concentrations the luminescence of aequorin becomes independent of $[Ca^{2+}]$ (Allen *et al.* 1977). This means that even if we were able to inject enough aequorin to detect a resting glow, it might not be possible to establish the corresponding intracellular $[Ca^{2+}]$.

*Limitations imposed by the kinetics of the aequorin reaction.* An important factor that must be taken into account in the interpretation of luminescent signals from amphibian muscle fibres is the limited speed with which aequorin responds to sudden changes in $[Ca^{2+}]$. Hastings, Mitchell, Mattingly, Blinks & van Leeuwen (1969) studied the kinetics of the aequorin reaction from this standpoint with rapid-mixing techniques, and found that after abrupt rises or falls in $[Ca^{2+}]$, the light intensity approached its new level exponentially and with a rate constant of about 100/sec at 20 °C (half-time about 6 msec) for all concentration changes examined (both rising and falling). Loschen & Chance (1971) later reported that the rate constant was considerably higher and also dependent on $[Ca^{2+}]$, but the significance of this difference is clouded by their having worked at relatively high temperatures and with solutions that contained substantial concentrations of EDTA (for further discussion see Blinks *et al.* 1976). Until this apparent discrepancy is resolved, we shall operate under the worst-case principle and assume that the results of Hastings *et al.* apply to our experiments. In that case the time course of the rising phase of the aequorin response will always have been influenced strongly by the kinetics of the aequorin reaction (see Beeler, van Leeuwen & Blinks, 1970). The extent to which the declining phase of the aequorin response will have been distorted depends on the conditions of the experiment. Under most conditions, the rate of decline of aequorin responses is lower than the maximum rate with which aequorin luminescence can be extinguished by chelating $Ca^{2+}$ *in vitro*. The difference is never great, however, and in twitches it can be narrowed or eliminated by warming to 25 °C or above. Tetanic aequorin responses (Text-fig. 6) decay with a rate constant about half the maximum imposed by the kinetics of the aequorin reaction.

As one would expect, warming increases the intensity of the luminescence of

aequorin, as well as the speed with which it responds to changes in $[Ca^{2+}]$. Thus, the observation that the amplitude of the aequorin response increases with increasing temperature (Text-fig. 3) does not necessarily signify that warming increases the calcium concentration change associated with excitation-contraction coupling. The intensity of aequorin luminescence at a given $[Ca^{2+}]$ has a $Q_{10}$ of 1·4–1·6 over the range of interest (Prendergast *et al.* 1977). This is about the same as the factor by which peak light intensity increases with temperature in the aequorin responses of Text-fig. 3.

*Implications of the non-linear relation between $[Ca^{2+}]$ and light intensity.* As Baker, Hodgkin & Ridgway (1971) have pointed out, the non-linear nature of the relationship between $[Ca^{2+}]$ and the intensity of aequorin luminescence means that the light signal recorded from an aequorin-injected cell will be influenced not only by the total amount of free calcium ion in the cytoplasm but also by its distribution. Since over a fairly wide range (see Allen *et al.* 1977) the light intensity varies approximately in proportion to $[Ca^{2+}]^{2·5}$, the introduction of a given amount of Ca into a solution of aequorin leads to the emission of much more light if the $[Ca^{2+}]$ is locally high than if the ion is distributed uniformly throughout the solution. The over-all light signal will be dominated by luminescence from the regions of highest $[Ca^{2+}]$.

Distances within the sarcomere are such that if $Ca^{2+}$ distribution were governed by diffusion alone, gradients would virtually disappear within a millisecond or two. However, diffusional equilibration must take place much more slowly than that because of the binding of Ca by intracellular structures and soluble macromolecules (Bassingthwaighte & Reuter, 1972). Thus during twitches and the initial parts of tetani, substantial gradients of $[Ca^{2+}]$ must exist in the sarcoplasm. The highest concentrations will be achieved immediately around the sites of Ca release (presumably the terminal cisternae) and it will be these regions from which most of the light is emitted and in which most of the aequorin consumption takes place.

We have already argued that the aequorin consumption associated with a single twitch cannot lead to a significant *over-all* depletion of the photoprotein injected into the muscle cell. However, the likelihood that luminescence, and therefore aequorin consumption, occurs predominantly in the immediate vicinity of the sites of Ca release raises the possibility that temporary *local* depletion of aequorin might have a significant influence on the aequorin responses recorded in successive contractions. In order to assess the potential importance of local aequorin depletion we must estimate how long local inhomogeneities of aequorin concentration might be expected to persist within the muscle cell. The most convenient model is probably that of multiple microscopic spherical zones of depletion around the sites of Ca release. The time required for replenishment of these zones will be a function of their size, and therefore, for a given amount of aequorin consumption, of their number. The worst case, i.e., that in which replenishment was slowest, would be that in which all of the aequorin consumption in a sarcomere occurred at a single place. If the unit of cell volume is taken to be 1 cubic sarcomere length, or about 10 $\mu m^3$, and the fraction of aequorin consumed in a twitch one part in 100,000 (see above), then the volume of sarcoplasm totally depleted of aequorin would be $10^{-4} \mu m^3$, or a sphere some 60 nm in diameter. If we assume that the aequorin in the zones of local depletion is in free diffusional equilibrium with that in the rest of the cell, and that the diffusion coefficient estimated from the macroscopic spread of luminescence ($5 \times 10^{-8}$ $cm^2$/sec) also applies at this level, we can estimate the time required for replenishment of the spherical zone of depletion from the nomogram provided by Crank (1956, p. 86). The aequorin concentration at the center of a 60 nm sphere of total depletion would be 96 % restored by diffusion in less than 0·1 ms. (Even if a sphere containing 1 % of a cubic sarcomere length were totally depleted of aequorin, it would take only about 7 msec for 96 % repletion.) Since the diffusion into multiple smaller spheres would take place still more

quickly, it seems safe to conclude that the local depletion of aequorin cannot have a significant influence on the recorded aequorin response unless local diffusion is restricted by some mechanism that does not affect the macroscopic spread of aequorin in the cell.

*Interpretation of aequorin signals from muscle fibres*

*Twitches.* We concluded just above that the peak light intensity in a twitch is probably influenced primarily by the amount of Ca released from the terminal cisternae. Since this is only one of a number of factors influencing the calcium transient in the sarcoplasm bathing the myofilaments, one should not necessarily expect a direct correlation between peak light intensity and the force developed in isometric twitches. If in twitches the $[Ca^{2+}]$ in the vicinity of the myofibrils exceeds the level required to saturate troponin, then the contractile response should be influenced primarily by the duration of the calcium transient, not by its amplitude. In this circumstance one would expect a poor correlation between the amplitude of the aequorin signal and the force developed in twitches. Other mechanisms could lead to the same effect, however, and thus it is not safe to conclude from the poor correlation observed (e.g., Text-fig. 5) that saturation occurs.

The form of the declining phase of the aequorin response in a twitch must be determined in a complicated way by the diffusion, binding, and sequestration of Ca, combined with the factors discussed on pp. 314–315. If the geometrical relations among the sites of Ca release, binding, and sequestration are constant, it seems reasonable to attribute *changes* in the time course of the declining phase of the aequorin response to *changes* in the rate of Ca sequestration by the sarcoplasmic reticulum, though little else about the characteristics of the uptake process can be deduced directly from the form of the aequorin response.

*Tetani.* The interpretation of aequorin signals in tetani is simplified to some extent by the long duration of the aequorin response and the slowness with which light intensity changes during all but the initial part of the tetanus. These features suggest that at any given locus in the cell the free Ca in the sarcoplasm must come virtually into equilibrium with saturable Ca binding sites. Under such circumstances significant gradients of $[Ca^{2+}]$ are not likely to exist over distances as short as a sarcomere length. However, if the rates of Ca liberation or uptake are not the same in different parts of the cell, significant gradients of $[Ca^{2+}]$ might exist over distances of hundreds or even tens of microns, so it cannot be assumed that the $[Ca^{2+}]$ is the same everywhere in the cell even during the plateau of a tetanic aequorin response.

The fact that during a tetanus at optimal length there may be large changes in the intensity of the aequorin response without corresponding changes in force is most easily explained by assuming that the contractile mechanism becomes saturated throughout the cell at a $[Ca^{2+}]$ well below the level that is usually achieved in tetanic contractions. Although none of the observations presented in this paper is inconsistent with this interpretation, some well established aspects of the behaviour of amphibian skeletal muscle fibres appear to be in conflict with it. For example, the maximum force generated in potassium contractures is appreciably greater than in tetani (e.g., Hodgkin & Horowicz, 1960). Furthermore, the maximum force developed by single fibres in tetani at optimum length can be increased as much as 10–25 % by agents such as caffeine (Rüdel & Taylor, 1971), nitrate (Taylor, 1976)

or $Zn^{2+}$ (Lopez, Wanek & Taylor, 1977) all of which greatly increase the tetanic aequorin response (unpublished). These observations might be explained if at least part of the cross-section of the muscle was normally less than fully active during tetani not only at short fibre lengths (Taylor & Rüdel, 1970) but at the optimal length as well. However, if this were the case, one would expect changes in the intensity of the aequorin response to be accompanied by changes in force during a tetanus at the optimal length, which they are not. We are not certain how this apparent contradiction will be resolved, but in any case it should be reemphasized that changes in the amplitude of the aequorin response give an exaggerated impression of the changes in $[Ca^{2+}]$ responsible for them. If the sarcoplasmic $[Ca^{2+}]$ falls on the steep part of the $Ca^{2+}$ concentration–effect curve for aequorin (Allen *et al.* 1977), a fivefold change in light intensity (e.g., Text-fig. 4) corresponds to a less than twofold change in $[Ca^{2+}]$. The light signal will be dominated by the emission from those regions where $[Ca^{2+}]$ is highest, and for a given increment in the amount of Ca released, free $[Ca^{2+}]$ will rise most sharply in just those regions where the Ca binding sites on troponin have already been saturated.

For the reasons discussed earlier in this section it is not possible to draw conclusions about the degree of activation in twitches from comparisons of the amplitudes of the aequorin responses recorded from the same fibre during twitches and tetani. Fibres are not rare in which the amplitude of the aequorin response in a rested-state twitch is several times greater than that during the plateau of a well fused tetanus. This almost certainly means that the amount of Ca released during the twitch is much greater than that released by any single action potential during the plateau of the tetanus. It does not necessarily mean, however, that the $[Ca^{2+}]$ at the level of the myofilaments is as high during the twitch as during the tetanus.

*$K^+$-contractures.* The upper records of Text-fig. 12 indicate that under the conditions of our experiments force development and light first became detectable at the same $[K^+]$ (17·5 mM). The striking difference between the shapes of the curves for force and light in Text-fig. 13 reflects the non-linear nature of the relation between $[Ca^{2+}]$ and the rate of the aequorin reaction, coupled with the fact that only the mechanical response appears to have reached saturation.

The biphasic nature of the aequorin responses triggered by exposing the fibre to high-$K^+$ solutions is probably a manifestation of a phenomenon that has been noted on mechanical records by others, and explained by Costantin (1971) as follows. The initial rapid rise in force reflects depolarization of the surface membrane with electrotonic spread into the T system. The subsequent dip reflects partial repolarization of the surface membrane due to spontaneous inactivation of delayed rectification (i.e., to a decrease in potassium permeability) in the face of a continued high chloride permeability. The final slow rise in force occurs as $K^+$ diffuses into the T tubules and depolarizes them directly.

The results from the experiments with potassium depolarization reveal rather dramatically the extent to which the relation between force and light can be influenced by the brevity of a twitch. In 30 mM-$K^+$ a force nearly equal to that of a rested-state twitch was developed in association with a light intensity only about a tenth as great as in the twitch. The difference presumably reflects the influence of two

factors: (1) local gradients of $[Ca^{2+}]$ are likely to be much greater in the twitch, and (2) the development of force was presumably limited by the short duration of activity in the twitch.

*Estimation of intracellular $[Ca^{2+}]$*

Previous approaches to the 'calibration' of light signals from aequorin-injected cells have depended on the detection of a resting glow and the injection of Ca-EGTA buffers to determine the buffered $[Ca^{2+}]$ that just failed to alter the resting glow (Baker *et al.* 1971). Estimates of $[Ca^{2+}]$ during activity have then sometimes been made on the basis of the assumption that the intensity of the aequorin signal is proportional to the square of the $Ca^{2+}$ concentration. This is now known to be a dangerous oversimplification; the $Ca^{2+}$ concentration–effect curve is sigmoid on a log-log plot, and has a maximum slope of 2·5 (Allen *et al.* 1977).

In the absence of a detectable resting glow it is of course not feasible to use the null method to establish a point on the $Ca^{2+}$ concentration–effect curve for aequorin-injected skeletal muscle fibres. D. G. Allen and J. R. Blinks (unpublished) have recently developed an alternative method for use with preparations of the type used here. They measured the total amount of light emitted when the cell membranes were lysed with a detergent under optical conditions identical to those used to record physiological aequorin responses. This enabled them to express the light intensity during the plateau of a tetanus as a fraction (about $2 \times 10^{-3}$) of that which would have been obtained had all of the aequorin in the cell been exposed instantly to a saturating $[Ca^{2+}]$. From a calcium concentration-effect curve for aequorin determined *in vitro* in a solution containing 150 mM-KCl and 1 mM-$Mg^{2+}$ at pH 7·0, and expressed in terms of the same fraction, it was estimated that the tetanic light output corresponded to a $[Ca^{2+}]$ of approximately $5 \times 10^{-6}$M. A signal equal to the dark count of our photomultiplier would have corresponded to a $[Ca^{2+}]$ of about $4 \times 10^{-7}$M. *In vitro*, the ratio of light output in the absence of $Ca^{2+}$ to that in saturating $[Ca^{2+}]$ was about $1{\cdot}3 \times 10^{-7}$. In our experiment this would have corresponded to a dark count of about 1/sec, clearly below our ability to resolve the signal with the apparatus used. At present, all we can say about the resting $[Ca^{2+}]$ is that it can have been no higher than about $2 \times 10^{-7}$M.

*The influence of stimulus frequency*

One of the most prominent aspects of the interval–response relationship is the descending staircase of peak light intensity that occurs when repetitive stimulation is begun after a period of rest. Since the possibility that it might reflect the local consumption of aequorin has been ruled out (pp. 315–316), we feel that the descending staircase must reflect a progressive decrease in the amount of Ca released in successive twitches. The progressive decrease in the rate of decline of the individual aequorin responses that occurs simultaneously with the descending staircase probably reflects a gradual slowing of the sequestration of Ca by the sarcoplasmic reticulum, an effect that may serve to increase the time that effective $Ca^{2+}$ concentrations are maintained at the level of the myofilaments.

A progressive decline in the amount of Ca released and a progressive decrease in the rate of Ca sequestration in successive twitches could have a common origin in a progressive displacement of Ca from sites of release to sites of uptake in the sarco-

plasmic reticulum. Winegrad (1968, 1970) has proposed, on the basis of autoradiographic and ultrastructural evidence from frog skeletal muscle, that the terminal cisternae are the sites of storage and release of most of the Ca involved in excitation-contraction coupling, and that the Ca binding responsible for relaxation occurs in the longitudinal elements of the sarcoplasmic reticulum. His autoradiographic measurements indicate that once Ca has been sequestered by the longitudinal elements it is returned to the terminal cisternae only rather slowly (half-time 28 sec after a long tetanus at 4 °C). On the basis of these measurements, Winegrad (1968) predicted that there might be a 'gradual depletion of calcium in the terminal cisternae during a series of twitches at a moderate frequency'. He also concluded that 'a twitch occurring when the calcium content of the intermediate cisternae and longitudinal tubules is elevated should have a prolonged active state' because the rate at which isolated vesicles of sarcoplasmic reticulum take up Ca is inversely related to the amount of Ca that they contain (Makinose & Hasselbach, 1965; Weber, Herz & Reiss, 1966). Winegrad went on to point out that the mechanical responses of muscle do not correspond to these predictions particularly well. The fact that the aequorin responses correspond very much better is in accord with our view that their amplitude depends primarily on the amount of Ca released from the terminal cisternae, while the force developed in a twitch is probably determined by both the amplitude and the time course of the Ca transient in the vicinity of the myofilaments. The force developed will thus be influenced not only by the amount of Ca released, but also by the amount already bound to potential 'sinks' within the cell, by geometrical factors, and in particular by the avidity with which Ca is sequestered by the sarcoplasmic reticulum.

Connolly, Gough & Winegrad (1971) have also accounted for many of the features of post-tetanic potentiation in terms of the redistribution of Ca from sites of release to sites of sequestration. The results shown in Text-fig. 8 are in accord with their interpretation. However, another factor that might be important in determining the mechanical response to a Ca transient is the initial state of the myofibrils. If a Ca transient of a given size encountered myofibrils with a residual (but mechanically subthreshold) amount of Ca already bound to them, it might produce a stronger contraction than if the myofibrils were totally Ca-free (see Sandow, 1965). Finally, it is conceivable that the sensitivity of the myofibrils to $Ca^{2+}$ might change from moment to moment as a result of phosphorylation of component proteins (Mannherz & Goody, 1976) or some other chemical change, and that such alterations might contribute to phenomena such as those illustrated in Text-figs. 5 and 8.

### *The influence of extracellular* [$Ca^{2+}$]

In frog muscle the influx (Bianchi & Shanes, 1959; Curtis, 1970; Frank & Winegrad, 1976) and the efflux (Shanes & Bianchi, 1960; Curtis, 1970; Kirby, Lindley & Picken, 1975; Frank & Winegrad, 1976) of Ca are low at rest and substantially increased by excitation. The changes in influx and efflux induced by activity are similar enough so that the total cellular Ca content is not altered greatly or for long (Curtis, 1970; Frank & Winegrad, 1976). However, the precision and temporal resolution of available flux measurements are not sufficient to indicate whether the balance between influx and efflux might be altered enough to influence the availability of Ca

for excitation-contraction coupling during the course of a tetanus. Although in skeletal muscle the *redistribution* of intracellular Ca is likely to be of primary importance in the twitch-to-twitch modulation of Ca transients, the possibility must be considered that changes may also occur in the *total amount* of Ca available to participate in the cycle. Such changes might be partly or wholly responsible for the more gradual changes in light intensity that occur during tetanic stimulation – the secondary rise in luminescence on one hand, and tetanic fade on the other. The fact that the secondary rise in luminescence still occurs when the muscle is bathed in a solution virtually free of $Ca^{2+}$ (Text-fig. 14) would appear to rule out a gradual accumulation of Ca during activity as the primary cause of this phenomenon. However, the fact that tetanic fade is accentuated in the $Ca^{2+}$-free solution suggests fade might reflect the net loss of Ca from the cell.

### *The influence of fibre length*

If our interpretation of the significance of the aequorin response in twitches is correct, the amount of Ca released by the action potential is first increased, then progressively reduced as the muscle fibre is stretched above slack length. Although other factors are clearly of major importance, changes in Ca release may play a significant role in determining the influence of fibre length on the kinetics of the twitch. Even if the cytoplasmic $[Ca^{2+}]$ reaches saturating levels during twitches at all fibre lengths, changes in the amount of Ca released would be expected to have an influence on the duration of activity, and therefore on the extent of shortening or the amount of force developed in a twitch. The fact that force and light intensity rise together during tetani at high fibre lengths (see Text-fig. 11) does not necessarily imply that the contractile mechanism is not saturated at these lengths. At fibre lengths above the optimum, force may creep upward in this way as a result of the development of inhomogeneities in sarcomere length (Huxley & Peachey, 1961; Gordon, Huxley & Julian, 1966). If this is the basis of the upward creep, the reduction of force at long sarcomere lengths might result entirely from diminished filament overlap (Gordon *et al.* 1966) despite the fact that there is a concomitant reduction in the Ca transient.

The mechanism of the decreased release of Ca at high fibre lengths is unknown, though it seems possible that reversible distortion of the junctional region between the terminal cisternae and the transverse tubules could be responsible. Distortion of this sort has been demonstrated by electron microscopy (Frank & Winegrad, 1976) and associated with a reduction in the Ca efflux during activity (Sopis & Winegrad, 1967; Frank & Winegrad, 1976). The recovery of the aequorin response after a highly stretched fibre is returned to slack length indicates that no permanent damage is produced by stretch. The fact that the recovery is not immediate suggests that the change in the light signal with stretch is not the result of some sort of optical artifact. The lack of an effect of stretch on the time course of the decline of the aequorin response is difficult to interpret because of the altered geometrical relations among sites of Ca release, binding, and sequestration in the cell.

The decreased intensity of the tetanic aequorin response at low fibre lengths may in part be due to decreased Ca release in the core of the fibre. It has previously been observed (Taylor & Rüdel, 1970; Costantin & Taylor, 1973; Taylor, 1976) that

differences in sarcomere length, myofilament orientation, and shortening velocity develop between the peripheral and central myofibrils of muscle cells that are allowed to shorten greatly during tetani, and this has been attributed to decreased activation of the core of the muscle fibre.

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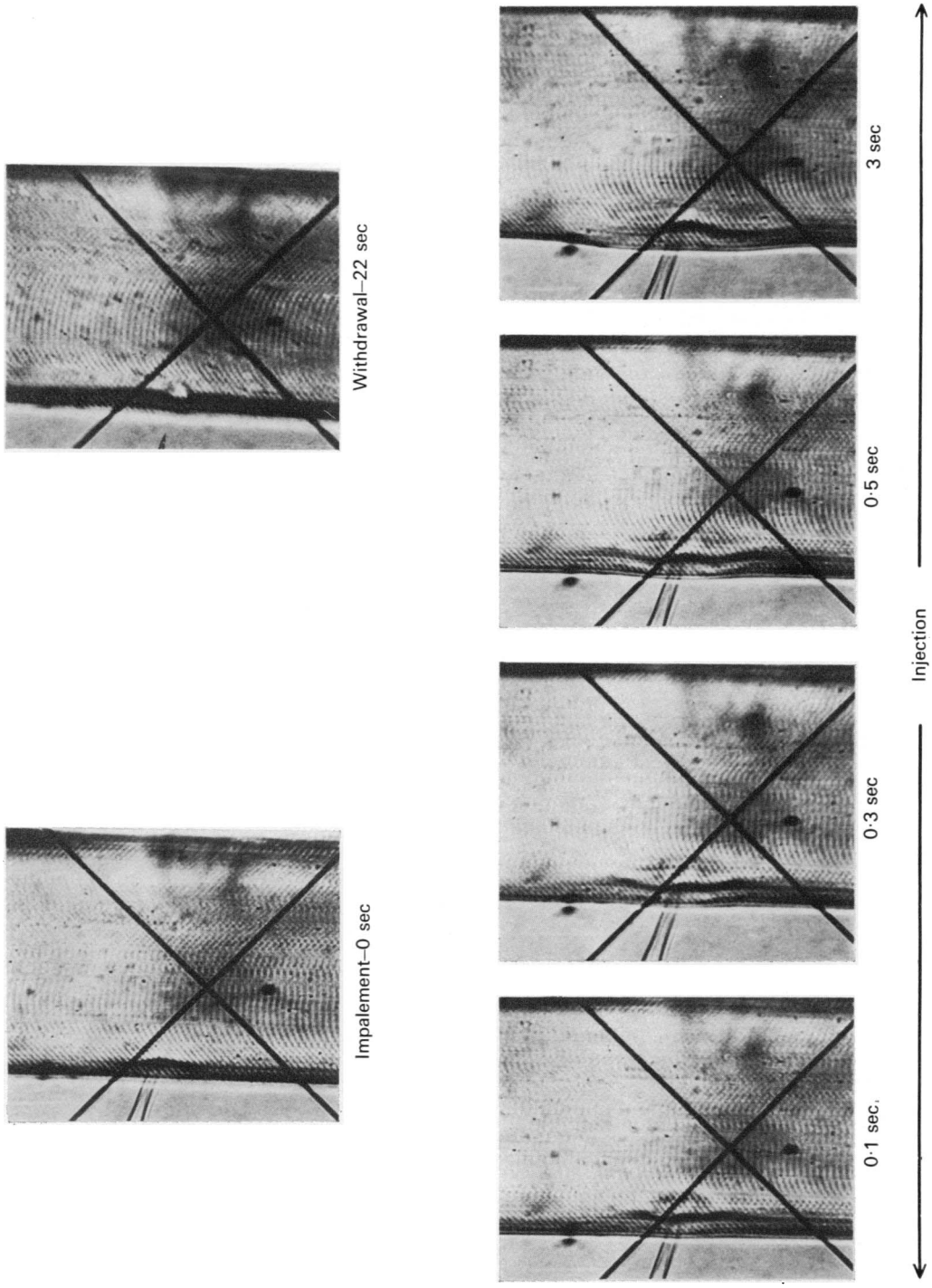


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## EXPLANATION OF PLATE

Microinjection of aequorin. Single fibre from iliofibularis muscle of *Xenopus laevis*; striation spacing 2·7 $\mu$m, fibre width 101 $\mu$m, temperature 15 °C. Frames from cine film taken at 20 frames/sec. Injection pressure between 4 and 6 atm.