Phosphate Starvation and the Nonlinear Dynamics of Insect Fibrillar Flight Muscle

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ABSTRACT The nonlinear mechanical dynamics of glycerinated insect fibrillar flight muscle are investigated. The most striking nonlinearity reported previously, which often resulted in oscillatory work being limited to frequencies below those of natural flight, disappears if 5 mM or more orthophosphate is added to the experimental solutions. We show that two further asymmetric nonlinearities, which remain even though phosphate is present, are predicted by cross-bridge theory if one takes account of the expected distortion of attached cross-bridges as filament sliding becomes appreciable. Adenosine triphosphate and adenosine diphosphate have opponent effects upon the mechanical rate constants, suggesting a scheme for the sequential ordering of the events comprising the cross-bridge cycle.

INTRODUCTION

In a recent paper (Thorson and White, 1969) we suggested and tested possible explanations for the work-producing mechanical dynamics of insect filbrillar flight muscle. Our idea was based on alteration by strain of the rate constants for attachment and detachment of cross-bridges between the thick (A) and thin (I) filaments. We showed that, for small perturbations, the delayed tension changes following length changes in such a system agreed well with those of the muscle; moreover, the hypothetical rate constants for the cross-bridge cycle, determined solely by fits to the mechanical data, predicted a cycling rate for the cross-bridges which was compatible with experimental measurements of the rate of adenosine triphosphate (ATP) hydrolysis in fibrillar muscle.

In this paper we restrict our interpretation of the contractile mechanism still further by describing the nonlinear mechanical dynamics of the muscle in response to length changes exceeding the above near-linear range. Rapid

length changes of more than a few tenths of a per cent induce tension transients which exhibit both asymmetric nonlinearity and, at slightly higher amplitudes, damped isometric oscillations. These effects have been reported previously (Tregear, 1967; Abbott, 1968; Steiger, 1969; Schädler et al., 1969), but most of the earlier work has been done using sinusoidal length changes over a limited range of frequencies, mainly with the aim of determining the maximum amount of mechanical work that is obtainable from the system and relating this to the usage of ATP (Pringle and Tregear, 1969; Steiger and Rüegg, 1969). Since these nonlinearities are potentially rather strong clues to the underlying mechanism we have made a more extensive study of them. The chief new property of the nonlinear behavior reported here lies in our demonstration that orthophosphate, in millimolar concentration, can alter the nonlinear dynamics drastically.

Since our main aim has been to characterize the transition from the linear to the nonlinear dynamics in a form readily comparable with cross-bridge theory (Huxley, 1957; Thorson and White, 1969; Julian, 1969; Podolsky et al., 1969) we have emphasized transient rather than sinusoidal responses. The reason for this is that we find it more convenient and informative to solve the simultaneous nonlinear differential equations of a multiple-cross-bridge viscoelastic sarcomere under ramp changes of length than under sinusoidal length changes (Thorson and White, in preparation). In this paper the theoretical interpretation of the data is restricted to a qualitative description of the effects expected due to distortion of cross-bridges during large-amplitude length changes, based upon a simplified cross-bridge model.

Although cross-bridge theory allows one to test hypotheses about the rules for bridge attachment and detachment without specification of the precise stages of the cycle at which ATP and adenosine diphosphate (ADP) are bound, one hope has been that these stages might be identified with the help of the theory. We shall show that several recent results, including our own, are compatible with the following generalization: ADP reduces the rate at which tension can change, whereas ATP increases it. Applied to the cross-bridge cycle, this notion places an interesting constraint upon the sequential ordering of ATP binding, ADP unbinding, and the state of the cross-bridge during which it contributes to interfilament shear force.

MATERIAL AND METHODS

Fibers from *Lethocerus cordofanus* dorsal-longitudinal flight muscle have been used, about 8 wk after glycerol extraction. The glycerination procedure is as described by White (1970).

The mechanical apparatus consists of a servo-controlled vibrator for applying length changes to the muscle, and a tension transducer. The vibrator and ancillary electronics have been described previously (Jewell and Rüegg, 1966; White, 1970)—

they are capable of producing length changes of 200 μm (4% of the 5 mm length of muscle always used) in less than 1 msec. The tension transducer that we built and used was designed by Huxley and Simmons (1968). This is a variable-capacitance transducer constructed of glass. Its capacitance is measured with the circuit described by Cambridge and Haynes (1959). This transducer is a considerable improvement on others that we know of for measuring rapid tension changes, having, in our case, a resonant frequency of 2.7 kHz, a stiffness of 4 μ/g, and a damping factor of about 0.5 with the muscle-attachment probe in place. (The performance quoted by Huxley and Simmons, 1968, applies to the transducer without any added structure for muscle attachment.)

Because microdynes are convenient units at the macromolecular level, we have converted all measured tensions to microdynes per A filament. The conversion is based upon the number of fibers in the preparation (usually five) and the mean value 6.3×10^5 A filaments/fiber measured in *Lethocerus cordofanus* by Chaplain and Tregear (1966).

The response of muscle tension to both step and sinusoidal length change has been investigated. The step length changes were controlled from the output of a D-A converter connected to a PDP-8I computer (Digital Equipment Corp., Maynard, Mass.), with a program written by Dr. R. H. Abbott. This enables a sequence of length changes to be applied to the muscle, each length being held for a selected duration; the velocity of length change between levels is selected as well. The high-amplitude sinusoidal length changes were controlled by a wave-form generator (Feedback Ltd., Crowborough, Sussex, U. K., type TWG 300). For both the step and the high-amplitude sinusoidal experiments the length and tension outputs were displayed on a Tektronix 502A oscilloscope (Tektronix, Inc., Beaverton, Ore.) and photographed directly. A continuous paper-chart record (Devices Penrecorder, Devices Instruments, Ltd., Welwyn Garden City, Herts., U. K., type M4) was also kept of each experiment. The sinusoidal response at low amplitudes, in the region of linear response of the muscle, was determined using a Solartron Transfer-Function Analyser (Solartron Electronics Group, Ltd., Farnborough, Hants., U. K. type JM1600). This measures the amplitude and phase of the tension with respect to the input length at a given frequency. The analyzer was controlled by the PDP-8I computer, enabling the response over a range of frequencies to be measured very rapidly, with a program written by Dr. R. H. Abbott.

The fibers were immersed in solutions in a temperature-controlled bath. Bundles of five fibers, 5 mm long, were studied at 18°–20°C except where otherwise stated.

We have considered it essential to control at least the following parameters: concentrations of Ca^{++}, $MgATP^{--}$, $MgADP^{-}$, ($HPO_4^{--} + H_2PO_4^{-}$), pH, and ionic strength. All of our solutions have contained 5 mM ethylene glycol bis(β-aminoethyl ether)N,N,N',N'-tetraacetic acid (EGTA). The actual composition of the solutions was determined with the use of Perrin and Sayce's (1967) computer program, taking into account the ionic species of Table I and the association constants given there. We have adjusted ionic strength by changing the KCl concentration. The most commonly used solutions in these experiments, together with the calculated concentrations of various ionic species and the total ionic strength of each solution, are given

in Table II. The 0.32 μM Ca^{++} concentration in the activating solutions was chosen since at higher levels of activation the "high-tension state" (Jewell and Rüegg, 1966) often occurred.

Fibrillar flight muscle has, when relaxed, a much higher stiffness than other striated muscles. This makes it possible to define a rest length for a set of fibers on the apparatus as that length at which the tension in the relaxed muscle is just zero. For this reason it has been standard practice (see, e.g., Abbott, 1968; White, 1970) to find rest length and then to increase length by 1 % so as to provide a steady state of stretch activation about which perturbations are studied. We adopt this method here but with

TABLE I

IONIC SPECIES AND ASSOCIATION CONSTANTS

No.	Ionic species	$\log_{10} K$*	Source‡	No.	Ionic species	$\log_{10} K$	Source
1	Hhistidine	9.16	SC	20	MgATP	4.0	SC
2	H_2histidine	15.22	SC	21	MgHATP	8.62	SC
3	H_3histidine	17.04	SC	22	CaATP	3.6	SC
4	Mghistidine	2.1	SC	23	CaHATP	8.46	SC
5	Cahistidine	1.4	SC	24	HADP	6.35	SC
6	HEGTA	9.46	PCR	25	H_2ADP	10.34	SC
7	H_2EGTA	18.31	PCR	26	CaADP	2.78	SC
8	H_3EGTA	20.99	PCR	27	MgADP	3.11	SC
9	H_4EGTA	22.99	PCR	28	MgHADP	7.87	SC
10	MgEGTA	5.21	PCR	29	KADP	1.15	ATP value
11	MgHEGTA	12.22	PCR	30	NaADP	1.17	ATP value
12	CaEGTA	11.00	PCR	31	HPO_4	12.36	SC
13	CaHEGTA	14.18	PCR	32	H_2PO_4	19.56	SC
14	KEGTA	0.96	EDTA value	33	H_3PO_4	21.68	SC
15	NaEGTA	1.8	EDTA value	34	$KHPO_4$	12.81	SC
16	HATP	6.5	SC	35	$NaHPO_4$	12.91	SC
17	H_2ATP	10.55	SC	36	$MgHPO_4$	14.86	SC
18	KATP	1.15	SC	37	$CaHPO_4$	14.56	SC
19	NaATP	1.17	SC				

* K = total association constant (i.e., for H_3histidine, $K = [H_3\text{histidine}]/[H]^3 \cdot [\text{histidine}]$) with dimensions molar^{-n} (n an integer).

‡ Sources: SC, Sillen and Martell (1964); PCR, Portzehl et al. (1964).

an important qualification: the following experiment using phosphate solutions (see Table II) clarifies the significance of the above procedure in terms of the "steady state of stretch" upon which step changes of length are imposed. In Fig. 1, the open triangles show successively the near-zero passive (i.e., not activated by Ca^{++}) tension defining rest length or zero strain, and the tensions resulting from 0.5 and 1 % strain after allowance for 1 min of stress relaxation (White, 1967). From this passive "operating point" 0.2, 0.5, and 1 % rapid steps are applied and the "steady tension" reached in about 100 msec (as illustrated below in Fig. 4) is plotted as the filled triangles. These tensions are "steady" in the context of the time scale of the experiments of this paper, in which we ignore the small stress relaxation that would occur if our brief test steps were maintained for minutes rather than seconds.

The open and closed squares of Fig. 1 repeat the above sequence for the same fibers in activating solution (0.32 μM Ca^{++}), with identical length settings; some tension is developed at the passive rest length, and a similar nonlinear tension-length curve is found for the long-term and short-term steady tensions. Note that the nearly linear short-term tension-strain curves (closed symbols) both extrapolate to a zero-tension strain at just over 0.5 %. In various experiments this point ranged from 0.5 to

TABLE II

CONSTITUENTS OF PHOSPHATE (P_i) AND NONPHOSPHATE (0-P_i) ACTIVATING (*A*) AND RELAXING (*R*) SOLUTIONS

Solutions	P_i-*A*	P_i-*R*	0-P_i-*A*	0-P_i-*R*
	mM	*mM*	*mM*	*mM*
Na_2ATP	10	10	10	10
$MgCl_2$	12	12	10	10
EGTA	5	5	5	5
$CaCl_2$	3	0	3	0
KCl	20	20	45	45
K_2HPO_4	13	13	0	0
KH_2PO_4	7	7	0	0
Histidine Cl	0	0	20	20
Calculated concentrations of selected complexes and free ions				
Ca^{++}, μM	0.32	*	0.32	*
Mg^{++}, mM	1.20	1.19	1.35	1.31
$MgATP^{2-}$, mM	8.34	8.32	8.55	8.52
ATP^{4-}, mM	0.70	0.70	0.63	0.65
$MgHPO_4^-$, mM	2.39	2.37	‡	‡
HPO_4^{3-}, mM	6.29	6.30	‡	‡
$H_2PO_4^{2-}$, mM	9.97	9.98	‡	‡
Ionic strength, mM	118	115	117	114
pH	7.0	7.0	7.0	7.0

* A contamination of 1 μM Ca^{++} was included in the calculations, giving free Ca^{++} of 6.31 and 6.32 $\times$ 10^{-11} M, respectively.

‡ Similarly, a contamination of 1 μM phosphate was included in the calculations of the nonphosphate solutions, resulting in values of about 0.1 μM $MgHPO_4^-$ and about 0.8 μM total orthophosphate ion concentrations. A contamination of 1 μM ADP was added to all the calculations.

0.7 %. Therefore, for short-term length changes less than 1 % above the "1 %" operating point, the short-term steady tension behaves as though the muscle were a linear spring stretched initially by only 0.3–0.5 %. In particular, note that activation by Ca^{++} in this case has simply changed the stiffness of this spring. This point is of critical importance in relating the data of this paper to analyses of the nonlinear dynamics of the cross-bridge cycle, since the predicted dynamics following step changes of length depend very much upon the steady state of stretch activation upon which they are imposed (Thorson and White, in preparation).

Drift in the tension transducer was controlled by frequent slackening of the fibers

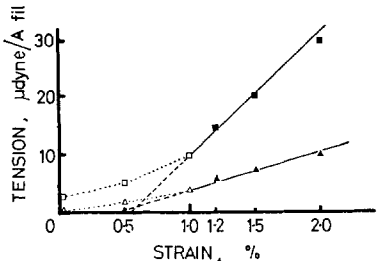


FIGURE 1. Analysis of the long-term and short-term steady states. Length-tension curves for the data of Fig. 4 were drawn as described in the text. Squares: fibers in activating solution; triangles: fibers in relaxing solution.

until they exerted zero tension. In the experiments illustrated in Figs. 1 and 4 the base line was determined in this way before and after each set of records. In the other experiments the base line was determined at the start and end of each experiment, but only occasionally between these times. The base lines for most of the data are known with less certainty than for the data of Figs. 1 and 4; inspection of our continuous slow paper-chart records shows that the tensions of the fibers in the activating solutions at the standard 1 % extension were in the range 5–12 μdynes/A filament, and those in the relaxing solutions in the range 2–6 μdynes/A filament.

RESULTS

Effect of Orthophosphate Ions (P_i) on the Response

Preliminary experiments, in which we used phosphate buffer, produced mechanical dynamics which differed grossly from other measurements, particularly with respect to the step responses of Steiger (1969). Since our aim has been to characterize the mechanical dynamics for comparison with cross-bridge theory, we considered it essential to find the origin of such differences.

Fig. 2 illustrates the response obtained to a step length change of 0.5% applied to a bundle of fibers held at a steady length 1% above rest length in phosphate and nonphosphate activating solutions. Even the qualitative differences between the two results are striking. In both there is an initial transient which is not well reproduced on these slow records, but which can be seen in the faster records of later figures. In the presence of 20 mM orthophosphate (P_i) the tension then reaches a steady level characteristic of the new length in approximately exponential fashion in response to either a step increase or a step decrease in length. In the absence of P_i there is a large transient increase in tension in response to an increase in length which then decays to a steady level approximately the same as that obtained in the presence of P_i. We have termed the momentary high tension the "phosphate-starvation transient" (PST) in order to emphasize its dependence upon the absence of P_i. After the subsequent step down there is also, following the very rapid initial effects, a comparatively slow rise in tension to a peak which then decays to the

original steady tension—in other words there is a momentary increase in tension in response to either a positive or a negative length change.

That this difference is associated with the difference in phosphate concentration, rather than the difference in histidine which was used as the buffer in the absence of P_i, is shown by repeating the experiment in a solution containing both histidine and phosphate, keeping the ionic strength constant. The P_i response is obtained. Further, the use of Trizma base (Sigma Chemical Co., London) or PIPES (piperazine-N, N'-bis [2-ethane sulfonic acid]) instead of histidine as pH buffer results in the non-P_i response, as long as P_i is kept at low concentrations.

In one experiment using histidine-buffered activating solution, we varied the P_i concentration using values of 0.1, 0.2, 0.5, 1, 2, 5, 10, and 20 mM. The full phosphate-starvation transient was measured at P_i concentrations of 1 mM

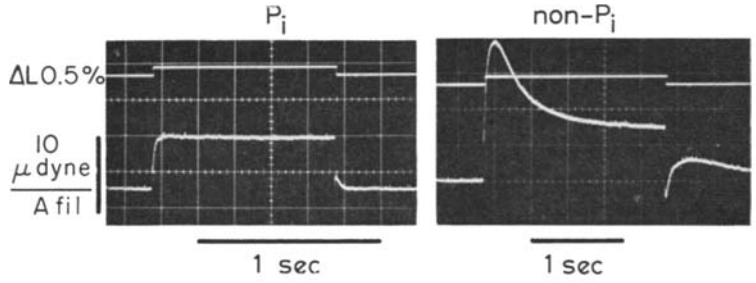


FIGURE 2. Comparison of the tension wave forms caused by step length changes in phosphate and nonphosphate activating solutions. (Solutions are as in Table II, except that the concentration of KCl in the nonphosphate solution was 53 mM.) The upper trace is of muscle length, and the lower of muscle tension.

and below, but was noticeably reduced at 2 and 5 mM P_i, and not measurable at 10 and 20 mM P_i.

The phosphate-starvation transient is not due to secondary effects of P_i binding free Ca^{++} or Mg^{++} in the solution since we controlled the concentrations of Ca^{++}, Mg^{++}, and $MgATP^{--}$. It is not due to a difference in the ionic strength, since this was equated in the P_i and non-P_i solutions, and similar qualitative differences are seen at all ionic strengths in the range 80–170 mM. Phosphate starvation transients are also found with ATP concentrations between 5 and 20 mM and in the presence or absence of either myokinase or a creatine phosphate/creatine phosphokinase ATP-regenerating system, suggesting that the phenomenon is not due to diffusional barriers preventing the central myofibrils of a fiber obtaining sufficient ATP—as might be the case if there were an indirect effect of P_i on the rate of hydrolysis of ATP.

The striking asymmetric nonlinearity of the response in the absence of P_i is shown even more clearly when the duration of the step change is decreased.

Fig. 3 *a* compares the response obtained in the non-P_i activating solution with that obtained in both non-P_i relaxing solution and the P_i-activating solution. The response in the non-P_i activating solution again illustrates the phosphate starvation transient. Notice especially the responses obtained to 50-msec upward and downward pulses of length change. To both there is a *positive* transient increase in tension lasting longer than a second. The magnitude of the transient is usually greater following a downward pulse; in this case, a brief decrease in length results in a long-lasting increase in tension. The magnitude of the PST increases as the duration of the pulse increases for a

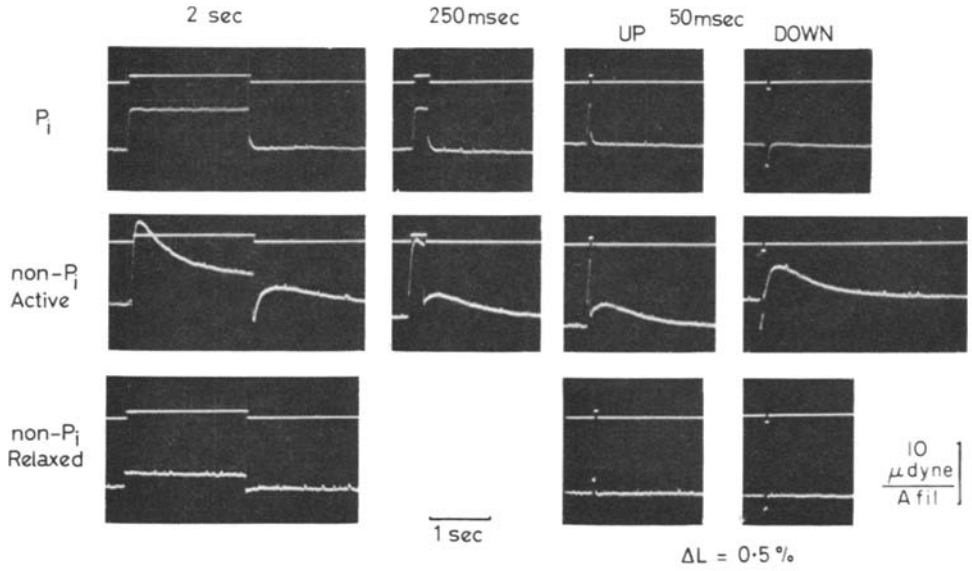


FIGURE 3 *a*. The effect of pulse duration on the response of the muscle in the phosphate and nonphosphate activating solutions and nonphosphate relaxing solution of Table II. The last two columns compare a 50 msec upward pulse with a 50 msec downward pulse.

downward pulse of length change. Larger PST's are obtained when the last change of length is a stretch.

Fig. 3 *b* again illustrates the response to short pulses, and shows the tension changes occurring during the pulse on a faster time base.

Effect of Step Amplitude

For comparison with theory, we are particularly interested in the manner in which the near-linear dynamics at small length changes become nonlinear at slightly larger length changes. Figs. 4 and 5 illustrate the response obtained in the P_i and the non-P_i solutions to step length changes of 0.2, 0.5, and 1.0% for both the activating and relaxing solutions. There are two sets of figures for each of the four responses. The first is recorded with a slow time base and

illustrates the entire response. The second is at a faster sweep speed, and illustrates the rapid events more clearly. In the latter records the sweep is triggered to cross the oscilloscope screen twice for each exposure, once to illustrate the step up and the second time to illustrate the step down; the two sweeps are not contiguous but triggered so as to display the steps up and down conveniently on one photograph.

All of the active responses in Figs. 4 and 5 contain an initial approximately 1 msec transient change of tension (not well resolved in the slow records) followed by a slower transition to the equilibrium tension associated with the new length. The latter takes seconds in the non-P_i condition and tens of

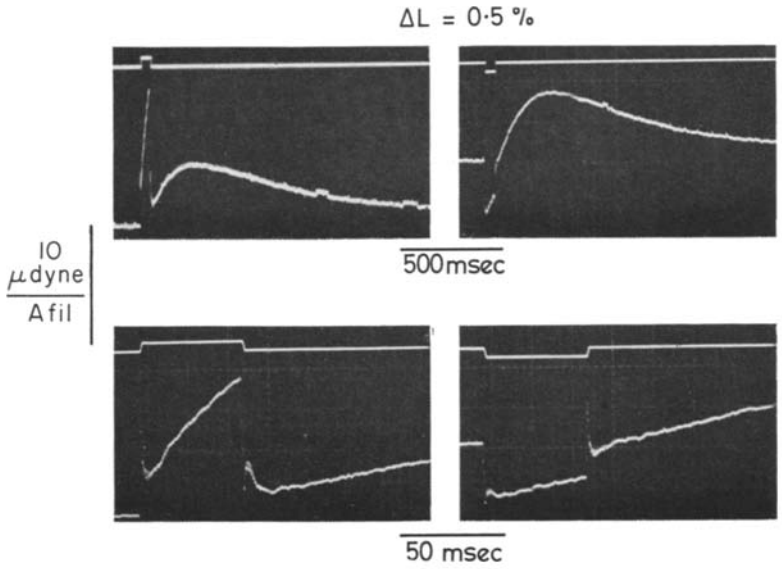


FIGURE 3 *b*. Phosphate starvation transients as in Fig. 3 *a*. The response of the muscle in nonphosphate activating solution to upward and downward length pulses (0.5%, duration 50 msec) at two different time-base velocities is shown.

milliseconds with P_i present. As step amplitude is increased the P_i response exhibits several characteristic nonlinearities; the initial transient becomes larger for the step down than for the step up, and the rate of attainment of equilibrium following the step up increases with step amplitude. Moreover, the approach to equilibrium following the step down becomes quite complicated.

The non-P_i step responses are rather well described as P_i responses with the PST's discussed above superimposed upon them. However, the generalization that for large amplitudes the step-down initial transient exceeds the step-up transient, which we have not seen violated in P_i, is often violated in non-P_i solutions, as Fig. 5 *a* shows clearly.

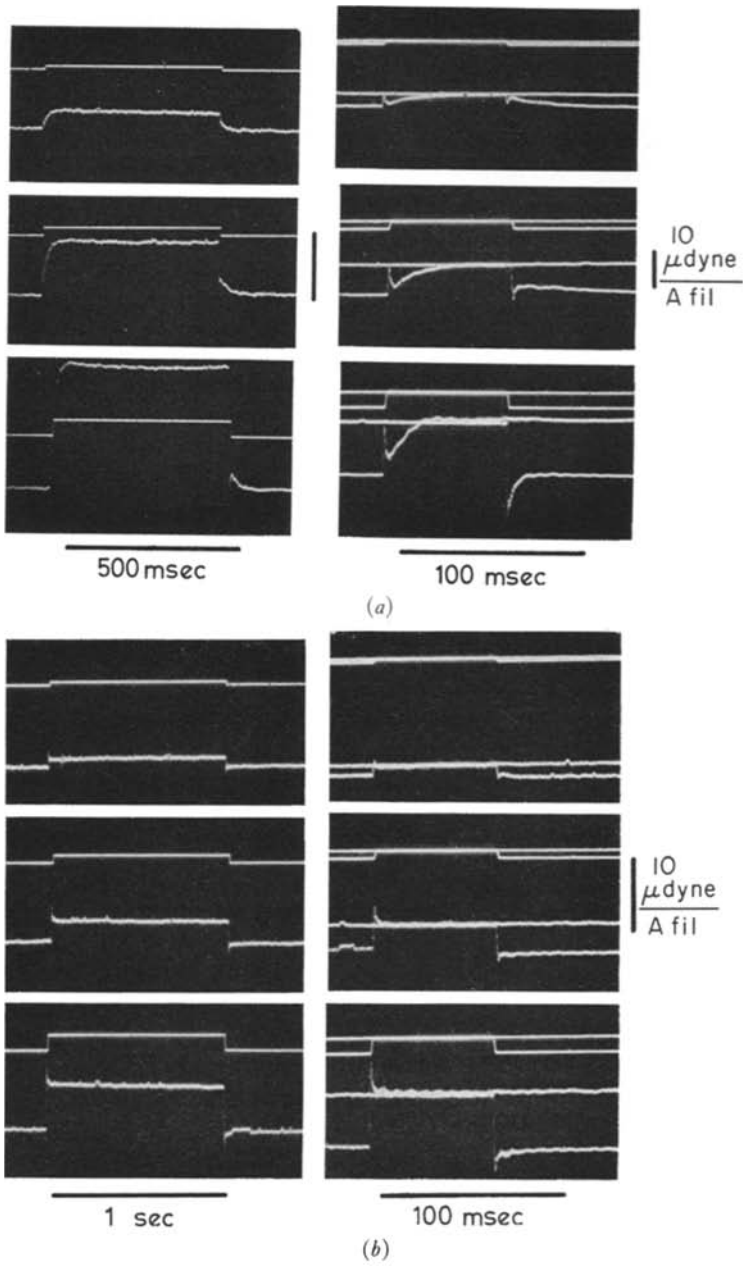
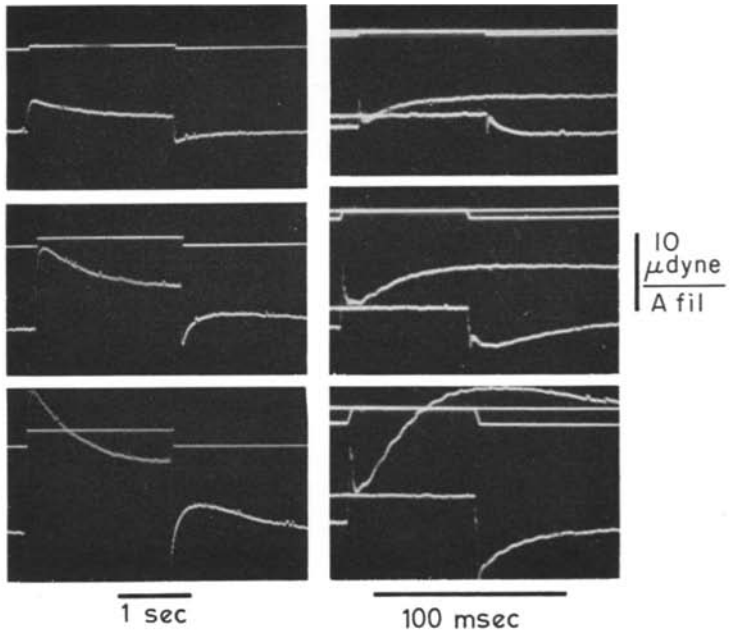
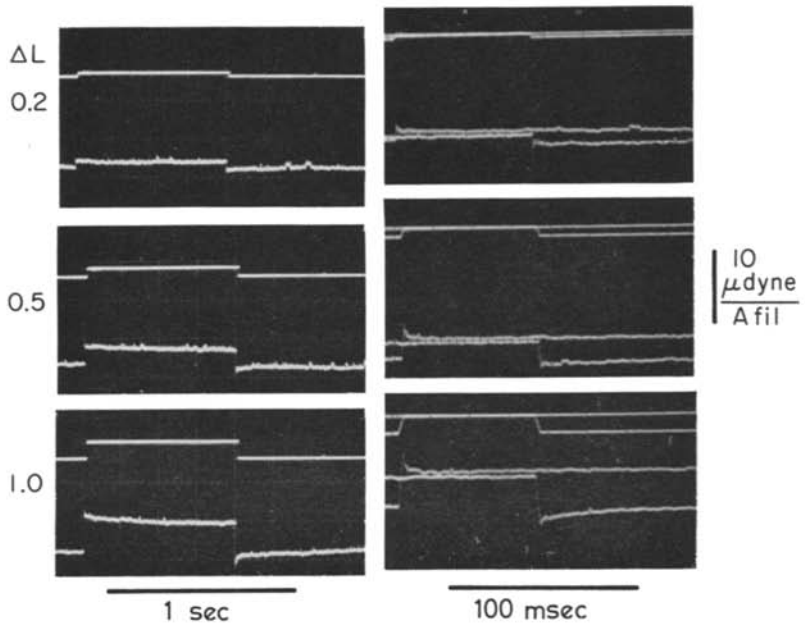


FIGURE 4. Tension wave forms in response to step changes of length of 0.2, 0.5, and 1.0% in phosphate activating (*a*) and relaxing (*b*) solutions. The two sets of records for each solution are given at different sweep speeds to show both the rapid and the slow events. The "steady" tension change in the responses of (*a*) and (*b*) correspond to the solid symbols of Fig. 1. Solutions contained 10 mM phosphate and 30 mM KCl, otherwise they are as in Table II. Double traces in the fast-sweep response are explained in the text. Vertical bar: 10 μdyne/A filament.



(*a*)



(*b*)

FIGURE 5. Step responses, measured as in Fig. 4, in nonphosphate solutions. KCl concentration was 20 mM, otherwise as Table II. Response amplitude has fallen between the slow and fast recordings at 0.5%, but the early portion of the wave form is typical.

Ramp durations in Figs. 3, 4, and 5 are 1 msec. The heights of the initial transients vary if one changes the duration of the ramp in the range 0.5–2 msec. This is of importance to our interpretation of these transients below, since the result suggests that the filaments undergo some relative motion during the 1-msec ramps.

Isometric Oscillations

As reported by Schädler et al. (1969), very large amplitude steps of strain produce an oscillatory response in the tension record. These they termed isometric oscillations. Fig. 6 illustrates such responses to a 2% step of strain obtained in P_i-activating solution at two different temperatures, the tension oscillating at about 11.5 and 19.5 Hz, respectively. These oscillatory responses occur in both P_i and non-P_i activating solutions. In the non-P_i solutions the oscillations are superimposed upon the phosphate-starvation transient.

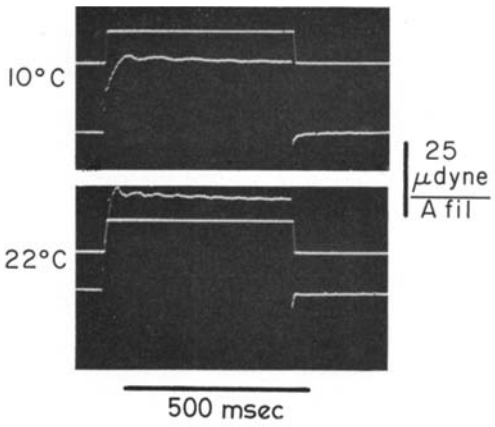


FIGURE 6. Isometric oscillations of tension. Step responses were measured in phosphate activating solution for 2% changes of length at 10° and 22°C. The solutions of Table II were used. Vertical bar: 25 μdyne/A filament.

We have observed the isometric oscillations in both the presence and absence of myokinase and in the presence and absence of a creatine phopshate/creatine phosphokinase ATP-regenerating system. This result suggests that the oscillations are not critically dependent upon an oscillation of local ADP or ATP availability at sites associated with actin-myosin interaction.

Response to Sinusoidally Varying Length Changes

In Fig. 7 *a* the frequency response of the muscle is compared in the P_i and the non-P_i activating and relaxing solutions under low-amplitude (0.2% peak to peak) sinusoidal length changes. The results are plotted in the form of a Nyquist plot.

In contrast to the gross effect of phosphate starvation on the large-amplitude response, the small-signal plots of Fig. 7 in P_i and non-P_i solutions show comparatively little difference in shape. There is some phase lead and attenuation of gain at very low frequencies in P_i starvation. At these low amplitudes the power output available from the muscle is reduced by P_i. This differs from the result obtained at higher amplitudes (see below). Notice, however, that the large-amplitude asymmetric nonlinearity (positive tension transient following both step up and step down of muscle length) tends to disappear as length changes are made very small; the 0.2% step response of Fig. 5 *a* shows that the overshoot (which we have termed the PST)

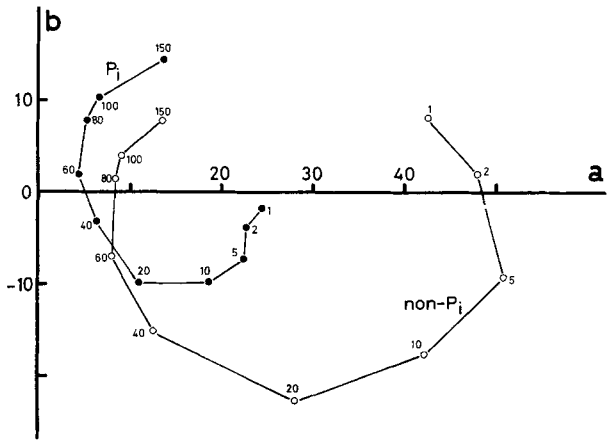


FIGURE 7 *a*. Comparison of the sinusoidal response of one set of fibers in phosphate and nonphosphate activating solutions (Table II). The amplitude of length input was 0.2% peak to peak. The data are presented in the form of a Nyquist plot, the measurement frequencies, read clockwise, being 1, 2, 5, 10, 20, 40, 60, 80, 100, and 150 Hz for both curves. Unit divisions on both the real and imaginary axes are in microdynes/A filament-per cent strain.

is matched by an undershoot on the step down. Thus P_i does affect the small-signal response, but in a manner perhaps not noticed previously since frequencies below 1 Hz have not usually been employed; the decay of a PST which takes several seconds corresponds to the phase lead at the low frequencies (due to a "zero" of the small-signal transfer function at a few hundredths of a cycle per second; see, e.g., Machin [1964]). It is not necessarily implied that the remaining small-signal dynamics are attributable to a simple first-order process, as careless interpretation of Fig. 7 might allow. In fact the overshoot and undershoot following the steps up and down in Fig. 5 *a* appear to have different shapes.

Results of low-amplitude experiments using three different ionic strengths are presented in Fig. 7 *b* to illustrate the strong effect of ionic strength upon

the active muscle. The low-frequency active tension increases greatly in amplitude as the ionic strength decreases. If the ionic strength is reduced below the values shown, the fibers enter the "high-tension state" (Jewell and Rüegg, 1966). This occurs at a higher value of ionic strength in P_i than in

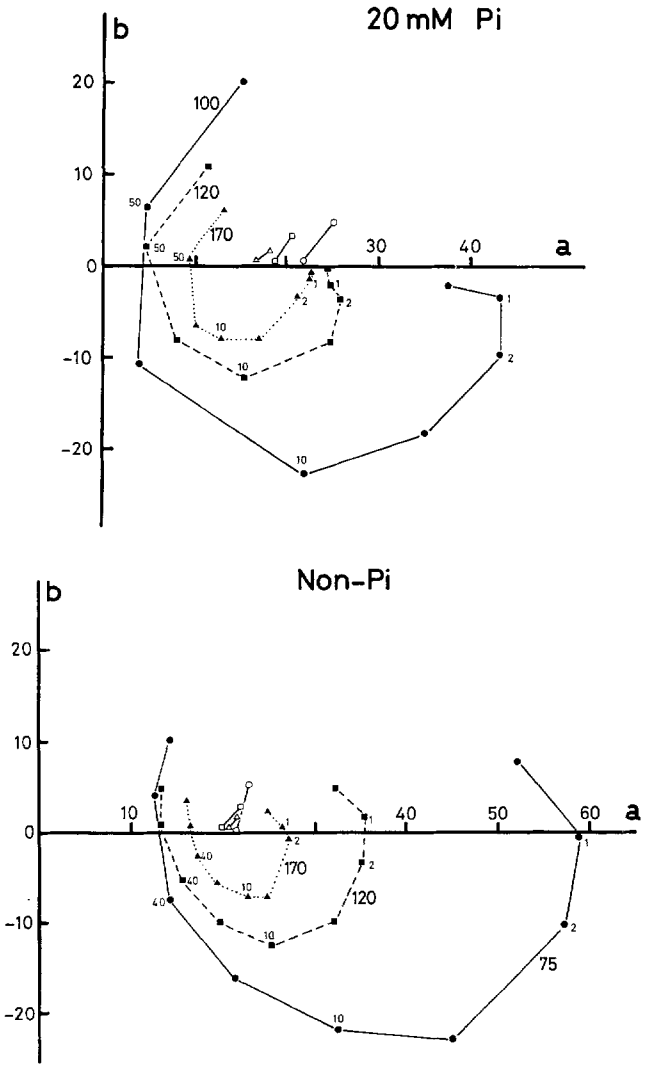


FIGURE 7 *b*. The effect of ionic strength (values are in millimoles per liter) on the sinusoidal response in phosphate and nonphosphate activating (closed symbols) and relaxing (open symbols) solutions. The ionic strength was altered by changing the KCl concentrations of Table II. The amplitude of length input was 0.2% peak to peak. The phosphate and nonphosphate data are from different sets of fibers. Measurement frequencies: P_i solutions, 0.5, 1, 2, 5, 10, 20, 50, and 100 Hz; non-P_i solutions, 0.5, 1, 2, 5, 10, 20, 40, 80, and 120 Hz. For the relaxing solution data only the extreme values are plotted. Unit divisions are as in Fig. 7 *a*.

non-P_i solutions. The effect of ionic strength in the step response takes the form of a change in the amplitude of the delayed tension. In this muscle as in vertebrate striated muscle, the ATPase activity of the isolated actomyosin gel decreases as the ionic strength increases (Maruyama and Pringle, 1967).

In Fig. 8 the large-amplitude sinusoidal responses in P_i and non-P_i solutions are compared. The tracings adjacent to the photographic record are included to indicate the direction of movement of the instantaneous length-tension locus about the loop. The spots associated with each record were obtained both before and after the sinusoidal response at that frequency was

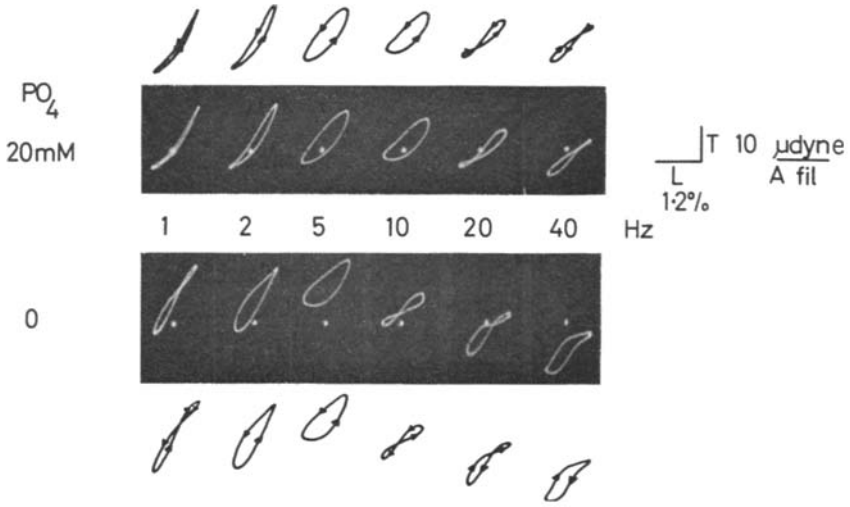


FIGURE 8. High-amplitude (nonlinear) sinusoidal response in phosphate (20 mM P_i) and nonphosphate (20 mM histidine) solutions (Table II) at various applied frequencies, plotted as length-tension loops. Amplitude of applied length was 1.2% peak to peak. The tracings adjacent to the photographic record indicate the direction of movement of the instantaneous length-tension locus. The spots associated with each record indicate the tension of the fibers in the absence of oscillation, and were obtained by exposure of the film both before and after the oscillatory response was recorded.

determined and indicate the tension in the fiber in the absence of oscillation. The loops collapse in the absence of P_i at frequencies near 10 Hz. When P_i is present work is obtained at frequencies above 20 Hz. In the non-P_i solutions the loops are strongly displaced from the equilibrium tension in the absence of any oscillation. If the fibers are producing positive work (i.e., the loop is anticlockwise) then the displacement is towards greater tensions, whereas if the work produced by the fibers is negative then the displacement is towards lower tensions. This effect is less marked, though still present, in the P_i solutions. There is also a much greater tendency for the non-P_i solutions to cause open figure-eight loops (as, e.g., in the 10 Hz record of the non-P_i response); these often cannot be observed at all in the P_i solutions, although

in Fig. 8 the P_i response does form a figure eight before collapsing near 40 Hz.

Fig. 9 illustrates, for the non-P_i solution, the time-course of tension and length at 5 Hz, showing the transient effects as the oscillation is switched on and off. Note how quickly (in fact during the first cycle) the average value of the oscillating tension has increased.

Rüegg et al. (1971) have also investigated effects of P_i. They report an increase in the frequency of maximum work per cycle using sinusoidal length changes with amplitudes in the range 0.5–2%, and also a reduction in the contractile tension in the presence of P_i. With 5 mM P_i they could detect no change in the ATPase activity of their fibers from that in the absence of P_i. The only overlap with our work is the effect of P_i on the response to large-amplitude sinusoidal length changes. However, an implication of their paper is that P_i increases the frequency of maximum work even at very low amplitudes. R. H. Abbott (personal communication, 1972) has observed

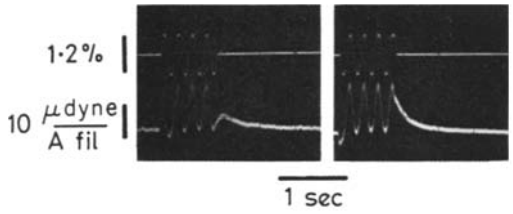


FIGURE 9. High-amplitude sinusoidal responses at 5 Hz in nonphosphate solution of Table II. Length (upper trace) and tension (lower trace) were recorded separately as functions of time.

this effect at 0.2%. This is contrary to our observations at the low amplitudes; it is possible that P_i has two effects—a change in the basic rate constants as well as a reduction in the phenomenon responsible for the PST—and that the magnitudes of the two effects are variable.

Opponent Effects of ATP and ADP upon the Rate Constants

In the course of the controls reported above, in which damped isometric oscillations of tension were observed in the presence and absence of either myokinase or a rephosphorylating system, we found that the frequency of these oscillations was correlated with the ATP concentration used. This effect of ATP, from 5 to 20 mM, is illustrated in Fig. 10. Moreover, use of solutions with increased ADP usually led to somewhat reduced frequencies.

If the isometric oscillation frequency were a measure of the rate at which cross-bridge force (and hence cross-bridge attachments) can change, then other such measures might be expected to obey similar rules. Under *driven* oscillations, where length is forced sinusoidally and tension observed, the activated frequency response may reflect these rates (Thorson and White,

1969). Indeed, Abbott (1968) found that the activated frequency response exhibited a slowing of the primary rate constant (under a first-order transfer-function description) as ADP concentration was increased. Estimates of the rate constant for delayed tension in his data vs. ADP concentration are also plotted in Fig. 10.

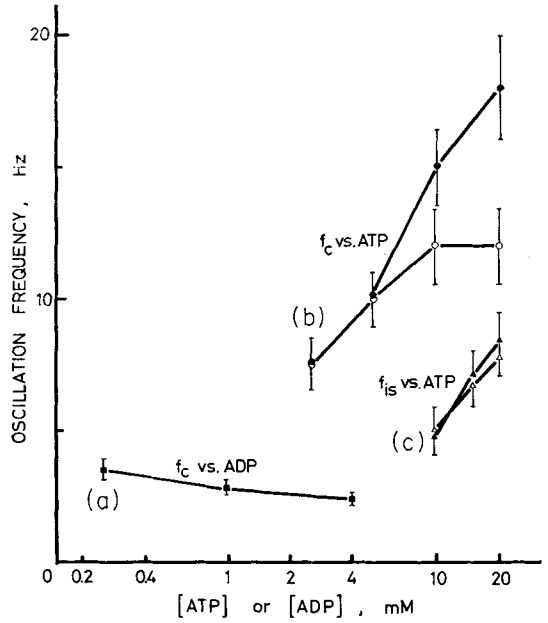


FIGURE 10. Variation of the frequency of maximum negative viscous modulus (f_c) vs. ADP and ATP, and isometric oscillation frequency (f_{is}) vs. ATP. Conditions: (*a*) f_c vs. ADP; 5 mM $MgCl_2$, 5 mM ATP, 4 mM EGTA, 50 mM KCl, 20 mM histidine (pH 6.9), 10 mM sodium azide, 3 mM $CaCl_2$ (original data from Abbott, 1968). (*b*) f_c vs. ATP: $MgCl_2$ and ATP in equimolar concentrations, 20 mM phosphate, and KCl adjusted to maintain the ionic strength at about 150 mM. (*c*) f_{is} vs. ATP: 12 mM $MgCl_2$, 10 mM histidine, 30 mM KCl in all solutions; 20°C. The ATP concentration was varied. In (*b*) and (*c*) open and closed symbols denote separate experiments. f_c is related approximately to the rate constant α for the exponentially delayed tension by $\alpha = 2\pi f_c$. Vertical bars denote the range of uncertainty in estimation of the frequencies from Nyquist and tension plots. The lower frequencies in Abbott's (1968) experiments are probably due to the use of fibers that had been glycerol-extracted a few months before use.

In further support of the above generalization (that ADP reduces, and ATP increases, the rate at which tension can change), Steiger and Rüegg (1969) reported that fibrillar muscle is capable of large-signal work at frequencies of the order of 25 Hz if ATP concentrations as large as 15 mM are provided, whereas ATP concentrations of 5 mM result in reduced high-frequency performance. Although our results show that such high ATP concentrations are unnecessary for high-frequency work if one avoids ortho-

phosphate starvation, the ATP dependence of Steiger and Rüegg is compatible with the generalization we suggest here. We have therefore measured the small-signal activated frequency response over a range of ATP concentrations. As shown in Fig. 10, the rate constant of a first-order low-pass filter approximating the data increases monotonically with ATP concentration from 2.5 to 20 mM. Although ionic strength was not controlled by the computations described in Materials and Methods for the experiment of Fig. 10, we did compensate approximately with KCl and estimate, using Perrin and Sayce's (1967) program, that ionic strength did not vary by more than 10% from 150 mM. As the frequency-response plots of Fig. 7 in solutions of varying ionic strength show, such a change would not measurably affect the small-signal rate constant.

DISCUSSION

Phosphate Starvation

We have shown that free orthophosphate ions have a critical effect upon the mechanical performance of glycerinated fibrillar flight muscle. At 18°C, with driven sinusoidal length changes of 1% peak to peak, the frequency of maximal work production is about doubled by the addition of 10 mM P_i.

The phenomenon is clearer at high amplitudes of stretch than with small stretches. For example, Jewell and Rüegg's (1966) Fig. 10 was obtained with a length change of about 0.07% strain, under which conditions the effect ought to have been very small. Comparison with our Fig. 5 *a* suggests that their record would not demonstrate this effect, and it is possible that they missed the slight reduction in tension that would have occurred after a second or more.

Sacktor and Hurlbut's (1966) demonstration of about 8 mM total PO_4 content in fly fibrillar flight muscle, although not proof that similar *free* P_i concentrations are present in either the fly or in our muscle, suggests that we have found in P_i a physiological requisite for normal work production by the muscle.

Surprisingly, the means by which P_i starvation destroys the capacity for high-frequency chemomechanical energy conversion is apparently *not* via the slowing of a limiting rate constant for stretch-induced delayed tension. In fact, in small-signal analyses we do not find correlation of this rate constant with P_i concentration. Rather, the means of destruction of the capacity for large-amplitude work is the introduction of second harmonic in tension in response to sinusoidally driven length—i.e., figures of eight (see Fig. 8) develop at rather low frequencies if P_i starvation is not avoided. Correlates of this effect in our step-response experiments are the striking phosphate-starvation transients (PST's) seen following 0.5% or larger step changes of length. The fundamental requirement of work production, in mechanical

terms, is a negative viscosity—delayed tension changes following length changes, in an identical direction. Phosphate starvation, however, is associated with peculiar delayed tension transients which are positive in response to length changes of either sign.

Earlier studies of the large-amplitude performance of glycerol-extracted fibrillar muscle have drawn attention to the problem that this preparation may be unrepresentative of the operation of the living muscle. Pringle and Tregear (1969), using 5 mM ATP, no P_i, histidine, or tris(hydroxymethyl)-aminomethane (Tris) buffer, and generally no myokinase, found that work production became negative due to figure-eight effects at frequencies not much above 10 Hz. The natural flight frequency is of the order of 25 Hz. Steiger and Rüegg (1969), on the other hand, using histidine pH buffer and myokinase but no P_i, showed that work per cycle remains positive to frequencies of the order of the flight frequency if the ATP concentration is made high (15 mM) and Ca^{++} concentrations of 1 mM or larger are used. The myokinase system, of course, buffers against ADP buildup and the associated slowing of the rate constants which ADP would produce.

Our result, showing that high-frequency work is obtainable at 18°C with 0.32 μM Ca^{++}, 10 mM ATP, and no myokinase as long as one avoids P_i starvation, further alleviates the worry that glycerol-extracted muscle may be a poor mechanical model. If it could be shown conclusively that there are in fact a few millimoles per liter of free P_i in the live sarcoplasm, the situation would be clarified. Meanwhile, we conclude that studies of fibrillar muscle ought to employ at least 5 mM P_i, even if only for comparison's sake.

Quite aside from the above comparative problem, the effects of P_i starvation offer potential clues concerning the contractile mechanism. Why should it be that removal of P_i permits large positive transients of tension following length changes of either sign? Our hypotheses fall into two classes: first, the fact that any rapid change of length induces a PST suggests that a "stirring effect" may be operating. One must then imagine that Ca binding, troponin disinhibition, or another requisite for the activation of contractile force is facilitated momentarily by change of length of the muscle in either direction. Local interfilament motion might relieve a diffusion barrier or alter critical steric relationships. Moreover, this postulated release of extra activation must be such that it does not occur when 5 mM P_i is present.

A second class of hypotheses is phrased in terms of the cross-bridge cycle of attachments and detachments (see, e.g., Huxley, 1957; Thorson and White, 1969). Extra force arises in the isometric case if the cross-bridge detachment rate is reduced, since then the equilibrium is biased toward attachments. Following an increase of length, stretch activation may increase attachment rate (Thorson and White, 1969). If detachment rate were then momentarily reduced or attachment rate momentarily further increased in the absence of P_i, a PST-like overshoot of tension would result. The reason that this view is

interesting is that following the splitting of ATP, both ADP and P_i must at some point in the cycle be released by the relevant site or sites. If the release of P_i is reversible, the rate of this step could be reduced by the presence of P_i. However, it is not at all clear that the PST following a decrease in length can be explained similarly. In any event, both of the above lines of reasoning suggest further study of the P_i-starvation phenomenon—particularly as to its specificity for the PO_4 ion.

Opponent Effects of ADP and ATP upon the Cross-Bridge Cycle

In our earlier paper we suggested a possible mechanism for the working of insect flight muscle based upon a two-state contractile cycle of cross-bridge activity in which the bridges were either attached to the I filaments and generating tension, or detached and exerting no tension. We assumed that changes of strain in the thick filaments produced proportional changes in the probability per unit time of attachment (p_a). The sum of the rate constants p_a (for attachment) and p_d (for detachment) then determines the over-all rate constant for the delay of tension following length changes. We tested this model against the experimentally determined dependence of ATP hydrolysis upon strain of the muscle fibers by assuming that one ATP molecule was hydrolyzed during each mechanical cycle, using parameters evaluated from the mechanical data. The quantitative agreement obtained encouraged us to ask more detailed questions of the link between the hydrolysis of ATP and the cross-bridge cycle. The data of Fig. 10 show correlations between the mechanical rate constants and ATP and ADP concentrations.

Since in the mechanical cycle of attachment and detachment ($p_a + p_d$)—in the small-signal case at low strains, approximately equal to p_d—is the rate constant limiting tension changes, and since ADP reduces this rate, the sequence of events outlined in Fig. 11 is suggested. If the states to the right-hand side of the dotted line of Fig. 11 corresponded to bridge attachment and concomitant shear-force generation, then increased availability of ADP could favor the back reaction between AM-ADP and AM, possibly reducing net detachment rate (see White, 1972). This restriction is stronger than might appear at first sight. For example, note that the mechanical data of Fig. 10 would *not* be explained in terms of such p_d control if ADP were to come off before the cross-bridge attains the state in which it is both attached *and* pulling, for then the availability of ADP would not have the effect of stabilizing shear-producing attachments and, in certian cases, reducing detachment rate.

Similarly, p_d would be increased if ATP binding were associated with the detachment process as shown in Fig. 11. This idea is then compatible with the increased mechanical rate constant observed as ATP concentration is increased. Such multiple-state cycles require special analysis (White, 1972).

A superficial investigation of the cycle would suggest that if ADP causes a

reduction in p_d then the rate of hydrolysis of ATP, which is equal to the average cycling rate $p_a p_d/(p_a + p_d)$, should be inhibited by ADP. Experimentally the opposite is sometimes found—ADP can cause activation of the ATPase activity of fiber bundles (Abbott and Mannherz, 1970; Maruyama and Pringle, 1967). However, if increase of filament strain causes an increase

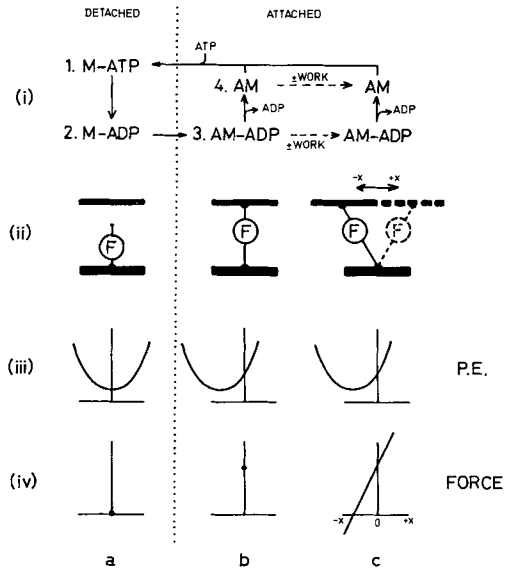


FIGURE 11. The indicated correspondence between the biochemical and mechanical events comprising a cross-bridge cycle. The evidence suggesting this view is discussed in the text. (*i*) Biochemical events relating myosin (M) and actomyosin (AM). (*ii*) Schematic structural states of a cross-bridge, (*ii*)*a* representing the detached state, (*ii*)*b* the attached state at the conformation of attachment, and (*ii*)*c* attached states after filament sliding. (*iii*) configuration potential energy vs. distortion of a cross-bridge, showing the net displacement of the equilibrium position which corresponds to the "detached ↔ attached-and-pulling" transition. (*iv*) the corresponding local cross-bridge interfilament shear force. Two attached states (3 and 4) are included to represent the notion (see text) that although attachment may remain possible after the release of ADP, interfilament shear force ought to occur prior to that event. The term "distortion" is intentional—although each attached bridge makes a small contribution to shear force, its motion during attachment is "imposed" upon it by the states of the other bridges and the external load.

in p_a, which we have suggested (Thorson and White, 1969) as an hypothesis for strain activation of the muscle, then it is found that a reduction in p_d can be associated with an increased rate of hydrolysis of ATP, over a range of variation of p_d; that is, strain is increased by the increased cross-bridge shear force exerted by the extra cross-bridges which attach as p_d decreases (Thorson and White, in preparation). Therefore both the ATPase and tension depend-

encies upon ADP concentration are qualitatively compatible with the implication of Fig. 11 that ADP ought to be released after, rather than before, the bridge begins to produce interfilament shear force.

The in vitro experiments most comparable with the considerations of Fig. 11 are those by Taylor and his coworkers (Finlayson and Taylor, 1969; Finlayson et al., 1969; Lymn and Taylor, 1970; Taylor et al., 1970). Taylor's analysis leads to the view that the splitting of ATP occurs while it is bound to the myosin rather than to the actin-myosin complex, and that the dissociation of ADP and P_i from the myosin-ADP-P_i complex is considerably accelerated by interaction with actin. Thus detached bridges ought to split ATP (our evidence alone, of course, does not preclude the splitting of ATP after the myosin has complexed with the actin), store the resultant energy in a particular configuration, and release it during attachment. Our notion that attached (force-producing) bridges ought initially to have ADP bound to them, that this attachment is stabilized by ADP retention, and that the binding of ATP is associated with detachment is therefore consistent with Taylor's view. It is of course also compatible with the "plasticizing" (relaxing and hence detaching) effect of ATP and, at least qualitatively, with the known effects of ADP as discussed above. A candidate role for P_i in the scheme of Fig. 11 is not obvious because of the complexity of the phosphate phenomenon.

Interpretation of the Mechanical Response

As shown in the superimposed step responses of Fig. 12 *a*, one of the clear asymmetric nonlinearities exhibited by the muscle in phosphate is that the early upward tension transient during the upward ramp of length is smaller than the corresponding downward transient at the end of the step.

Since these early transients were found to vary in magnitude with ramp direction in the region 0.5–2 msec, the transients are not associated simply with elastic components in series with structures which are held totally rigid by viscous forces at these velocities. Therefore some internally viscous structures are changing length during the ramps or there is some interfilament motion. In the latter case, the cross-bridges which are attached at the beginning of the ramps will be distorted and contribute partially to the transient tension change. This contribution will depend upon the way the potential energy of a cross-bridge (and the related shear force) varies with its distortion.

The above asymmetry is then qualitatively interpretable in terms of cross-bridge distortion: prior to the step increase of length a small fraction of the bridges is attached (tension is low); distortion of these bridges produces a particular contribution to the upward tension transient. However, just prior to the step reduction of length, the larger steady tension is associated with a larger fraction of attached bridges; the reduction of length causes these to be distorted in the opposite sense. The greater number of bridges attached at

this instant of time can thus cause an elastic change in tension which is correspondingly greater than that in response to the earlier length increase.

This phenomenon is illustrated in Fig. 12 *b* by a calculation chosen to show the effects of distortion without introducing the complexity of the visco-

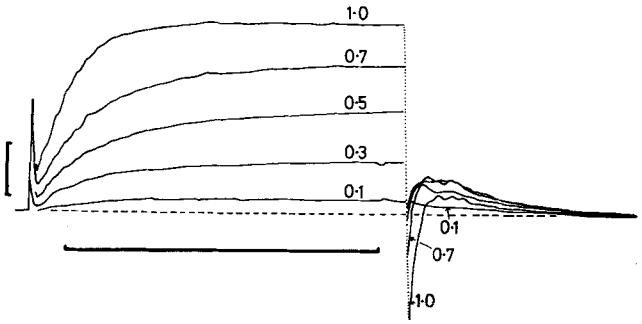


FIGURE 12 *a*. Tracings of step responses in phosphate solution; per cent amplitude of applied step length change is indicated on the figure. Horizontal bar: 200 msec; vertical bar: 10 μdynes/A filament. The phosphate solution of Table II was used.

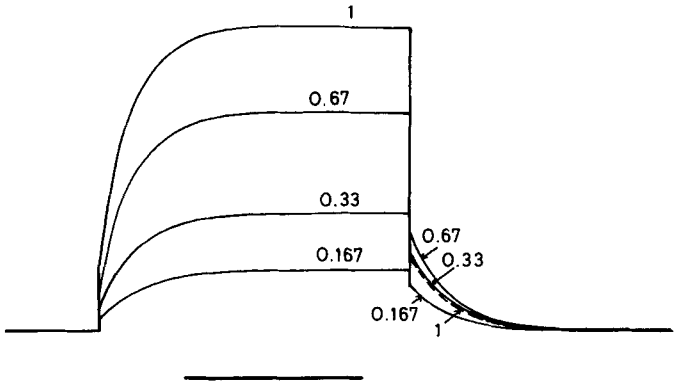


FIGURE 12 *b*. Calculated step responses for the cross-bridge model of Thorson and White (1969) without distributed parameters or viscoelasticity, but with the additional property that the shear force exerted by a cross-bridge varies linearly with its displacement from the orientation at attachment. The calculations and the values of the constants used are described in the Appendix.

elastic sarcomere. In our earlier analysis (Thorson and White, 1969) we were able to ignore the effect of distortion of a cross-bridge upon the shear force exerted during attachment, because only very small distortions occur in the small-signal case. Here the distortions are larger, and we have added the plausible property that the tension exerted by an attached cross-bridge varies, in this case linearly, with its distortion due to interfilament motion. This relationship is shown schematically in Fig. 11. In addition to the development of

the transient asymmetry as step height is increased, the calculation in Fig. 12 *b* reproduces a further feature of the nonlinear response; that is, the delayed rise of tension following the early upward transient is generally more rapid than the corresponding return to equilibrium following the step down. Since this aspect of the response of the model depends largely upon the sum (p_a + p_d), the assumption of stretch control of p_a produces the indicated asymmetry.

The mechanical behavior of the muscle is clearly more complicated than that of the "single bridge with distortion" calculation of Fig. 12 *b*. However, in our study of the full nonlinear equations of a multiple-cross-bridge viscoelastic sarcomere (Thorson and White, in preparation), we find that the two types of asymmetry interpreted in Fig. 12 remain. The full calculation allows one to account first for the detailed dynamics of the passive (i.e., unactivated) muscle and then to examine the effects of bridge distortion on force and detachment rate (p_d) which suffice for the complicated nonmonotonic active waveforms. Once these are simulated, interest attaches to conditions under which the model will exhibit amplitude-dependent isometric oscillations, and to the parameters which affect the model in the way P_i starvation affects the muscle.

Our working hypothesis concerning strain activation (Thorson and White, 1969) specifies some means by which muscle stretch can strain the A filaments. Since Ashhurst (1971) finds no evidence of a structure connecting the A filaments to the Z line in *Lethocerus* flight muscle, we ought to state that evidence of such connections in *Lethocerus* is now available (White, in preparation).

Julian's Interpretation of Fibrillar Muscle

Julian (1969) has investigated the behavior of Huxley's (1957) formulation of the cross-bridge cycle in certain nonconstant-velocity conditions. He showed that Huxley's system in series with a suitable elastic component accounts well for the time-course of development of tension of vertebrate striated muscle in isometric tetanus, for which Jewell and Wilkie (1958) had shown that Hill's (1938) formulation did not suffice. The idea in question was that the steady-state force-velocity curve ought to apply at each instant of a nonconstant-velocity transient, the fallacy of which is illustrated very well by Julian's calculation of the dynamics.

Julian goes on to modify Huxley's model so as to simulate the delayed tensions following stretch of insect fibrillar muscle. He adopts, with us, the notion that stretch changes p_a. However, following Jewell and Rüegg's (1966) suggestion of a delay in Ca^{2+} binding, he postulates an exponential delay limiting the stretch-induced changes of p_a. Appropriately eliminating his series elastic component (fibrillar muscle is very stiff), Julian shows that his model then responds to length steps in a manner much like that of fibrillar muscle.

Julian's implication is therefore that simulation of a delayed increase in

Ca^{2+} binding following stretch is the basis of the delayed tension in his calculation. If this were the case, Ca^{2+} binding would be a distinct alternative to our earlier suggestion (Thorson and White, 1969) that the delayed tension arises because the fraction of cross-bridges attached cannot change at rates beyond those limited by the instantaneous sum of the average attachment and detachment rate constants ($p_a + p_d$), or ($f + g$) in Julian's notation. In fact, it is clear in Julian's Fig. 15 that delayed tension is changing considerably more slowly than is his Ca^{2+}-binding-delayed attachment rate constant. To make this important point clear, we have repeated Julian's calculation both with and without his delayed Ca^{2+} binding. As Fig. 13 shows, most of the delay in tension remains even if Ca^{2+} binding is instantaneous. (The dips following the transient peaks are altered, but they can readily be adjusted by changes in the arbitrary rules governing detachment rate vs. distortion of the bridges.) Therefore, since Julian's values for the parameters f and g were evolved to fit a variety of data, we can only interpret our demonstration (in Fig. 13) that they are appropriate for fibrillar-muscle delays as further support for our original view. The dynamics of Ca^{2+} binding could of course be effective or crucial, but there is as yet no evidence to say so.

Comparison of Figs. 12 and 13 illustrates several important points. Julian has permitted p_d to reach very large values as the attached cross-bridges are distorted during the step change of length. This effect of course causes very rapid transient detachment and simulates very well the observed tension dip following the early tension transient.

Although we agree that p_d might be large at this instant, we have not included the variation of p_d with distortion in our calculations in Fig. 12 for two reasons: first, the resulting agreement with the transients in the data is potentially misleading unless one also includes the powerful viscoelastic properties of fibrillar muscle. These dominate the early transient response of the passive muscle (White, 1967; White and Thorson, 1969; Fig. 4 *b*, this paper) and must affect to some extent the activated response as well. Our observation that the early transient peak varies with ramp rate is also compatible with viscous effects, though changes of detachment rate with distortion as in Julian's model can in principle produce similar phenomena. Hence it seems premature to account for the complicated dynamics entirely via the kinds of cross-bridge distortion and changes of attachments currently considered. Second, the purpose of our calculations has been simply to show the power of distortion-dependent bridge force alone (with p_d constant) in accounting for the asymmetric nonlinearity, and not to fit the transient data to the extent one can do with a model as complex as Julian's. Julian's formulation is of course similarly valuable in that one can study the consequences of bridge-attachment dynamics (with p_d controlled by distortion) in the absence of the complexity of the indicated viscoelastic effects.

Finally, one can locate in Julian's calculation the two phenomena we have illustrated in Fig. 12. The rise of tension following the initial transient and dip is more rapid than the corresponding decay on the step down as our postulated (p_a + p_d) rate limitation predicts. Moreover, the initial transient is smaller following the step up than the step down in our repetition of Julian's

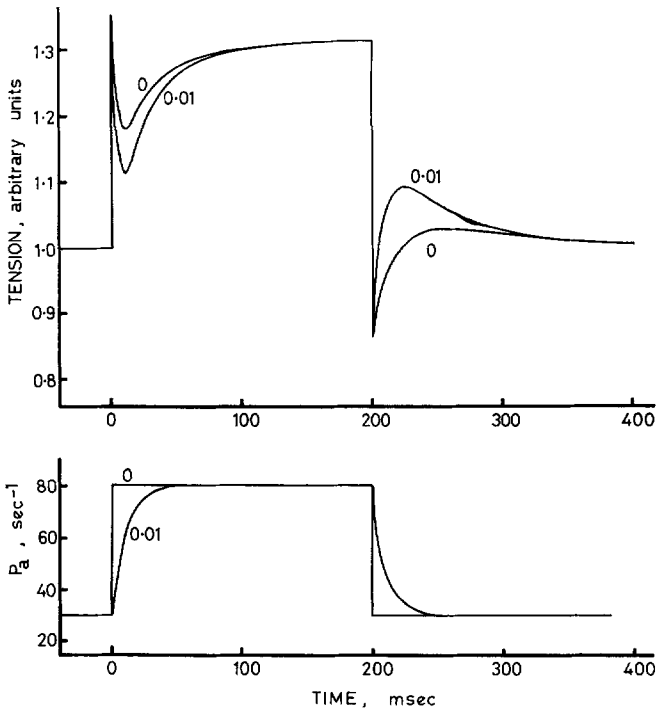


FIGURE 13. The time-course of tension (above) and attachment rate constant (below) as determined via numerical integration of the equations for Julian's (1969) analysis of insect fibrillar flight muscle. Curves labeled 0.01 include Julian's postulate that the stretch-induced change of p_a may lag stretch with an exponential delay of 0.01 sec. The curves labeled 0, for which this delay has been eliminated, show that the delayed tension change remains—its basis is the time taken for the fraction of attached cross-bridges to change, as in our earlier proposal. The wave form resembles, but is incommensurate with, the step response of the muscle, as discussed in the text.

calculation,[1] in agreement both with the asymmetry in the data and with our analysis of the effects of cross-bridge distortion.

SUMMARY

(*a*) The mechanical dynamics of glycerol-extracted insect fibrillar flight muscle, from the giant water bug *Lethocerus cordofanus*, have been investigated

[1] In Julian's (1969) Fig. 15, this relationship between the up and down transients is erroneously reversed (Julian, personal communication, 1972).

with step and sinusoidal length changes in the range 0.2–2%. For rapid length changes less than 0.2% the "stretch activation" underlying work production in flight is manifest as a nearly linear negative viscosity. When length changes greater than 0.2% are applied, several highly informative nonlinearities are superimposed upon the linear response.

(*b*) These nonlinearities include asymmetry both of an early transient peak in tension and of the rate of development of delayed tension. At the largest step changes of length applied, damped isometric oscillations of tension, with temperature-dependent frequencies, occur.

(*c*) We show that marked qualitative differences in the nonlinear behavior of glycerol-extracted flight muscle, reflected in comparisons of our, and previous, measurements, depend upon the presence or absence of millimolar concentrations of orthophosphate ions in the experimental solutions. Phosphate starvation induces a powerful asymmetric nonlinearity wherein any change of length, whether an increase or decrease, causes a transient *increase* of tension. Since provision of 5 mM or more free phosphate ions eliminates this maladaptive property—permitting the muscle to do cyclic work near the normal flight frequency—and since as much as 8 mM orthophosphate has been reported in analysis of live fibrillar muscle, free phosphate may be an essential factor in the living muscle.

(*d*) The dependence of the mechanical performance upon ionic strength T is shown to require careful control of the contents of the experimental solutions. We describe a method for keeping Γ constant while taking account of the calculated concentrations of 37 different complex species.

(*e*) In a variety of mechanical situations, increased ATP concentration increases the rate at which tension can change and increased ADP concentration reduces this rate. We demonstrate that this generalization, viewed in terms of the sequence of events comprising the cross-bridge cycle, can place stringent restrictions upon the particular phases of the cycle at which ATP is bound and at which ADP is set free.

(*f*) Adding to our earlier model of the cross-bridge cycle (derived from the small-signal mechanics) the plausible assumption that shear force due to attached cross-bridges varies when interfilament motion becomes appreciable, we show that two of the above asymmetric nonlinearities are predicted qualitatively. Finally, we repeat Julian's (1969) calculation of the dynamics of insect fibrillar muscle in order to show that the basic work-producing property in his model arises not from his postulated delay in Ca^{++} binding, but rather in accordance with attachment and detachment rates as in our earlier proposal.

APPENDIX

The mathematical description of cross-bridge dynamics used to obtain the theoretical responses of Fig. 12 *b* is based upon the model of Thorson and White (1969). In that paper we considered only the response of the muscle to small applied length changes,

and made the assumption that the shear force produced by a cross-bridge was constant during attachment, since relative sliding of the A and I filaments was small with respect to cross-bridge length. Here we remove this restriction. An attached cross-bridge will be distorted (see Fig. 11) due to relative filament sliding, and we describe this distortion by the amount of sliding, x, since attachment occurred. Thus all attaching bridges have zero distortion (i.e., $x = 0$) at attachment. We assume that the shear force exerted by a cross-bridge varies linearly with distortion, as shown in Fig. 11, (*iv*), *c*, and also that all detached bridges have equal probability of attachment, with $x = 0$.

Muscle tension is equated to bridge shear force, and we take muscle length (L) to represent the strain which controls the probability per unit time of attachment (p_a). We assume that p_a is directly proportional to L, the constant of proportionality (Q) being an activation coefficient.

$$p_a = Q \cdot L.$$

If $n(x, t)$ is the fraction of all cross-bridges which is attached and has distortion x at time t, then for constant L

$$\frac{\partial n(0, t)}{\partial t} = Q \cdot L[1 - n(0, t)] - p_d \cdot n(0, t)$$

in which p_d is the probability per unit time of attachment, here taken as independent of both distortion and time. Just prior to each step the steady-state attached fraction is

$$n(0, \infty) = \frac{Q \cdot L}{Q \cdot L + p_d}.$$

If a displacement (step up or down) is now applied which produces a distortion x_d at $t = 0$, the time-varying tension is

$$T(t) = k \cdot [n(x_d, t) \cdot (x_d + x_0) + n(0, t) \cdot x_0]$$

in which k is the slope, and x_0 the absolute value of the intercept of the force vs. distortion curve of Fig. 11,

$n(0, t)$ is determined by the differential equation above with $n(0, 0) = 0$

$n(x_d, t)$ is determined by $\frac{\partial n(x_d, t)}{\partial t} = -p_d \cdot n(x_d, t)$ with $n(x_d \cdot 0) = n(0, \infty)$ prior to the step.

The responses illustrated in Fig. 12 *b* correspond to the values $p_d = 20$/sec, $Q = 6$/(sec-% strain), $x_0 = 120$ A, and the steps of strain are 0.167, 0.33, 0.67, and 1 %, producing corresponding values of x_d (1 % corresponds to 120 A); the horizontal bar represents 200 msec.

Tension units are arbitrary, determined by the choice of k. The measured tensions

in Fig. 12 *a*, and counts of cross-bridge density (two pairs per 146 A in a half sarcomere of 1.2 μ; Reedy, 1968) suggest that if x_0 is 120 A, k is about 3×10^{-9} dyne/A of distortion. Interestingly, the corresponding value of configurational potential energy ($\frac{1}{2}kx_0^2$) per cross-bridge at the configuration of attachment is about 6 kcal/mole, on the order of estimates for the cell's energy of hydrolysis of ATP.

It should be clear that this calculation is designed to demonstrate (see Fig. 12) that the cross-bridge cycle including only bridge distortion can account qualitatively for two of the chief asymmetric nonlinearities measured. To make this point, we have intentionally left out the probable dependence of p_a on x and the viscoelastic effects, both of which are powerful when taken in conjunction with our hypothesis of control of p_a through strain. Too many nonlinearities spoil the intuition, if they're all stirred in at once.

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