Comments on
"Quantal Mechanisms in Cardiac Contraction"

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We appreciate that Sir Andrew Huxley wrote the following critique of the preceding Special Article at our request.

The Editor

The phenomenon to which Pollack gives the name “stepwise shortening” (though he claims that similar steps occur also during passive lengthening) is clearly of great interest if it is genuine; indeed, Pollack claims that it requires fundamental rethinking of current ideas about the mechanism of contraction. Before any such conclusion can be reached, however, three questions must be answered:

First, is the phenomenon genuine? Two of Pollack’s methods have been severely criticised, and in my view he has not answered those criticisms.

Second, if the phenomenon is genuine, is it related to events in the contractile mechanism itself? The fact that it is claimed to occur during stretch and release of unstimulated fibers, and even in fibers so far extended that there is no overlap of thick and thin filaments, suggests that passive elements are responsible.

Third, if in some cases the steps do arise in the contraction process, does this imply that current theories are wrong? They could be manifestations of the phenomenon well known under the name “isotonic transients”.

I will address myself to these three questions in that order. I may as well say at the outset that I am not convinced that the phenomenon is real. The deviations from smooth shortening are small (order of ± 0.1% of sarcomere length) and none of the methods used by Pollack and his collaborators has been shown to be free from errors sufficient to give the appearance of “steps” of this magnitude.

Are the Steps Genuine?

Pollack and his collaborators claim to have demonstrated “stepwise shortening” by four methods: (1) measuring the position of the first-order diffracted beam when laser light is passed through the preparation, (2) direct measurement of the periodicity of the striations in high-speed cine micrographs of a fiber, (3) throwing an image of the striation pattern onto an array of photodiodes that are scanned sequentially, giving a signal the frequency of which (inversely proportional to striation spacing) is measured with a “phase-locked loop”, and (4) throwing images of two markers attached to the fiber on to arrays of photodiodes and measuring the movements of the images electronically. I will consider these four methods in turn. I shall restrict my remarks to experiments on skeletal muscle fibers; Pollack also claims that stepwise shortening occurs in cardiac muscle, but in this case there are additional sources of error (eg, discontinuity of striation pattern at intercalated discs) and possibilities of real fluctuations in shortening speed (asynchronous activity in different cells) that do not exist in skeletal fibers.

1. Measurement of Diffraction Angle

Rüdel and Zite-Ferenczy have shown, to my mind conclusively, that the intensities of the beams diffracted by the striations are largely governed by “Bragg angle” effects, and this conclusion has recently been confirmed by Brenner and Sundell et al using different methods; a review by Zite-Ferenczy et al in press.

These effects arise as follows. Because of slight stagger in the relative positions of adjacent myofibrils, the striation planes are not exactly perpendicular to the fiber axis, the amount of deviation from the perpendicular being fairly uniform within regions referred to as “domains” but varying from one domain to another within each fiber. Each domain gives a strong diffracted beam only if the angle between the incident beam and the striation planes has the correct value (the “Bragg angle”), which depends on the striation spacing and the wavelength of the light. Different domains have slightly different values of the striation spacing and their contributions to the diffraction pattern therefore do not coincide. This gives rise to the well-known fine structure within each diffracted beam, due partly to the contributions from different domains appearing as separate elements and partly to interference between them where they overlap. The position of the first-order beam measured in methods such as that used by Pollack is a mean of the positions of these elements of the fine structure weighted according to their intensities. When a fiber shortens (or is stretched), things happen that will alter the relative intensities of the fine-structure elements: First, longitudinal movement of the fiber will bring domains into or out of the beam of light, and second, any change in tilt of the striation planes will bring the angle of incidence of the

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beam closer to or further from the Bragg angle, at which the contribution from that domain is a maximum. Hence, the mean diffraction angle measured by Pollack will have, superposed on changes straightforwardly due to change of striation spacing, small changes due to changes in relative intensity of the finestructure elements.

Pollack dismisses this group of phenomena, saying “direct tests of the Bragg mechanism have proved un-supportive. Since the critical angle is wavelength dependent, each wavelength should reveal a different set of Bragg planes. But they do not. The pattern of reflections remains wavelength insensitive.” He refers to work by Leung\(^7\) here; in fact, however, the range of wavelengths used by Leung was small (ratio of 1.08 between longest and shortest) that only minor changes of intensity of individual fine-structure elements are to be expected, and such are indeed visible in Leung’s Figure 1. The mean wavelength used was close to 0.5 \(\mu\)m in air (therefore about 0.36 \(\mu\)m within the fiber), and the striation spacing was 3.3 \(\mu\)m, so the Bragg angle would be \(\frac{\pi}{2} \arcsin(0.36/3.3) = 3.1^\circ\). A change of wavelength by a factor of 1.08 is therefore equivalent to a change of angle of incidence about equal to 0.08 \(\times\) 3.1\(^\circ\) = 0.25\(^\circ\). Rüdel and Zite-Ferenczy\(^8\) varied the angle of incidence over a much wider range (± 12\(^\circ\)) and showed that the half-width of individual peaks was 1–2\(^\circ\), so that a change equivalent to 0.25\(^\circ\) would not be expected to have much effect. (Leung saw differences between the fine structure patterns in the various diffracted beams, which in my view were probably due to Bragg-angle effects. Leung, however, attributed them to myofibrils not being all parallel to the fiber axis but being inclined by angles up to 5\(^\circ\); this interpretation appears, however, to be untenable, both because such inclination is not seen either in fresh fibers or in longitudinal sections and because it would cause “arcing” of the lines composing the diffraction pattern.)

It seems to me inevitable that “Bragg-angle effects” of these kinds will occur; but it is difficult to estimate their magnitude, and it is not immediately clear whether they can account for any appreciable proportion of the “steps” seen in the records of Pollack and others. An observation that strongly implies that some of the “steps” seen by diffractometry do arise from such effects is that a “step” seen with one of the two first-order beams is often not seen with the other;\(^9\) Pollack himself has confirmed this observation,\(^9\) though he claims that the 19% of occasions when the “steps” shown by the two beams did coincide is more than can be explained by chance. An investigation where it has been shown decisively that large and spurious “steps” can arise from Bragg-angle effects is that of Goldman and Simmons\(^10\) (referred to but not discussed in Pollack’s article).

When striation spacing was measured by a method equivalent to Pollack’s and the fiber (a relaxed skinned fiber) was made to shorten at a steady speed, large “steps” were sometimes seen. These were always eliminated when the angle of incidence of the laser beam on the fiber was oscillated through 1–2\(^\circ\) (peak-to-peak) at a frequency of about 3 kHz, so as to average out the Bragg-angle effects (the segment of fiber illuminated was not altered by the oscillation). Goldman\(^11\) has also built a diffractometer that uses white light to average out Bragg-angle effects; it is a null instrument in which both the deviation and the dispersion due to diffraction by the striations are cancelled by another stage of diffraction through an acousto-optic device that acts as a diffraction grating with variable spacing. Using this device on skinned fibers from the frog, Goldman saw only smooth decrease of sarcomere length during active and passive shortening.

Until Pollack uses some means of eliminating Bragg-angle effects equivalent to these instruments devised by Goldman, I shall not place any reliance on the genuineness of “steps” appearing in his records of the diffraction angle. It will also be necessary to show that a “step” has not been introduced by one of the other sources of error in this method demonstrated by Altringham et al.\(^12\)

2. Measurement of Striation Spacing By Cine-Microscopy

Pollack claims to have confirmed the reality of the steps seen with his diffractometer method, by direct measurement of striation spacing in successive frames of high-speed cinemicrograph films.\(^13\) The cine photographs were taken with the same laser light that was used for the simultaneous recording of the position of one of the first-order diffraction beams. Their records (three fibers) do indeed show “steps” occurring simultaneously in the two types of record, but the two methods cannot be regarded as independent. The periodicity seen in the image of a striated structure formed with laser light is straightforwardly the periodicity of the interference bands formed by the undeviated light and the two first-order diffracted beams. Hence, there will necessarily be a close correlation between the values of striation spacing found on the one hand by measuring the cine micrographs and on the other by measuring the deviation of one of the first-order diffracted beams. The photographic result obtained with laser light is therefore affected in the same way by the same main sources of error as the diffraction method. Perfect agreement is not to be expected for many reasons, eg, that the deviation of only one of the two first-order beams was recorded while both contribute to determining the spacing in the microscope image.

In order for micrography to give a faithful image, not subject to the same errors as diffractometry, it is necessary to use incoherent light (ie, not laser light) and to use a large-aperture cone of illumination from the condenser (impossible with laser light if a substantial field of view is to be covered). Using ordinary light, the striations in a living fiber are invisible in these conditions (there are only phase differences, not intensity differences, in the image) but good and contrasty images of the striations are obtained with polarized light or with two-beam interference microscopy.\(^14\) (The Nomarski system for differential interference contrast is not suitable for muscle since the image is
due partly to the high refractive index of the A bands and partly to their birefringence.

For these reasons, I do not regard the direct photography method as used by Pollack and his collaborators as a confirmation of the diffractometer results in any real sense.

3. "Phase-Locked Loop" Method

Pollack admits that "The PLL method inherently generates translation-induced fluctuations" and that these occur at the frequency with which sarcomeres enter or leave the region of measurement. I would prefer to say that the fluctuations are inherent in the particular equipment used by Pollack rather than in the method itself since they could have been eliminated by appropriate filtering before averaging the frequency signal. Pollack claims that "with signals of reasonable quality" the amplitude of these fluctuations in the measurement of sarcomere length "remains 2 nm or less," but they were much larger when "one of a number of conditions [not specified] had not been carefully controlled."

Even an amplitude of 2 nm peak-to-peak is not acceptable. Superposed on genuine shortening of the appropriate speed, the slope of the record would fluctuate between zero and twice the mean, and in this case the apparent step size would be times the fluctuation amplitude, i.e., 6.3 nm, which is close to the modal value in some of the histograms in Figure 4 of Granzier and Pollack. The peak-to-peak amplitude of the deviation from a smooth curve in the left-hand trace of Figure 2 of Pollack’s article in this volume is 3.8 nm, which is very close to the figure of 3.6 nm that I deduced for the error fluctuation amplitude from various values published by Pollack. The statement by Granzier and Pollack that, in a test using a grating, the peak-to-peak amplitude of the fluctuation was approximately 1 nm but "grew considerably when illumination levels were made subnormal" is not reassuring.

These calculations alone would show that it is not safe to assume that the translation-induced fluctuations are too small to be the origin of the "steps", but evidence that the "steps" do, at least sometimes, originate in this way was given by Altringham et al., who used Pollack’s equipment measured the speed of translation of the fiber and showed that "one oscillatory cycle in the sarcomere-length record corresponds to the translation of one sarcomere past a given point in the optical field." This is a strong indication that in such cases the steps are an instrumental artifact. Pollack suggests that Altringham's records have excessive noise and are not typical of those obtained by Pollack himself, but he does admit to believing that in the experiment illustrated in the authors' Figure 2b, Altringham et al. appear to be dealing with genuine sarcomere-length signals; these records are, however, among those where Altringham showed that each cycle corresponds to translation of the fiber by a distance equal to the length of a sarcomere, showing that the steps are artifactual.

This discussion recapitulates some of the arguments used by Altringham et al and by Pollack and myself in adjoining articles in Nature. In Pollack’s article in this volume, he does not even refer to these published criticisms of his PLL method. He does make a reference to the 1984 articles of Altringham et al and myself but only in connection with the "progressive shift in the sampled population" of sarcomeres due to fiber translation, a distinct source of possible error that is indeed raised by Altringham et al but not discussed by me in my 1984 article.

My view is that "steps" of the oscillatory character shown in Pollack’s Figure 2 and obtained by his phase-locked loop method should not be accepted as genuine unless accompanied in each case by records showing that a) the frequency of the oscillations is not close to the frequency with which striations pass a given point in the field and b) that translation of the same region of the fiber, without change of length, does not give rise to appreciable fluctuations in the sarcomere-length signal. These are simple controls which ought to be done as a matter of course; for example, the first could be easily achieved by recording on the second beam of the oscilloscope the output of one of the photodiodes in the array on to which the image of the striation pattern is projected.

4. Measurement of Distance Between Markers Attached to the Fiber

In principle, this is a very straightforward method. In the form used by Pollack and his collaborators, however, the movements of the images of the two markers are measured electronically. The apparatus has so far only been described in outline and no controls (e.g., displacement of the fiber without change of length) have been published, so it is impossible to judge what errors it may be subject to. Three key questions are the following.

a. Is the surface of the Ringer’s solution above the fiber open to the air? If so, ripples on the surface will displace the images of the markers, causing spurious changes in the apparent length of the segment.

b. How is the "marker profile" defined? The relevant feature is the drop of intensity within the image of the marker, so a background intensity must be chosen from which the observed intensities on the elements of the photodiode array are to be subtracted. An error in the choice of this background level, or non-uniformity of the background, would cause errors in the electronically estimated center of the profile.

c. Is the illuminating system such that the striations are visible? If so, as dark and light bands enter and leave the regions imaged onto the photodiode arrays, the estimated centers of the markers will shift, giving rise to translation-induced fluctuations at a frequency of one per sarcomere length of translation, just as in the phase-locked loop method.

5. Conclusion Concerning Genuineness of the “Steps”

Pollack lays much stress on the fact that he has observed steps by four different methods. However, two of these methods (Sections 1 and 3 above) are
subject to errors that have been criticized in published papers, and Pollack has not adequately answered these criticisms. Method 2 automatically gives results closely correlated with those of method 1 and is therefore subject to the same errors and is not an independent confirmation; and method 4 has only been published in outline and is subject to several possible sources of error. Pollack has not published adequate control records showing, for example, absence of fluctuations when a fiber is moved along bodily through the recording region; nor has he published records of movement of the fiber during the recording of "steps." Given these uncertainties in all of his methods, I do not regard the fact that all four show non-uniform shortening as evidence for the reality of the fluctuations. Results from a single, reliable method, with publication of full controls, would be much more convincing.

My conclusion is that the phenomenon cannot at present be accepted as genuine.

Do the Steps (If Genuine) Originate in the Contractile Mechanism?

From Pollack's first claim of stepwise shortening in 1977 until 1982, all accounts of "steps" were based on observations on stimulated preparations either shortening isotonically or undergoing local shortening while both tendons were held stationary. It was perhaps natural at that stage to suppose that the steps were due to some feature of the contractile process itself. The position was totally changed, however, when two short abstracts, followed by a full paper, described "steps" during passive length changes in fibers that were not stimulated and even in some cases at degrees of extension where there is no overlap of thick and thin filaments. Further, these steps were claimed to be similar to those recorded during active shortening, and there was no change in character of the steps when a fiber, initially at a length with no overlap, was allowed to shorten passively past the point where overlap begins.

The prima facie interpretation of these observations (if one assumes that the "steps" are not instrumental artifacts) is that the steps are in all cases due to something that does not involve the contractile process at all. Nevertheless, Pollack devotes many pages of his article in this volume to supposed difficulties of current theories of contraction in explaining the steps before he even mentions his observation of steps in unstimulated fibers.

Pollack argues further that the steps in high-stretched fibers must be due to the "connecting filaments" and that, because these are slack when the sarcomere length is below, say, 2.5 μm, steps at such lengths must be due to the thick filaments themselves. I regard this as a very weak argument. First, there is no positive evidence that the connecting filaments are responsible when a fiber is stretched to zero overlap; it might just as well be some other passive element such as the sarcolemma, the sarcoplasmic reticulum, the transverse tubules, or even the tendons. Before it is worth discussing the part played by connecting filaments, the other possibilities should be excluded: tendons by recording movement of the fiber ends; the sarcolemma by using mechanically-skinned fibers; the sarcoplasmic reticulum and transverse tubules by using detergent-treated fibers.

Even if the phenomenon is shown to be genuine, there are many features that one would like to know before beginning to discuss its interpretation and its significance. I have in mind three in particular.

a. Are the fluctuations in shortening velocity genuinely stepwise or are they nearer to being sinusoidal? Some of Pollack's records do appear to show one or a few periods of nearly zero shortening speed (eg, Figures 3 and 4 of Pollack et al), while others look more nearly sinusoidal (eg, Figure 2 of Pollack's article in this volume, or Figure 7A of Granzier and Pollack, where the oscillations are so large that the slope of the shortening record actually reverses sign repeatedly). This raises my second question.

b. Do all the fluctuations in shortening velocity have the same origin? It might be, for example, that nearly-sinusoidal oscillations have their origin in passive elements of the muscle fiber while isolated steps or pauses might be related to the contraction mechanism.

c. What is the maximum segment length in which the phenomenon is detectable? Pollack emphasises that the "steps" are still conspicuous in segments up to 0.8 mm long but gives no indication whether they are still present when measured in longer segments, or how their amplitude or frequency of occurrence varies with segment length. I do not recall even any evidence that the fluctuations are not in phase throughout the length of the fiber; Pollack states that this is impossible because the imposed change of total length is smooth, but if the fluctuations originated in irregular stretching or shortening of the tendons, this is just what would happen.

In conclusion: It appears to me that even if the phenomenon is genuine, there is not yet enough evidence about its characteristics, or about which structure in the fiber it originates in, to make it worth while to discuss its nature. Any such discussion at the present stage is highly speculative. The evidence, such as it is, is against supposing that the contractile process is involved.

If in Some Cases the Steps are Genuine and Originate in the Contractile Mechanism, What Are the Implications for Theories of Contraction?

Oscillatory responses in the time course of shortening when the load on a muscle fiber is altered ("isotonic transients") have been well known for more than a quarter of a century. It has long seemed to me that fluctuations in shortening speed such as are claimed by Pollack might be expressions of the same phenomenon, and indeed we suggested that the oscillatory length changes we recorded might be related to the stepwise shortening observed by Frank in frog muscle." Frank's work was reported in more detail by
Emel'yanov et al\textsuperscript{28} and by Yefimov and Frank,\textsuperscript{29} and Pollack refers to this work (in this volume) in a way which shows that he regards his own observations as referring to the same phenomenon. Hence, I can claim that we put forward an explanation for “stepwise shortening” more than ten years before Pollack’s first report of it\textsuperscript{19} in 1977. Several of Pollack’s records of local shortening at the onset of stimulation (eg, Figures 4 and 5 in Pollack et al\textsuperscript{22}) resemble isotonic transients in showing (a) an initial period of shortening at well above the steady-state velocity, followed by (b) a period of greatly reduced velocity, and finally (c) a heavily damped oscillatory approach to the steady velocity. Pollack dismisses this suggestion on the grounds that his records appear more angular than the smoothly oscillatory isotonic transients recorded by others, but in view of the sources of error discussed earlier, I do not regard these details of Pollack’s records as well established. Very small random fluctuations superposed on smooth oscillations would be sufficient to give the appearance of sharp changes of slope.

As regards the oscillatory-looking records obtained by Pollack at somewhat greater time intervals after the onset of stimulation (eg, Figure 3, Jacobson et al\textsuperscript{16}), presumably some other parts of the fiber, or the tendons, are undergoing similar oscillations in opposite phase. In recordings of isotonic transients, the degree of oscillatoriness varies considerably from one fiber to another, and in some cases, with the load near to the isometric value, there is only very slight damping (eg, Figure 1 (a) of Armstrong et al\textsuperscript{14}), and any disturbance of steady shortening would be expected to set up lightly damped oscillations that would be superposed on nearly steady shortening. Such disturbances could arise in many ways, eg, through “give” in the tendon attachments (in which case the oscillations would be in phase throughout the length of the fiber) or through irregularities in the intracellular release of calcium (in which case more-activated parts of the fiber would oscillate in opposite phase to less-activated parts).

If it should turn out that some of Pollack’s phenomena in stimulated muscle are, in effect, “isotonic transients”, it will be ironic that he should claim that they undermine current theories of cross-bridge action, since these theories are largely based on “isometric transients”\textsuperscript{20}, which are alternative and equivalent expressions of the same processes as underlie isotonic transients.

Pollack lays much emphasis on the synchronizing of contractile events over substantial volumes within a fiber and, therefore, over very large numbers of myofilaments. He asserts that “the present theory contains no provision to synchronize the action of bridges,” but this is not true: In an isotonic transient response, for example, the whole of the fiber undergoes damped oscillatory changes of shortening speed that are synchronized by the step in applied load, and it is to be expected that similar oscillations will be set up, with synchrony over substantial volumes, if for example, as suggested in the preceding paragraph, the degree of activation is increased locally (eg, by non-uniform calcium release). An ironic feature of this claim of Pollack’s is that the theory that he proposes for explaining his “steps” contains no other mechanism that would cause filaments in different myofibrils, or in adjacent sarcomeres of a single myofibril, to act synchronously.

Conclusions
1. It is still quite uncertain whether any, and if so which, of the fluctuations in speed of shortening (or lengthening) recorded by Pollack and his collaborators, are genuine.
2. There are some of Pollack’s phenomena that, if genuine, must be related to passive elements in the fiber. Only if two (or more) distinct processes are postulated would it be possible to suggest that the contractile mechanism itself is involved.
3. If in some cases the contractile mechanism is indeed involved, it is probable that the “stepwise shortening” is a manifestation of the well-known isotonic velocity transient.

4. Until the phenomena have been recorded by much more reliable methods, it is altogether premature to discuss implications of “stepwise shortening” for theories of contraction.

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