

## THE DESCENDING LIMB OF THE FORCE–SARCOMERE LENGTH RELATION OF THE FROG REVISITED

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### SUMMARY

1. We studied the descending limb of the force–sarcomere length relation in single frog muscle fibres using sarcomere isometric contractions.

2. Sarcomere length was measured simultaneously with two independent methods: a laser diffraction method and a segment length method that detects the distance between two markers attached to the surface of the fibre, about 800  $\mu\text{m}$  apart. Both methods were used to keep sarcomeres at constant length during contraction.

3. Fibres were selected for low resting tension since it was known from previous experiments that for such fibres the force developed by fixed-end tetani is much higher than that predicted by the degree of filament overlap.

4. With fixed-end tetani, force decline with increase of sarcomere length was small between 2.0 and 3.0  $\mu\text{m}$ . At a sarcomere length of 3.0  $\mu\text{m}$ , force was about 90% of maximal.

5. With sarcomere isometric tetani, force was considerably lower than with fixed-end tetani. Force was maximal at about 2.1  $\mu\text{m}$  and decreased to zero at about 3.6  $\mu\text{m}$ . At intermediate lengths the descending limb was within 80 nm of the values predicted from filament overlap.

6. We investigated why force of fixed-end contractions was much higher than that generated by sarcomere isometric contractions.

7. During the force plateau of fixed-end tetani at sarcomere lengths longer than about 2.0–2.2  $\mu\text{m}$ , sarcomeres in the fibres mid-region were not isometric, but instead stretched slowly. By measuring the force–velocity relation it was shown that this slow stretch elevates active force well beyond sarcomere isometric force.

8. Stretch of the central region was also observed during the tetanic force rise. This was shown to result in an increase of passive force that grew larger at longer sarcomere lengths. At about 3.6  $\mu\text{m}$  the increase of passive force was similar to the total force generated by fixed-end contractions at this length.

9. Laser diffraction and segment length methods gave the same results, diminishing the chance that any systematic artifact underlies our findings.

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10. While earlier experiments from this laboratory carried out on fibres held at constant length during contraction did not reveal a linear descending limb, the present results support the linear descending limb as a characteristic feature of isometrically contracting sarcomeres.

#### INTRODUCTION

In the cross-bridge theory of muscle contraction (A. F. Huxley, 1957; H. E. Huxley, 1969), force is generated by functionally identical cross-bridges, the number of which varies linearly with the width of the overlap zone between thin and thick filaments. The force–sarcomere length relation is therefore predicted to have a linear descending limb, with maximal force at the sarcomere length with maximal overlap and zero force at the length where non-overlap starts.

The study by Gordon, Huxley & Julian (1966*a, b*) agreed well with this prediction. These investigators introduced a length-clamp technique that held the length of a 3–5 mm segment of the central region of the fibre at constant length during contraction. Tetanic force was found to rise fast initially, and then to creep slowly to a plateau. Since the slow climb of force was assumed to arise from the development of progressive dispersion of sarcomere length, Gordon *et al.* (1966*b*) emphasized the force value reached at the end of the fast rise phase. This force (obtained by back extrapolation of the ‘creep’ force; see Gordon *et al.* 1966*b*, Fig. 3) was found to decline with sarcomere length as predicted. This study is therefore considered important evidence in favour of the cross-bridge theory.

On the other hand, ter Keurs, Iwazumi & Pollack (1978) concluded that the increase of sarcomere length dispersion during the tetanus is not sufficient to explain the slow climb of force. Therefore, these investigators focused on the tetanic plateau force. The decline of plateau force with increase of length was found to be much less than predicted. (Henceforth, we refer to this gently descending curve as ‘flat’.) At a sarcomere length of 3.0  $\mu\text{m}$ , for example, where force is predicted to have decreased to 40% of maximal, measured force was as high as 90% of maximal. ter Keurs *et al.* (1978) concluded that their results were inconsistent with the theory.

This conclusion triggered a controversy. Since ter Keurs *et al.* (1978) did not use the length-clamp technique, but instead held fibres at constant length during contraction (Fig. 2 explains the difference between these two techniques), discussions focused on the possibility of sarcomere length variation during the tetanus that might have gone undetected (for review see A. F. Huxley, 1988). In subsequent studies a small region of the fibre (less than 1 mm) was kept isometric. Results obtained by plotting tetanic plateau force *versus* sarcomere length were close to the predicted linear descending limb (ter Keurs & Elzinga, 1981; Altringham & Bottinelli, 1985; Edman & Reggiani, 1987; Bagni, Cecchi, Colomo & Tesi, 1988). However, the force–sarcomere length curve obtained with fixed-end tetani in these studies was not as ‘flat’ as in the ter Keurs study (Pollack, 1983; ter Keurs, Luff & Luff, 1984). Therefore, these studies do not necessarily refute the conclusion of ter Keurs *et al.* (1978) that the force–sarcomere relation of the fibres used in their study is inconsistent with the cross-bridge theory.

The shape of the fixed-end curve may in fact bear on the shape of the sarcomere

isometric curve. For example, at  $3.0\ \mu\text{m}$ , sarcomere isometric tetani generated 15–20% less force than fixed-end tetani (Bagni *et al.* 1988, Figs 3C and 4C; Edman & Reggiani, Fig. 1). If the same difference were found for the fibres studied by ter Keurs *et al.* (1978), sarcomere isometric force would be much higher than predicted from filament overlap, i.e. sarcomere isometric force at  $3.0\ \mu\text{m}$  would be about 70% of maximal instead of the 40% predicted. Thus, in evaluating the generality of the shape of the force–sarcomere length relation, it is important to include fibres that produce ‘flat’ fixed-end curves. This was the objective of our study: to determine whether the linear descending limb is a characteristic feature of isometric sarcomeres of such fibres.

## METHODS

### *Fibres*

Single intact fibres were dissected from frogs (*Rana temporaria*; body length 4–5 cm) that were cold adapted at about  $4\ ^\circ\text{C}$  for 1–2 weeks prior to use. Frogs were quickly killed by decapitation. Fast-twitch fibres from the semitendinosus muscle were used (as described by ter Keurs *et al.* 1978). We selected fibres with a shallow passive force–sarcomere length relation, since it has been noted that those fibres give ‘flat’ fixed-end descending limbs (Pollack, 1983; Altringham & Bottinelli, 1985). Slack length of the fibres was about 10 mm. Small holes were punched in the tendons, with insect needles, 200–600  $\mu\text{m}$  away from the myotendinous junction, for attachment to the experimental apparatus.

Fibres were mounted horizontally in the experimental chamber between a servo-motor and a force transducer (AME 801E, Horten, Norway; natural frequency 5–7 kHz). Chamber volume was about 600  $\mu\text{l}$ . The walls of the chamber were transparent. Transparency was achieved by constructing the chamber out of small strips of glass that were glued together. Full transparency made it possible to illuminate the same part of the fibre from two directions: vertically for laser diffraction and imaging of the striations, and horizontally for the segment length system. After mounting of the fibre, the top of the chamber was covered by a small cover-slip to prevent diffraction-beam deflection by surface waves.

Experiments were performed at about  $2\ ^\circ\text{C}$ . (For details concerning the cooling system see Granzier, 1988.) During experiments temperature did not fluctuate more than  $\pm 0.1\ ^\circ\text{C}$ . Fibres were kept in a physiological salt solution of the following composition (in mM): NaCl, 115.5; KCl, 2.0;  $\text{CaCl}_2$ , 1.8;  $\text{MgSO}_4$ , 1.0;  $\text{Na}_2\text{HPO}_4$ , 6.3;  $\text{NaH}_2\text{PO}_4$ , 1; glucose, 5.0. The pH was 7.1 at  $2\ ^\circ\text{C}$ . The salt solution was refreshed after each contraction.

### *Diffraction*

In this method an on-line measurement of sarcomere length is obtained by using the principle of optical diffraction. A small region of the fibre was illuminated by a collimated, randomly polarized, He–Ne laser light beam (CW Radiation, Inc., LLS5R). Beam diameter (estimated from the position at which irradiance has fallen  $1/e^2$  of its peak axial value) was 400  $\mu\text{m}$  in most experiments, and occasionally 800  $\mu\text{m}$ . The diffraction pattern was collected with a long working distance (5.5 mm) bright field objective lens (NA 0.6). A telescope lens projected the diffraction pattern, after compression with a cylindrical lens, onto a photodiode array (Reticon RL 256 C/17).

Sarcomere length was determined from the distance between the zeroth and first-order diffraction maxima. The position of the first-order maximum was determined by locating the median position of the first-order intensity profile on the array. Locating the median was accomplished as described previously for locating marker position (Granzier, Myers & Pollack, 1987).

The first-order position signal was recorded on a digital oscilloscope with a 15-bit analog-to-digital converter (Nicolet, 4094/4851). The least significant bit of the A/D corresponded to 0.015 nm/sarcomere at a sarcomere length of  $3.6\ \mu\text{m}$ , less at shorter sarcomere lengths. Sarcomere length was determined from calibration gratings mounted in the chamber at the same level as the fibre. Using the least-squares method, we computed the linear equation that described the relation

between first-order peak position and the inverse of the known gratings spacing. The equation was determined for each experiment. The measured first-order position obtained from the muscle fibre was then converted to sarcomere length by using this equation. Resolution of the sarcomere length measurement was limited by noise. Root mean square (r.m.s.) sarcomere length noise (bandwidth 0–2 kHz) measured during tetanic contractions of five fibres (sarcomere length ranged from 2.0–3.4  $\mu\text{m}$ ) amounted to  $0.4 \pm 0.2$  nm/sarcomere ( $n = 20$ ).

#### *Segment length*

To diminish the likelihood of artifact, sarcomere length was also measured and controlled with a second method that is fully independent of diffraction. This method detects the distance between two markers positioned along the fibre surface. As markers we used thin cat hairs (cf. Granzier *et al.* 1987) that were mounted along the side surface of the fibres. At slack length, the marker closest to the force transducer was about 2 mm from the myotendinous junction, while the other marker was typically 800  $\mu\text{m}$  closer to the centre of the fibre. Thus, the segment was typically one-fourth the way along the fibre. For mounting of markers, the fibres were pre-stretched to an intermediate sarcomere length of about 2.9  $\mu\text{m}$ . Care was taken to attach the hairs perpendicular to the fibre axis. Alignment was subsequently checked during slow releases and stretches of the passive fibre, and also during short tetani. If the markers did not remain perpendicular to the fibre axis during any of these manoeuvres, their position was corrected.

Segment length was measured as described by Granzier & Pollack (1989). Segment length was converted into sarcomere length by measuring the sarcomere length within the segment using the optical microscope. The average of strings of about thirty sarcomeres were determined at different depths within the segment. We assumed that the measured degree of segment length change corresponded to the same degree of sarcomere length change. This assumption was tested by measuring sarcomere length simultaneously with the laser diffraction method; results were highly correlated (see Granzier & Pollack, 1989).

The segment length method had a time resolution of 260  $\mu\text{s}$ ; r.m.s. noise (bandwidth 0–2 kHz), determined from three fibres at a wide range of sarcomere lengths, was  $0.7 \pm 0.02$  nm ( $n = 10$ ) per sarcomere.

#### *Force and length control*

The servo-motor consisted of an electromagnetic puller, which produced linear motions over a range of 6 mm, and a digital control system which allowed us to switch between control of fibre length (motor position), force, first-order position, or segment length. These signals were first digitized by 12-bit analog-to-digital converters and then fed to a computer (Commodore 64). Any of the four signals could be chosen to control the motor. The chosen control signal was subtracted from a reference signal, giving an error signal that was sent to the amplifier that controlled the position of the electromagnetic puller so as to minimize the error. For a more complete description of the servo-system, see Granzier (1988).

r.m.s. noise (bandwidth 0–2 kHz) corresponded to  $0.9 \pm 0.5$  nm/sarcomere (mean  $\pm$  s.d.;  $n = 10$ ) during first-order position control, and to  $1.1 \pm 0.3$  nm/sarcomere ( $n = 10$ ) during segment length control. This noise is slightly larger than without sarcomere length control (see above); apparently the servo-system introduced some additional noise.

Force and length signals were digitized with 15-bit analog-to-digital converters of a four-channel digital oscilloscope. Signals were stored on floppy discs for subsequent analysis.

#### *Experimental protocol*

Fibres were mounted in the chamber and carefully aligned relative to the photodiode arrays, using two sets of high-resolution *X–Y–Z* translators on which the servo-motor and the force transducer were mounted. The markers were then attached to the fibre, and the various calibrations made. The laser diffraction system was used to measure the striation spacing within the segment delineated by the two markers.

Before each experiment we measured the major and minor fibre diameters with a stereo microscope set at  $80\times$ . Cross-sectional area, required to compute stress, was calculated assuming the fibre had an elliptical cross-section. Maximal active stress, measured at 2 °C, was  $283 \pm 33$  kN/m<sup>2</sup> (mean  $\pm$  s.e.m;  $n = 12$ ).

Fibres were stimulated with two platinum electrodes that ran parallel along either side of the full

length of the fibre. Pulses of constant current were used at a frequency of about 50 Hz. Frequency was adjusted to give maximal tetanic force in short test contractions at sarcomere spacings of about 2.0 and 3.0  $\mu\text{m}$ . Successive stimuli of the pulse train had opposite polarities; we found that this alternation prolonged the fibre's lifespan.

Pre-stretched fibres were tetanized while either fibre length, segment length or first-order position was kept constant. The duration of the tetanus was at least as long as required to obtain a force plateau. After contraction, the fibre was stretched to a new length, chosen in random order,

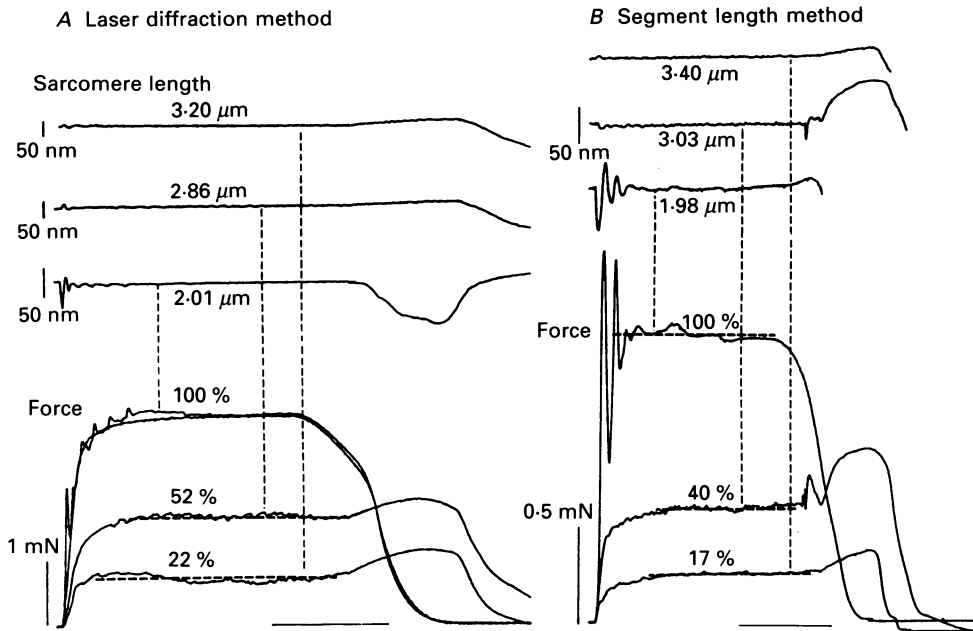


Fig. 1. Examples of sarcomere isometric contractions. Value shown above force trace is average relative force during the plateau, indicated by the horizontal dashed lines. *A*, results obtained with laser diffraction. Force generated by a fixed-end tetanus at a sarcomere length of 2.03  $\mu\text{m}$  (length measured during the force plateau) is also shown, superimposed on the sarcomere isometric tetanus that generated maximal force. *B*, results obtained with the segment length method. All results in *A* are from one fibre and the results shown in *B* are from a different single fibre. Maximal force, 440  $\text{kN/m}^2$  in *A* and 200  $\text{kN/m}^2$  in *B*; cross-sectional area,  $7.4 \times 10^{-3} \text{ mm}^2$  in *A* and  $7.5 \times 10^{-3} \text{ mm}^2$  in *B*; temperature, 2.3  $^{\circ}\text{C}$  in both experiments. Horizontal calibration bar denotes 0.5 s.

and following a rest period of about 200 times the duration of the previous tetanus, the fibre was again stimulated.

Plateau forces of fixed-end and sarcomere isometric tetani were measured, and the dependence of force on sarcomere length was investigated.

## RESULTS

### Diffraction

Sarcomere isometric contractions were generally of 'high quality': sarcomere length remained constant to typically within about 1–2 nm/sarcomere and force rose swiftly to a maximum within 0.1–0.3 s; Fig. 1*A* shows several examples. After the force maximum was reached, there were typically some small force fluctuations, but

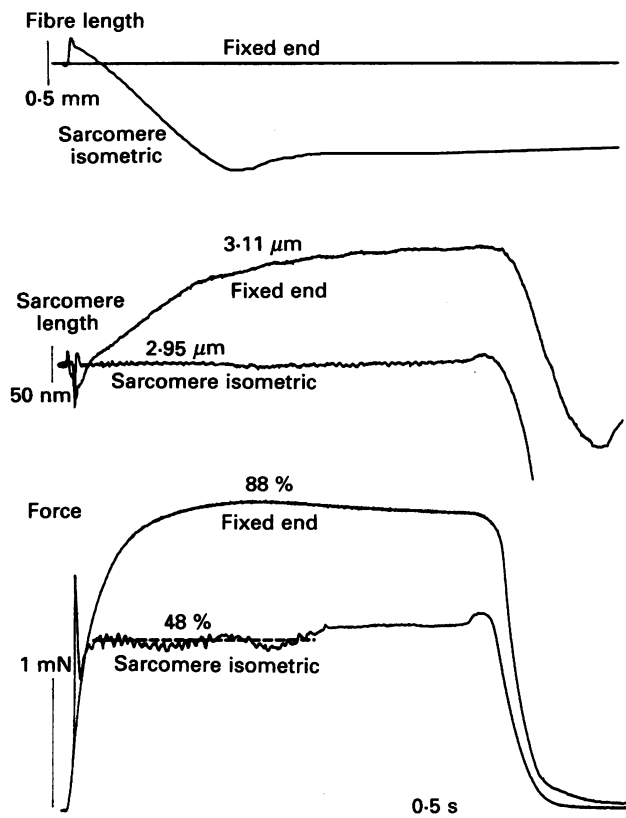


Fig. 2. Example of fixed-end and sarcomere isometric contractions. In fixed-end contraction, fibre length (plus the length of the tendons) is kept constant. Sarcomere length changes during contraction, due to stretching of the tendons and sarcomere length redistribution along the fibre. In the sarcomere isometric contraction, sarcomeres of a small region of the fibre are kept at constant length; to achieve this, fibre length is changed by the servo-system. The sarcomere isometric contraction generates much less force than the fixed-end tetanus. Results obtained with the diffraction method. Maximal force,  $280 \text{ kN/m}^2$ ; cross-sectional area,  $8.4 \times 10^{-3} \text{ mm}^2$ ; temperature,  $3.0^\circ \text{C}$ .

a clear plateau could always be discerned. A typical pair of contractions that both start out at the same fibre length, one kept fixed-end, the other kept sarcomere-isometric, is shown in Fig. 2. Force developed by the sarcomere isometric contraction was much less than that of the fixed-end contraction.

By varying the starting sarcomere length it was found that the difference in force between sarcomere isometric and fixed-end contractions was sarcomere length dependent. An example of this, with all results obtained from the same fibre, can be seen in Fig. 3. At sarcomere lengths less than about  $2.3 \mu\text{m}$ , the difference between fixed-end and sarcomere isometric force was negligible, but at longer lengths the force of the sarcomere isometric contraction was always lower. The force difference increased with sarcomere length; see Fig. 3C. The same result was found for all fibres (see below).

The difference between force obtained with sarcomere isometric and fixed-end

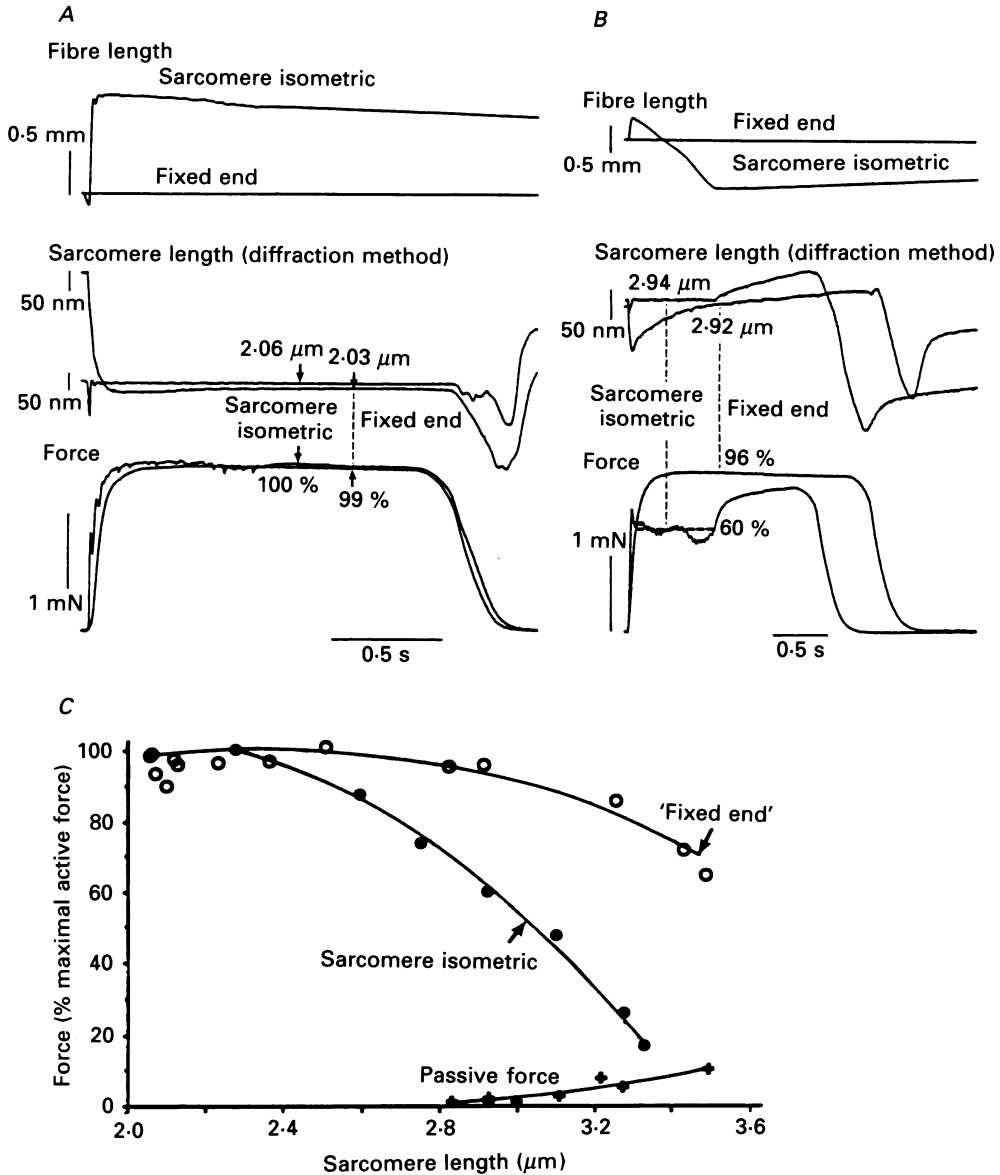


Fig. 3. Sarcomere length dependence of force difference between fixed-end and sarcomere isometric contractions. Results obtained with the diffraction method. *A*, at a sarcomere length of about  $2.0 \mu\text{m}$  the two types of tetani generate about the same amount of force. *B*, at longer lengths, sarcomere isometric contractions generate less force than fixed-end tetani. *C*, difference in force increases with sarcomere length. Sarcomere length plotted in *C* is the value 100 ms after the force plateau has been reached. All results were obtained from the same fibre. Maximal force,  $360 \text{ kN/m}^2$ ; cross-sectional area,  $5.9 \times 10^{-3} \text{ mm}^2$ ; temperature,  $2.3^\circ\text{C}$ .

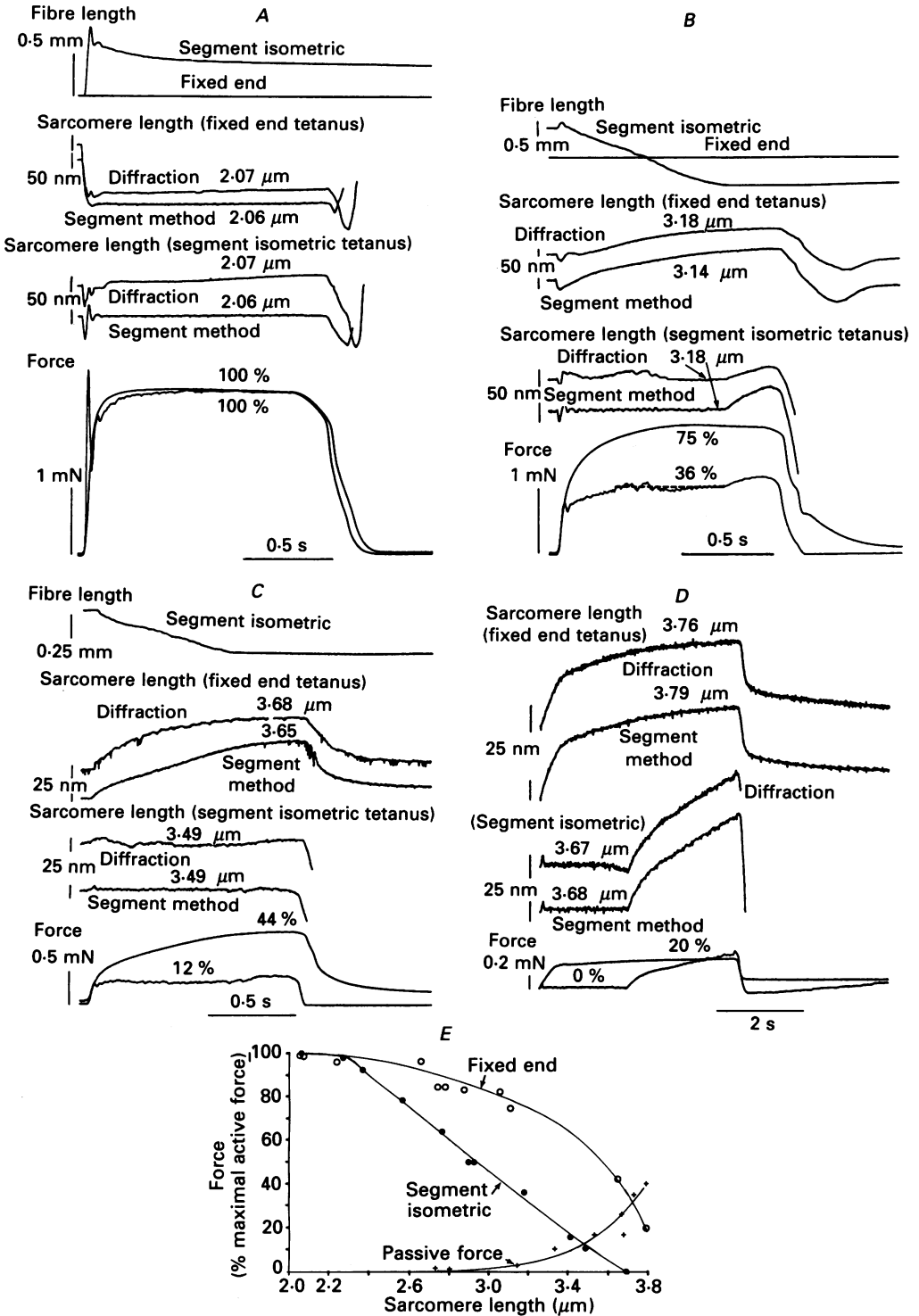


Fig. 4. For legend see facing page.



contractions could, in theory, originate from fibre translation. When sarcomere length is controlled, the fibre length changes (Figs 2 and 3). New sarcomeres move into the field of view while others leave. If the new sarcomeres are longer than the ones that leave, measured sarcomere length will increase; however, the servo-system is programmed to keep measured sarcomere length constant, and will thus shorten the fibre. Force will then diminish due to effects of shortening (Katz, 1939). Thus, translation combined with a consistent sarcomere length gradient along the fibre might explain why force is lower in sarcomere isometric tetani than in fixed-end tetani. To check this, we employed a second method that is insensitive to translation.

#### *Segment length method*

In this method the sampled sarcomere population remains consistent, so measurements are not affected by translation. Sarcomere isometric contractions were generally similar to those obtained with diffraction. Several examples, all from the same fibre, are shown in Fig. 1*B*. An initial sarcomere length oscillation was observed occasionally, mainly in contractions at short lengths, but afterwards the sarcomere length remained constant typically within about 1–2 nm/sarcomere. Examples of both fixed-end and segment isometric tetani, all from the same fibre, are shown in Fig. 4*A–D*, with respective forces plotted in Fig. 4*E*. As was found for diffraction, at sarcomere length less than about 2.3  $\mu\text{m}$  the difference between fixed-end and sarcomere isometric force was negligible. At longer sarcomere lengths, however, the force of the segment isometric contraction was always lower, and the difference increased with increasing sarcomere length.

In many of the segment length clamps, sarcomere length was measured simultaneously with laser diffraction. If there is a sarcomere length gradient along the fibre, translation that occurs during the segment length clamp (typically about 500  $\mu\text{m}$ ) will result in a diffraction-based sarcomere length signal that is different from that measured with the segment length method.

In general, the two sarcomere length signals were similar; see Fig. 4. In a total of sixteen dual measurements (sarcomere length range from 2.0 to 3.7  $\mu\text{m}$ ), sarcomere length measured by diffraction increased, on average, slightly during the period of segment length clamp:  $7.7 \pm 10.5$  nm. On the other hand, if translation were to underlie the low force levels measured in sarcomere isometric contractions obtained with diffraction, a sarcomere length increase of about 1300 nm can be calculated to be required (Granzier, 1988). The measured sarcomere length gradient along the tetanized fibre is thus much too small to explain the result.

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Fig. 4. Segment isometric contractions at different sarcomere lengths. *A*, *B*, *C* and *D* give four examples of pairs of tetani, superimposed, one fixed-end, the other segment isometric. Sarcomere length was measured simultaneously with the diffraction method. *E* shows that fixed-end and sarcomere isometric forces are similar at lengths less than about 2.3  $\mu\text{m}$ , while at longer lengths the force of the sarcomere isometric contractions becomes progressively less than that of fixed-end contractions. All results obtained from the same fibre. Maximal force, 450 kN/m<sup>2</sup>; cross-sectional area,  $5.5 \times 10^{-3}$  mm<sup>2</sup>; temperature, 2.3 °C.

*Pooled results*

The pooled results from all fibres are shown in Fig. 5*A*. Although there is a fair degree of scatter, the fitted curves (fourth-order polynomials) of the pooled results obtained with the two methods are essentially identical. Thus, systematic errors are unlikely.

Force generated by fixed-end tetani at sarcomere lengths longer than about 2.2  $\mu\text{m}$  is much higher than that generated by sarcomere isometric tetani. Figure 5*B* compares the fitted curves of the two types of tetani with the curve predicted from filament overlap. This predicted curve was determined from a thin filament length plus half Z-line width of 1.0  $\mu\text{m}$ , thick filament length of 1.6  $\mu\text{m}$ , and bare zone width of 0.15  $\mu\text{m}$  (Page & Huxley, 1963; Page, 1968, 1974; Suzuki & Pollack, 1986). Figure 5*B* shows that the descending limb of fixed-end tetani is clearly different from the predicted curve. On the other hand, the descending limb of sarcomere isometric tetani is close to predicted based on filament overlap. Linear regression analysis of the data on the descending limb (force less than maximal) indicated that force and sarcomere length were highly correlated (correlation coefficient 0.95 and 0.98 for results obtained with the diffraction method and segment length method respectively). However, the linear regression lines were found to lie at sarcomere lengths slightly longer than predicted: maximal and zero force were at lengths of 2.26 and 3.63  $\mu\text{m}$  for the segment length method and 2.13 and 3.70  $\mu\text{m}$  for the diffraction method. Also with higher-order polynomial regression analysis we found that the curves were slightly to the right of predicted: maximally 80 nm at intermediate lengths (between 2.3 and 3.4  $\mu\text{m}$ ), while at shorter and longer lengths the curves converged with the predicted force-sarcomere length relation; see Fig. 5*B*.

Figure 5*B* also shows several of the 'flattest' among the fixed-end curves published by others. These curves are similar to ours.

*Source of high force of fixed-end tetani*

It is clear from Fig. 5 that force generated during fixed-end tetani is higher than during sarcomere isometric contractions. Fixed-end tetani produce as much as 90%  $F_0$  (maximal force at optimal length) at a sarcomere length of about 3.0  $\mu\text{m}$ ; at 3.6  $\mu\text{m}$ , where non-overlap is reached, force remains as high as 50%  $F_0$ . We investigated why force of fixed-end contractions is much higher than that of sarcomere isometric tetani. First, passive force contributions were considered. In sarcomere isometric contractions passive force will remain constant since sarcomere length does not change (see also Discussion). In fixed-end contractions, on the other hand, sarcomere length often increased in the region under study (Figs 2-4) and the ensuing increase of passive force may explain the high forces associated with these sarcomeres.

The degree to which passive force increases during the force rise of fixed-end tetani will depend on the extent and velocity of stretch. The extent is shown in Fig. 6*A*. For those tetani in which the sarcomere length during the force plateau was between about 3.0 and 3.6  $\mu\text{m}$ , sarcomere length during the plateau was on average about 0.1  $\mu\text{m}$  longer than when the tetanus began; see Fig. 6*A*. As for velocity, we measured average stretch during the first 0.5 s of stretch (this period accounted for

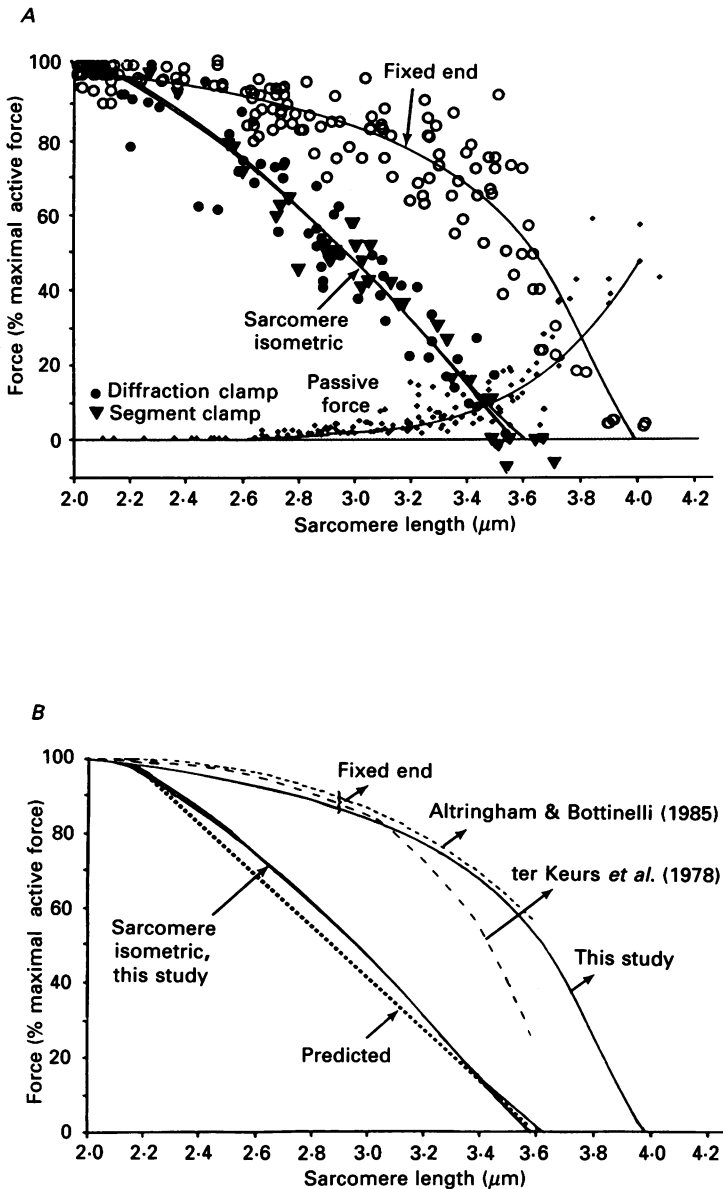


Fig. 5. Force-sarcomere length relations. *A*, pooled result from eleven fibres. Curves are fourth-order polynomial fits. Fixed-end results obtained with diffraction and segment length detection are shown with the same symbol; their individual fourth-order polynomial fits are indistinguishable. *B*, comparison of results with predicted descending limb based on filament overlap. Measured sarcomere isometric results (fourth-order polynomial fits) are much closer to predicted than are fixed-end results. Sarcomere isometric curves obtained with the two different methods are only slightly different at short length: the 'diffraction curve' is then below the 'segment curve'. Diffraction,  $y = -1250.9 + 1918.4x - 985.9x^2 + 218.1x^3 - 18.18x^4$ ; segment method,  $y = -2029.4 + 3112.5x - 1655.2x^2 + 381.2x^3 - 32.8x^4$ . Also shown are two curves obtained from fixed-end tetani as reported by ter Keurs *et al.* (1978) and Altringham & Bottinelli (1985).

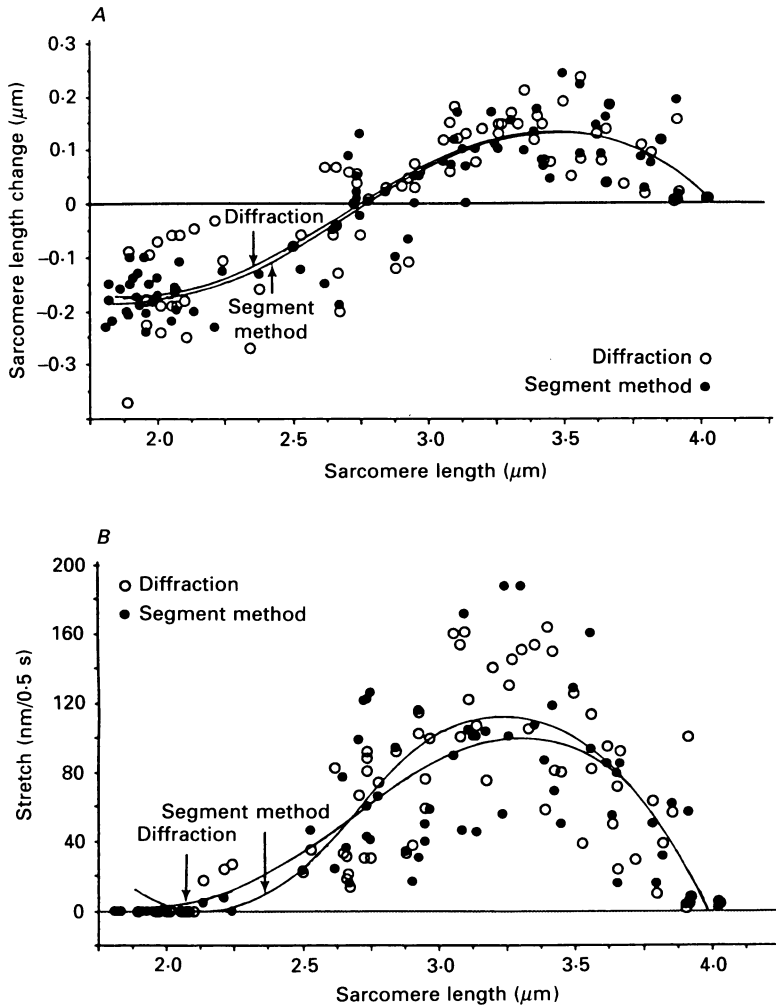


Fig. 6. *A*, local sarcomere length change during the force rise of fixed-end tetani. Plotted values represent differences between sarcomere length reached at the force plateau and the length measured prior to stimulation (negative numbers indicate shortening). *B*, amplitude of stretch that occurs during the first 0.5 s after start of stretch. (Zero indicates absence of stretch.) All curves are fourth-order polynomial fits.

most of the stretch: compare panels *A* and *B* of Fig. 6). Average stretch during this period was found to vary with sarcomere length; see Fig. 6*B*. At lengths between about 2.8 and 3.6  $\mu\text{m}$  it was on average slightly more than 80 nm/sarcomere; at lengths longer than about 3.6  $\mu\text{m}$  it decreased sharply.

To simulate this condition, passive fibres were stretched by 80 nm in 0.5 s (see inset of Fig. 7*A* for an example), and the ensuing elevation of passive force was measured. This experiment is similar to that performed by Gordon *et al.* (1966*a*). Passive force increased with sarcomere length; Fig. 7*A* shows that at lengths of about 3.6  $\mu\text{m}$  and longer the increase of passive force might explain the extra force generated during fixed-end contractions.

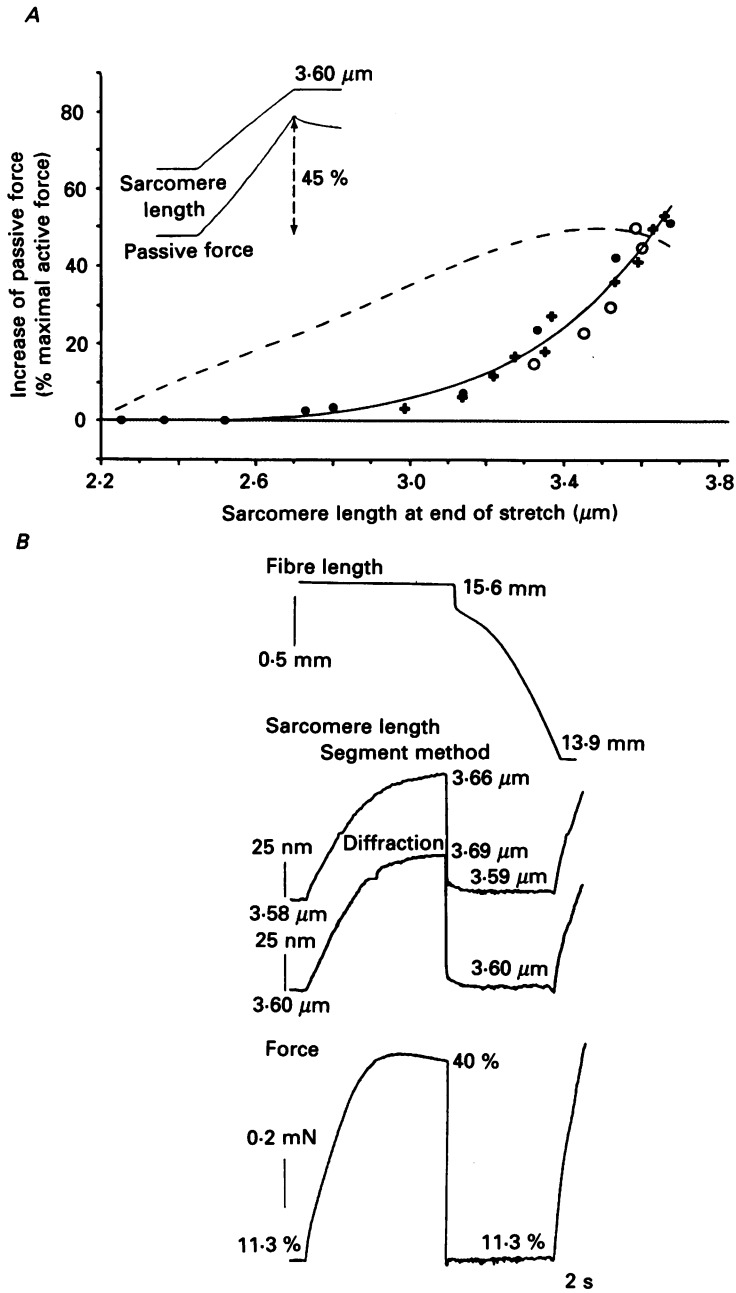


Fig. 7. *A*, increase of passive force arising from 80 nm stretch in 0.5 s. Experimental records depicted in inset explain the protocol. Sarcomere length at the end of the stretch is plotted against the increase of passive force during stretch. Results obtained from three fibres. Dashed line indicates difference between force generated by fixed-end tetani and sarcomere isometric tetani (determined from Fig. 5). *B*, quick-isotonic release during the plateau of a fixed-end tetanus at long length. See text for further details. Maximal force, 270 kN/m<sup>2</sup>; cross-sectional area,  $7.5 \times 10^{-3}$  mm<sup>2</sup>; temperature, 2.4 °C.

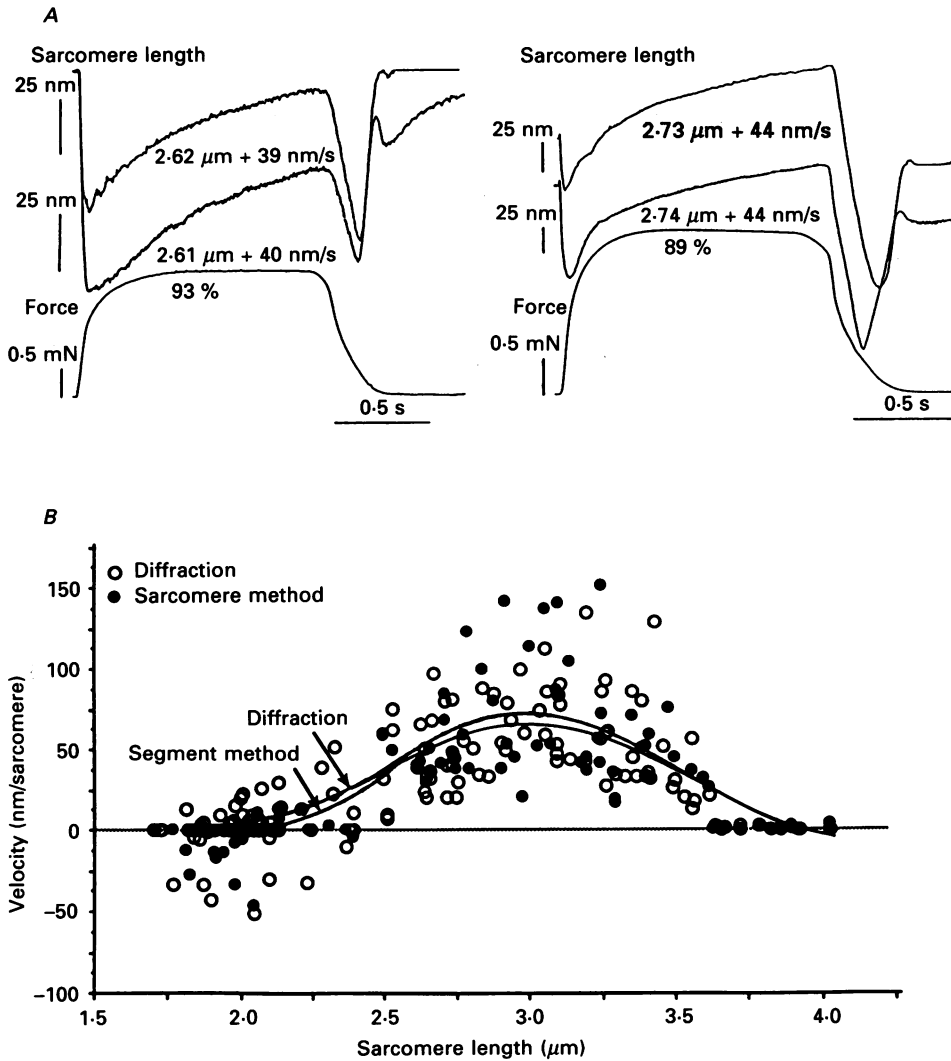
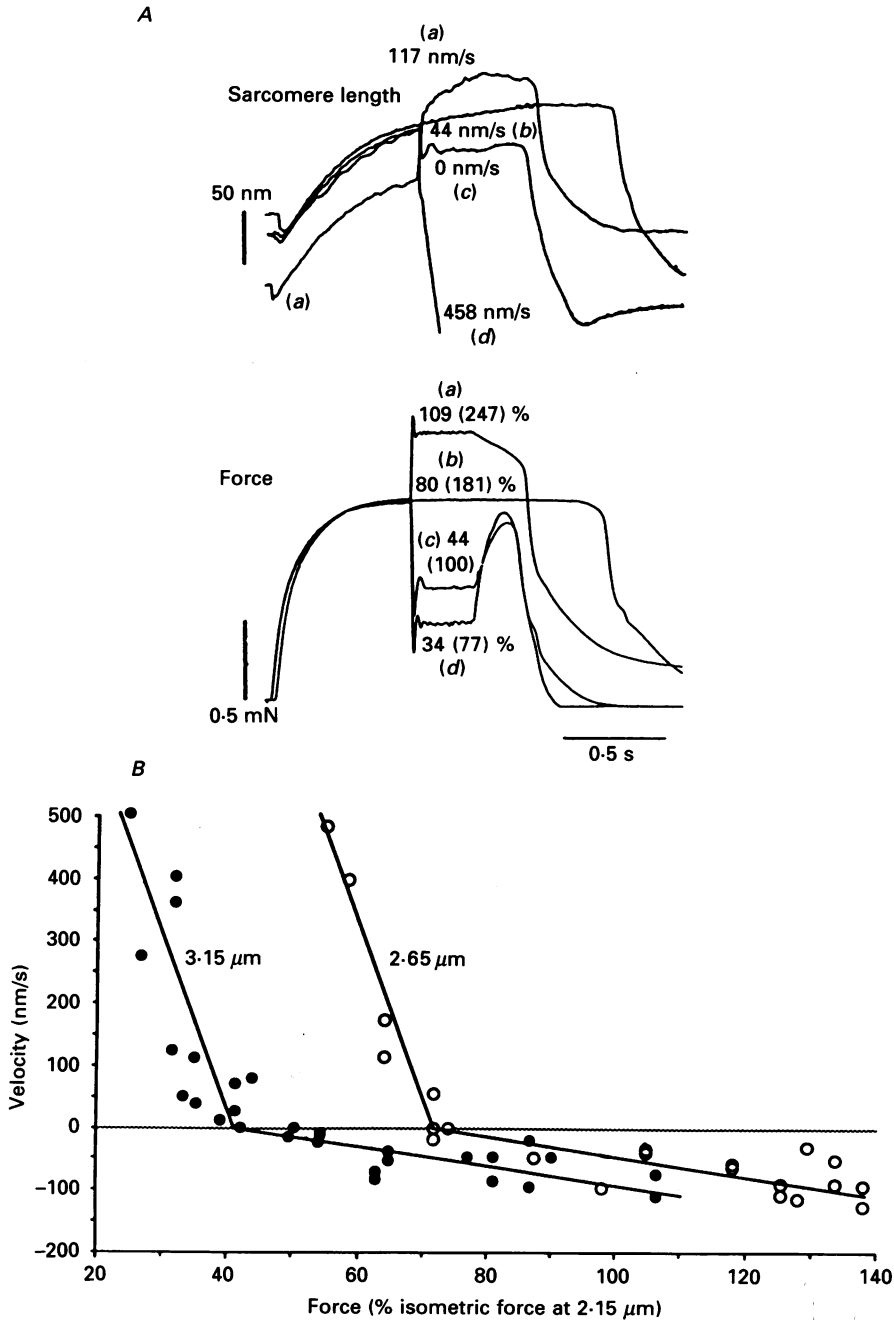


Fig. 8. Sarcomere stretch velocity during force plateau of fixed-end tetani. *A*, two examples. Sarcomere length is not constant during the plateau, but increases. Sarcomere length was measured simultaneously with the diffraction method (upper trace) and segment length method (bottom trace). *B*, pooled results. Abscissa shows sarcomere length measured 100 ms after the force plateau has been reached; ordinate gives concomitant sarcomere velocity (positive values indicate stretch). Curves represent fourth-order polynomial fits.

Fig. 9. Force-velocity relation for slow shortening and slow stretching. Results obtained with the segment length method. *A* shows representative records. Fibre was first held at a constant length. When force reached a plateau, load was changed to a new level and kept constant at that level. Sarcomere length during the force plateau prior to the load step was 3.15  $\mu\text{m}$ , except for contraction (*a*) in which it was set to 3.11  $\mu\text{m}$  to ensure that the length during the velocity measurement was close to 3.15  $\mu\text{m}$  as it was in the other contractions. Second sarcomere length trace and the second force trace from the top are



from a control tetanus, in which no load clamp was applied. The first value above each force trace represents force during load clamp as percentage of isometric force at 2.15  $\mu\text{m}$ ; second value represents force as percentage of sarcomere isometric force at 3.15  $\mu\text{m}$ . *B* shows pooled results obtained at two different sarcomere lengths. Lines are linear regression lines determined for either stretching or shortening. Negative values indicate stretch.

This explanation was confirmed with quick-isotonic release experiments in which fixed-end fibres were tetanized, and when force reached a plateau, force was suddenly reduced to the level of the passive force when stimulation began. If all of the force were passive, the sarcomere length attained after the load step would be the same as when stimulation began. On the other hand, if some of the force were active, sarcomeres would shorten after the load step to a length much shorter than when stimulation started. We tested fifteen tetani at sarcomere lengths between 3.6 and 3.7  $\mu\text{m}$ . Prior to the load step, sarcomeres stretched. During the load step they shortened abruptly: in twelve tetani they shortened to the same length as before stimulation, and in the remaining three tetani to a length slightly longer (maximally 35 nm/sarcomere). In no instance did they shorten after the load step to a length shorter than when stimulation started. An example is shown in Fig. 7*B*.

At lengths shorter than 3.6  $\mu\text{m}$ , stretch had only a small effect on passive force, and the elevated passive force could not account for the difference between fixed-end and sarcomere isometric force (Fig. 7*A*). Since we consistently found that sarcomeres in fixed-end tetani not only stretched during the force rise, but continued to stretch with low velocity during the force plateau, we investigated whether slow stretch might affect active force. (Stretch of the central region can also be inferred from the finding that the ends of the fibre shorten during fixed-end tetani; see Huxley & Peachey, 1961.)

Several tetani in which sarcomere stretch was observed during the force plateau are presented in Fig. 8*A*, while pooled results from all tetani are shown in Fig. 8*B*. At sarcomere lengths less than about 2.0  $\mu\text{m}$  or more than about 3.6  $\mu\text{m}$ , stretch was very small or absent, while at intermediate lengths, the sarcomeres under study were always being stretched during the force plateau. Average stretch reached a maximal value of about 60 nm/s at a sarcomere length of about 3.0  $\mu\text{m}$ . (The effect of such stretch on passive force is small at this length: passive force increases by only 1% of the maximal active force; H. L. M. Granzier, unpublished observations.) The same results were obtained with both methods; see Fig. 8.

We investigated the effect of stretch on plateau force by determining the force-velocity relation for slow stretching. This was done using load clamps at two lengths on the descending limb; see Fig. 9. Imposing loads that are much higher than the sarcomere isometric force resulted in stretch with only low velocity. At a sarcomere length of 3.15  $\mu\text{m}$ , for example, sarcomeres were isometric if the load was clamped to about 40% of the maximal force, while clamping to a load of 80% resulted in a stretch of only 60 nm/s. Thus, stretching by only 60 nm/s allows these sarcomeres to bear a force that is about twice the force generated under sarcomere isometric conditions. These findings are in agreement with the discontinuity in the gradient of the force-velocity relation found around zero velocity (Katz, 1939; see also Colomo, Lombardi & Piazzesi, 1988). Stretch, even with modest velocity, has a large effect on active force and may therefore be an important reason why active force in fixed-end contractions is higher than in sarcomere isometric contractions.

#### DISCUSSION

With fixed-end tetani, the descending limb of the force-sarcomere length relation was 'flat'. In contrast, sarcomere isometric contractions resulted in a curve that was



close to the linear descending limb predicted by the cross-bridge theory. The implications of these results are discussed below.

### *Passive force*

Sarcomeres in the fibre's central region were stretched during the force rise of fixed-end tetani. By stretching passive sarcomeres with amplitude and velocity similar to that observed, we estimated how much the passive force of the interrogated sarcomeres increased during contraction. This assumes that passive force is independent if the physiological state. This assumption has been tested for insect muscle (with conflicting results; see Chaplain & Frommelt, 1968; White, 1983), but not for vertebrates. However, it is unlikely that activation had a large effect on passive force in our study. This is based on the isotonic release contractions beyond overlap, as shown in Fig. 7*B*, which implied little or no change of passive force. Hence, at sarcomere lengths longer than about  $3.0 \mu\text{m}$ , passive force is an increasingly significant factor in explaining why force is much higher in fixed-end tetani than in sarcomere isometric tetani (Fig. 7*A*).

Passive force might also explain why the force-sarcomere length curve for fixed-end tetani is flatter in our study than in some others (Edman, 1966; Cecchi, Colomo & Lombardi, 1976; ter Keurs & Elzinga, 1981; Pollack, 1983). In fixed-end tetani, the end regions of the fibre, whose sarcomeres are generally shorter than in other regions (Huxley & Peachey, 1961), appear to determine the amount of force that is generated during the plateau (see also next section). Before the plateau is reached, passive force generated by the ends will have decreased, since the ends shorten considerably during the force rise. Altringham & Pollack (1984) found, for example, that representative sarcomeres in the ends shortened from an initial length of  $3.2 \mu\text{m}$  to a final length of about  $2.2 \mu\text{m}$ , while Huxley & Peachey (1961) reported shortening from about  $3.2$  to  $1.2 \mu\text{m}$ . Decrease of passive force will be small for the fibres used in our study since the passive force at the start of the contraction was low (Fig. 5). On the other hand, fibres whose force-sarcomere length relation has a steep descending limb show much higher levels of passive force (Pollack, 1983; ter Keurs *et al.* 1984); the decrease of passive force during shortening of the ends will therefore be much larger in these fibres. Total force generated by the ends during the plateau of fixed-end tetani will then be less, and the force-sarcomere length relation will have a steeper descending limb. Hence, differences in the steepness of the passive force-sarcomere length relation could underlie differences in shape of the descending limb determined with fixed-end tetani.

### *Stretch during the plateau of fixed-end tetani*

Sarcomeres in the fibre's central region stretched not only during the force rise but also during the force plateau of fixed-end tetani. This was confirmed with two independent methods (Fig. 8), so that it is unlikely that some artifact underlies this finding. Stretching of the interrogated sarcomeres while the total length of the fibre was kept constant implies that other sarcomeres must have been shortening. Most likely, these sarcomeres are located in the end regions of the fibre (Huxley & Peachey, 1961; Gordon *et al.* 1966*a*; Altringham & Pollack, 1984; Edman & Reggiani, 1984). Sarcomeres in the ends are apparently stronger than those in the centre.

As for the effect of stretch, the force-velocity relation shows that stretch with a

velocity similar to that observed during the force plateau increases active force far beyond isometric (Figs 8 and 9). Thus, the force of fixed-end tetani is not the isometric force of the sarcomeres in the fibre's central region, but instead a force that is greatly enhanced above their isometric value.

In conclusion, we found two factors that contribute to the high force of the interrogated sarcomeres during fixed-end tetani: enhancement of active force due to slow stretch, and, at lengths longer than about  $3.0\ \mu\text{m}$ , increase of passive force. Thus, contrary to the conclusions of previous work (see ter Keurs *et al.* 1978) the present study shows that the plateau force of fixed-end tetani does not represent the isometric force of the interrogated sarcomeres. To know this force, the sarcomeres must be kept isometric throughout contraction.

*The descending limb determined from sarcomere isometric tetani*

The position of the descending limb was close to that predicted by the cross-bridge theory, but not exactly the same. At intermediate sarcomere lengths the measured curve lies maximally 80 nm to the right of the predicted curve (Fig. 5B). The same result was obtained with both the diffraction method and the segment length method. It is unlikely therefore that the difference of 80 nm is somehow based on instrument artifact. The difference might originate from the fact that electrical stimulation results in activation that is slightly below maximal (2–3%) at  $2.15\ \mu\text{m}$ , while at longer lengths activation is maximal (Granzier, 1988). If this were the case, maximal force in Fig. 5 would be slightly underestimated, shifting the measured descending limb somewhat to the right of the predicted curve.

The difference between the measured and predicted descending limbs could also be due to residual variability of sarcomere length within the segment of the fibre that is investigated (Gordon *et al.* 1966b). Although we found almost identical curves (Fig. 5) with two methods that have a different sensitivity to variation in sarcomere length, we cannot exclude the possibility that a small degree of heterogeneity underlies the measured curvature of the descending limb.

Alternatively, the difference between the measured and predicted descending limbs might be explained if the prediction were not correct. For example, the first row of cross-bridges facing the bare-zone might be non-functional (Harrington, 1971), or filament lengths might be slightly different from those assumed. There is some uncertainty as to the exact length of the filaments. For example, some laboratories reported a thin filament length plus half Z-band width of a few hundredths of a micrometre shorter than the  $1.0\ \mu\text{m}$  assumed here (Huxley & Peachy, 1961; Brown, González-Serratos & Huxley, 1984), while others reported a value that is slightly higher than  $1.0\ \mu\text{m}$  (Page & Huxley, 1963). That thin filament length plus half Z-line width, or thick filament length, may be slightly larger than the values assumed in this investigation cannot be excluded, especially upon force development, during which any of these structures might elongate slightly (Huxley & Brown, 1967). Since sarcomere length is kept constant this would lead to slight increase of overlap.

A more extreme viewpoint is that the theory itself is not correct (Pollack, 1983), so that any such discrepancy between measured and predicted descending limb needs

no resolution. A recent theory (Pollack, 1984; G. H. Pollack, unpublished) accounts for both the flat fixed-end curve and the linear sarcomere isometric curve.

#### *Comparison with results of others*

For intact fibres, the position of the sarcomere isometric descending limb has been found to vary between almost identical to the linear descending limb predicted by the cross-bridge theory (Edman & Reggiani, 1987; Bagni *et al.* 1988) and one shifted to the right by maximally  $0.1 \mu\text{m}$  (Gordon *et al.* 1966*b*; ter Keurs & Elzinga, 1981; Altringham & Bottinelli, 1985). Thus the results of this study fall within the range reported by others.

As for skinned fibres, Fabiato & Fabiato (1978) investigated small bundles of myofibrils and found that the descending limb was close to predicted (based on filament lengths determined by Robinson & Winegrad, 1979) at both maximal and zero overlap, while at intermediate values the curve was about  $0.2 \mu\text{m}$  to the right of predicted. On the other hand, force generated by single myofibrils (Iwazumi, 1987) remained close to maximal until a length of about  $3.6 \mu\text{m}$  was reached, where force is predicted to be only about 20% of maximal (Robinson & Winegrad, 1979), while at longer lengths force dropped steeply. Thus, the force generated by myofibrils is higher than that generated by isometrically contracting sarcomeres of intact fibres.

A possible explanation for the high force generated by the myofibrils is that sarcomeres were not perfectly isometric during contraction. For example, sarcomeres might shorten somewhat during the force rise. Preliminary studies (Horowitz, Caljouw & Pollack, 1989) have shown that sarcomeres that shorten by less than 3% can develop considerably more force than sarcomeres that are held isometrically.

A second possibility is that the sarcomeres of the myofibrils shorten during the force rise, as above, but a few sarcomeres might shorten more than the others. The shortest sarcomeres might then continue to shorten during the force plateau, while the remaining sarcomeres are being stretched, as in the central region of fixed-end tetani of intact fibres (Fig. 8). This would lead to a large overestimate of isometric force (Fig. 9).

A third possibility is that the myofibrils might give the 'real' force-sarcomere length curve while the one obtained with intact fibres is somehow artificial. When overlap of intact fibres is reduced many factors change, e.g. interfilament spacing decreases (April, 1975), free calcium concentration decreases (Blinks, Rudel & Taylor, 1978) and overlap between thick filament and nebulin decreases (Wang & Wright, 1988). In theory, any of these factors might reduce force and result fortuitously in a linear descending limb. Against this explanation is the recent finding by Kishino & Yanagida (1988), who used an ultrasensitive force transducer to measure the force exerted on a single thin filament that was brought into contact with a surface coated with monomeric myosin. Thin filaments of different length were used ( $2\text{--}10 \mu\text{m}$ ) and the measured isometric force was found to vary linearly with thin filament length (Kishino & Yanagida, 1988, Fig. 4). Since thin filament length was the only variable in those experiments, while all other factors were kept constant, the linear relationship implies that overlap between thin filament and myosin is a highly relevant factor in determining the level of force. This supports the contention that the linear descending limb in intact fibres does not result fortuitously.

### Conclusion

The sarcomere isometric descending limb was found to be close to that predicted by the cross-bridge theory, although the fixed-end descending limb was largely flat. Therefore, a linear dependence of isometric force on overlap, or close to that, is not just a property of fibres whose fixed-end descending limb is already close to predicted (Pollack, 1983); instead, it appears to be a universal property of intact fibres.

A linear dependence of force on overlap is predicted by the cross-bridge model, but not solely by that model. The same dependence is expected by the S-2 based phase transition model (Harrington, 1971). And it is also predicted by a thick-filament shortening model (Pollack, 1984; G. H. Pollack, unpublished) under conditions in which sarcomeres are held strictly isometric. Thus, a linear dependence of isometric force on overlap is predicted by several contraction models, and all of these models are supported by this investigation.

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