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Energetics and "Efficiency" in the Isolated Contractile Machinery of an Insect Fibrillar Muscle at Various Frequencies of Oscillation*

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Zusammenfassung. Die in Form glycerin-extrahierter Fasern isolierte contractile Maschine des dorso-longitudinalen Muskels von *Lethocerus maximus* leistet oscillatorische Arbeit bei sinusoidaler Dehnung und Entdehnung in einem ATP-haltigen Medium und bei Frequenzen größer als 0,5 Hz. Eine Erhöhung der Frequenz erhöht die ATPase-Aktivität (oszillationsinduzierte oder dynamische Extra-ATPase) und die mechanische Leistung; eine Erhöhung über 4 Hz führte jedoch in früheren Experimenten oft zu einem totalen Verlust der Leistungsfähigkeit und der Extra ATPase und zur Zunahme der contractilen Spannung (High-tension state), vor allem dann, wenn die freie Calciumkonzentration sehr hoch war (10^{-5} M), bei Temperaturen über 25 °C oder bei relativ großen Oscillationsamplituden. Indem wir den Fasern bis zu 15 mM Mg-ATP und Myokinase (0.1 mg/ml) zuführten, vermieden wir das Auftreten eines "High-tension state". Dann aber war bei 30 °C und 10^{-5} M Ca^{++} die Rate der Arbeitsleistung und der ATP-Spaltung maximal bei einer Frequenz von 20—25 Hz; (das entspricht der Frequenz des Flügelschlages im lebenden Lethocerus). Bei einer Oscillationsamplitude von 2—3 % der Muskellänge ist dann die mechanische Leistung des Flugmuskels sogar von derselben Größenordnung wie in der lebenden, fliegenden Wasserwanze. Oberhalb der optimalen Frequenz nehmen Leistung und ATPase Aktivität parallel ab und verschwinden bei etwa 60 Hz getriebener Oszillation. Eine Erniedrigung der freien Calciumkonzentration oder der Temperatur erniedrigt die optimale Frequenz. Der Parallelismus in der Frequenzabhängigkeit von Leistung und ATPase Aktivität unter ganz verschiedenen Bedingungen ist eine Konsequenz des ziemlich konstanten Verhältnisses der Arbeitsrate und der ATP-Spaltungsrate, die ja beide von der Aktin-Myosin Interaktionsrate abhängen. Bei hohen freien Calciumkonzentrationen ist dieser Koeffizient allerdings weniger konstant, nämlich etwa 3—4 kcal/mol gespaltenes ATP bei einer Frequenz von 10 Hz, aber nur 1 kcal bei 2 oder 15 Hz.

Schlüsselwörter: Myofibrilläre Oscillation — Myofibrilläre ATPase — molekulare Bioenergetik — glycerinextrahierte Insektenmuskeln.

Summary. Glycerol extracted fibres from the dorsal longitudinal muscle of *Lethocerus maximus* represent the "isolated" contractile machinery and they produce oscillatory power in ATP salt solution when they are driven to oscillate at frequencies greater than 0.5 c/sec by sinusoidal stretch and release. Increasing

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the frequency increases the ATPase activity (oscillation induced or "dynamic" extra ATPase) and the output of power. But in previous experiments, an increase in frequency exceeding about 4 c/sec often leads to a complete loss of power output and to the development of the "high-tension state", especially when the free calcium concentration was high (10^{-5} M), at high temperatures (above 25°C) or at large amplitudes of oscillation. Avoiding the "high-tension state" by supplying the glycerinated fibres with up to 15 mM Mg-ATP or 0.1 mg/ml myokinase we are now able to show that the rate of ATP splitting and the rate of doing work are both optimal when the frequency was 20—25 c/sec (the wingbeat frequency of living Lethocerus) at 10^{-5} M Ca^{++} and 30°C, even at physiological oscillation amplitude (about 2—3% of the fibre length). The power output is then of the same order of magnitude as in live insect flight muscle. Above the optimum frequency, both power output and ATPase activity decrease in a parallel fashion and they vanish completely at about 60 c/sec. Decreasing either the free calcium concentration or the temperature reduces the optimum frequency. The parallelism of the frequency dependence of the ATPase activity and power output under various conditions is a consequence of the rather constant ratio of the rate of doing work and the rate of ATP splitting, both of which depend on the rate of actin-myosin interaction. At high calcium concentration the ratio or the mechano-chemical coefficient is about 3—4 kcal/mole of ATP split (i.e. 50%), at 10 c/sec oscillation but the efficiency is much less at 15 or 2 c/sec, indicating a less tight chemo-mechanical coupling.

Key-Words: Myofibrillar Oscillation — Myofibrillar ATPase — Molecular Bioenergetics — Glycerinated Insect Fibrillar Muscle.

Like living insect flight muscle *in situ* [11] isolated glycerol extracted fibres of the dorsal longitudinal muscle (DLM) of *Lethocerus maximus* suspended in ATP salt solution perform work when they are driven to oscillate by sinusoidal stretch and release at the appropriate frequency (Jewell and Rüegg [8]). In the functionally isolated contractile machinery of these fibres the rate of ATP splitting increases in proportion to the rate of doing work when the latter is increased by augmenting the extent of sinusoidal stretch and release in the range of 1—3% of the muscle length (Rüegg and Tregear [21]; Rüegg and Stumpf [19]). In the present paper the possibility is investigated whether there is a similar coupling between power output and ATPase activity at various frequencies of driven oscillation.

In a preliminary study of the effect of oscillation frequency on the ATPase of glycerinated insect muscle fibres [21] we found an increase of activity with increasing frequency up to about 2—3 c/sec, and under the conditions used the diffusional ATP supply was known not to be rate limiting (see Mannherz [12]). But at higher oscillation frequencies the results were rather erratic, since the fibres entered the "high-tension state" (see [8, 21]) associated with a loss of power output above about 4 c/sec, and at physiological amplitude (2—3% of fibre length) of oscillation. Evidence is accumulating [8, 12] that in the "high-tension state" the diffusional ATP supply is rate-limiting the ATPase

activity and, even more, the power output during driven oscillation, as the work done may be absorbed by an ATP-free fibre core.

Consequently, a reinvestigation of the relationship between power output, ATPase activity and efficiency at various frequencies of driven oscillation required first the establishment of optimal working conditions and, in particular, of an ATP supply which is not rate limiting. It was also advisable to "buffer" the ratio of ATP to ADP with myokinase and to control the ADP level, since ADP may sometimes increase the ATPase activity [14] and tension [5] of fibres.

Methods[1]

The dorsal longitudinal muscle of the tropical waterbug *Lethocerus maximus* was extracted with 50% glycerol, buffered with histidine and stored in the deep freeze as described earlier [8], Fibre bundles cut to a length of about 1 cm and consisting of 4—6 fibres were glued at L_0 to the ends of two vertical glass arms (0.5 cm apart) of the driven oscillation apparatus. One arm was connected to the RCA 5734 force transducer, the other one to a vibrator which generated a sinusoidal movement monitored by field-plates moving in a strong magnetic field. The length changes were displayed on the x axis and the tension changes on the y axis of the screen of a 502 A Tectronix oscilloscope. In some of the experiments the DC tension was continuously recorded during oscillation with a Schwarzer pen-writer. The horizontally mounted fibres were immersed in a perspex bath containing 0.3 ml solution and which could be rocked by means of a spring drive in order to provide gentle stirring. The apparatus and the temperature control system will be described in detail by one of us (G. St. [23]).

Solutions. Relaxation solution: 5 mM ATP, 5 mM Mg Cl_2, 40 mM KCl, 20 mM histidine buffer, 10 mM Na Azide, 4 mM EGTA. activating solution: 5 mM ATP, 5 mM $MgCl_2$, 40 mM KCl, 20 mM histidine buffer, 10 mM Na Azide, Ca-EGTA and EGTA (total EGTA 4 mM) in the appropriate ratio to give the desired level of free calcium (see [8]). In some of the experiments where the ATPase activity was high the concentration of ATP and $MgCl_2$ was increased to 12 mM or 17 mM and myokinase (0.1 mg/ml of the Boehringer preparation having 300 activity units) was added. The pH was adjusted to 6.5 and the temperature was controlled to $\pm$ 0.5 °C by controlling the room temperature, if not stated otherwise.

Analytical. The rate of ATP splitting was determined by measuring the phosphate liberated into the bath according to SCHÄDLERS [22] modification of the Marsh-method. The amount of phosphate release was found to be proportional to the incubation time (RÜEGG and TREGEAR [21]) and independent of the number of fibres in the range of 1—7 fibres (Table 1 in Ref. [20]). Fibre thicknes 80 μ.

The concentrations of ADP and AMP in equilibrium with ATP were determined (after incubation with myokinase and muscle fibres) by using the NADH coupled "optical test" preparation available from Boehringer Mannheim. For determination of the mechanical data (power, "delayed" tension, elastic and viscous modulus) see Fig. 9a.

[1] *List of abbreviations:* DLM, dorsal longitudinal muscle; EGTA, ethylene glycol bis- (β-aminoethyl ether)-N,N′-tetraacetate; ATP, adenosine triphosphate; ADP, adenosine diphosphate; P_i, inorganic phosphate; myokinase, adenylate kinase; L_0, resting length in situ.

Results

1. ATP Supply for Optimal Oscillatory Performance

Extracted dorsal longitudinal muscle fibres suspended in activating solution containing 5 mM ATP and 5 mM $MgCl_2$ and containing small Ca^{++} concentrations (10^{-7} M) are able to oscillate in the frequency range of 0,5 to 30 c/sec when they are sinusoidally stretched and released by 0.1 % of the muscle length (Jewell and Rüegg [8]).

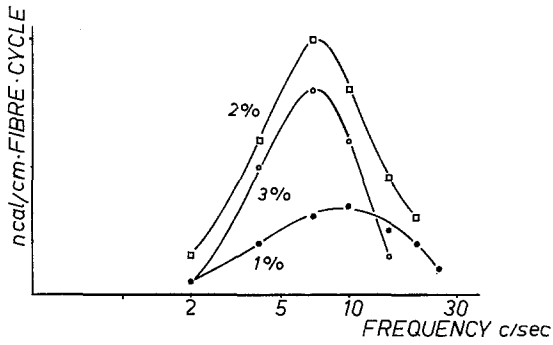


Fig. 1. *Oscillatory frequency optimum: Amplitude dependence.* The dependence of the work per oscillatory cycle on the frequency of driven oscillation at various amplitudes (in % length change peak to peak). The glycerinated fibre bundle was immersed in activating solution (see Methods) containing 10 mM ATP and myokinase, Ca^{++} 10^{-5} M, pH 6.5, $T = 27°C$

However, at larger free calcium concentrations and/or at large amplitude of driven oscillation (e.g. 2 % of the fibre length) sustained oscillation is only possible at frequencies below about 5 c/sec. At greater frequencies the fibres stop doing positive work after a few cycles, the mean tension raises and they enter the "high tension state" [8]. While these experiments are confirmed at low ATP concentration (5 mM Mg-ATP) we now find that addition of myokinase (0.1 mg/ml) or Mg-ATP (total conc. 12 mM or more) abolished the high tension state, and the frequency dependence of power output is then similar to that at smaller amplitudes (Fig. 1).

The decrease in high mean tension ("high tension state") and the restoration of power output by the addition of ATP and $MgCl_2$ (total concentration 12 mM) suggest that the "high tension state" is a diffusional artifact caused by the ATP starvation of fibres (Jewell and Rüegg [8]) rather than by the accumulation of ADP in the fibres (cf. Pringle [16]). This conclusion is supported by the observation that the lowering of the ATP concentration below a critical value causes

the development of high tension [8] and an increase in stiffnes and muscle viscosity associated with a loss of power output [8,12]. Such a critical decrease of the ATP concentration within the fibres suspended in 5 mM ATP-Mg media would indeed be expected to occur after increasing the amplitude and/or the frequency of driven oscillation, since this would lead to an increase in the ATP splitting rate [19] to such an extent that the diffusional ATP supply (Diffusion coefficient $= 3-5\times10^{-7}$ cm^2/sec; see [12]) became rate limiting.

Table 1. *The effect of myokinase on power output and ATPase activity of extracted DLM fibres during driven oscillation (3% length change). Fibres were incubated in activating solution containing 5 mM Mg ATP, 10^{-6} M Ca^{++} and pH 6.5, $T = 21°C$. The fibre bundle oscillated first without myokinase and then (after 30 min incubation with the enzyme) in presence of myokinase*

Oscillation frequency	2 c/sec		4 c/sec	
Myokinase (mg/ml)	0	0.1	0	0.1
Power output (μcal/min × cm fibre)	1.38	1.35	1.0	1.5
Extra ATPase[a]	0.37	0.38	0.42	0.49

[a] Oscillation induced ATPase in nmoles/min × cm fibre. The total ATPase activity was 0.75 (2 c/sec) and 0.8 (4 c/sec).

Addition of myokinase alone (0.1 mg/ml) to the activating solution containing 5 mM MgATP also improved the oscillatory performance (power output) and the oscillation induced ATPase activity at a driven oscillation frequency of 4 c/sec, but not at a frequency of 2 c/sec (Table 1).

At the lower frequency the ATP supply is known [12] not to be rate limiting since the ATP concentration difference between the medium and the fibre is less than 2 mM during isometric contraction and about 4 mM during driven oscillation. The effect of myokinase (at 4 c/sec) shows that this enzyme may constitute an ATP regenerating system under the conditions existing within the muscle fibre.

Without added myokinase, the ADP concentration would be at least 2—3 mM in the center core of the fibre (Mannherz [12]; Rüegg and Tregear [21]). This high ADP concentration ought to be considerably reduced by the myokinase reaction (synthesizing AMP and ATP) as the incubation medium contains only 0.5—1 mM ADP in a myokinase catalysed equilibrium with 5—12 mM ATP. Further evidence for an adequate ATP supply to the fibres in media containing 12 mM Mg ATP and 0.1 mg/ml myokinase is the finding that an increase

in the temperature (Fig. 8) or Ca^{++} concentration (Fig. 6) enhance the power output greatly in spite of the fact that these factors also increase the rate of ATP splitting.

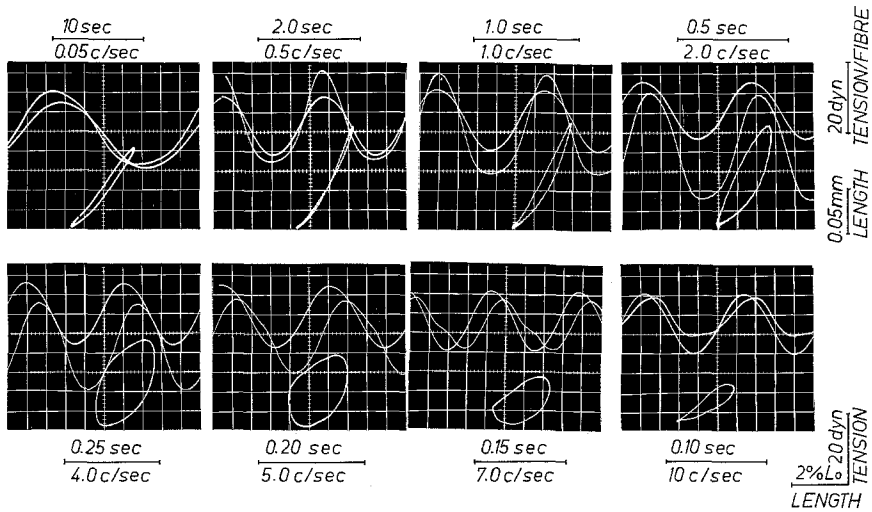


Fig. 2. *Oscillatory tension/length changes.* Length changes (above), tension changes (below) and phase-plane diagram (tension/length diagram) during sinusoidal stretch and release (driven oscillation) at an amplitude of 2% length change (peak to peak) and at the frequencies indicated (as c/sec). Note that tension changes are lagging behind length changes at 1—10 c/sec. The preparation (0.35 cm long bundle of 3 glycerinated fibres from the DLM) was immersed in activating solution containing 10 mM Mg-ATP, 5×10^{-6} M Ca^{++}, pH 6.5, $T = 22°C$

2. *The Frequency Dependence of ATPase Activity in Relation to Power Output during Driven Oscillation at Low Calcium Concentration*

Before the use of ATP regenerating systems the oscillatory performance was found to be more consistent at calcium ion concentrations producing half-maximum activation (about 10^{-6} M at pH 6.5 according to SCHÄDLER [22]) than at 10^{-5} M free calcium. Fig. 2 shows the sinusoidal tension changes and length changes during driven oscillation at various frequencies of stretch and release. Note that there is virtually no phase shift between tension and length changes at 0.5 c/sec. At higher frequencies the tension changes are lagging behind the length changes by a phase angle which increases with increasing frequency up to about 4—5 c/sec. Above this frequency a further frequency increase reduces the phase angle which becomes negligible above about 10—15 c/sec. The work per cycle i.e. the area of the Lissajous figure of

the tension length cycle exhibits a similar frequency dependence while the optimum for power output (i.e. work × frequency) occurs at about 5—6 c/sec.

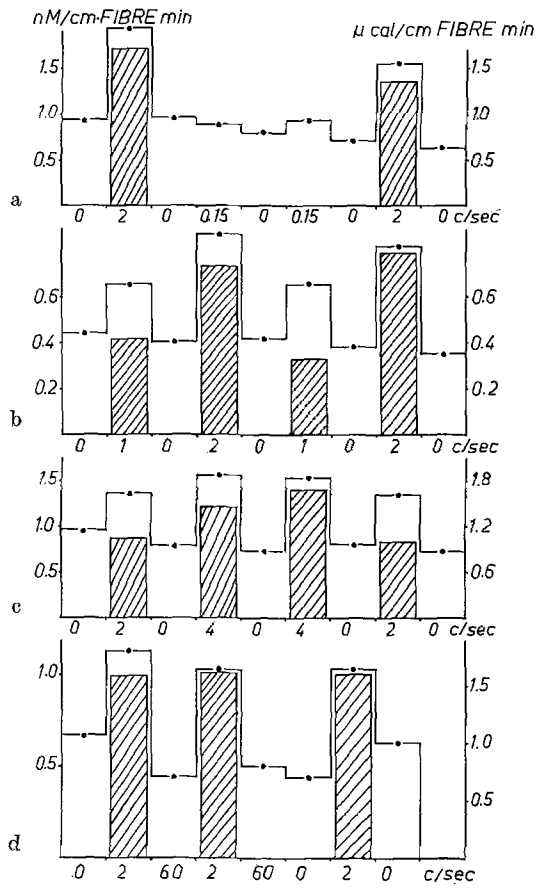


Fig. 3. *Oscillation-induced ATPase (extra ATPase).* The dependence of ATPase activity and power output (shaded columns) of glycerinated fibres (DLM) on the frequency of sinusoidal stretch and release (driven oscillation). ATPase activity in nmoles P_i released in 1 min by 1 cm length of fibre (left ordinate); power output (right ordinate) in microcalories per minute. The preparations were incubated for successive periods of about 20 min duration; incubation periods in which the fibres were stretched and released about a mean length (about 101 % L_0) alternated with periods in which the fibres were kept isometrically at the mean length. The oscillation amplitude was about 2.5 % length change, the reference oscillation frequency was 2 c/sec in all experiments and the test frequency was 0.15 c/sec in Exp. a, 1 c/sec in b, 4 c/sec in c and 60 c/sec in d. Throughout, the incubation medium (20—22 °C) contained activating solution with 10^{-6} M Ca^{++}, pH 6.5

In order to see whether the oscillatory performance, in particular the rate of work output, depended on the rate of energy release, we studied the frequency dependence of the ATPase activity (Fig. 3). The fibres were kept either isometrically at about 101—102 % L_0 in activating

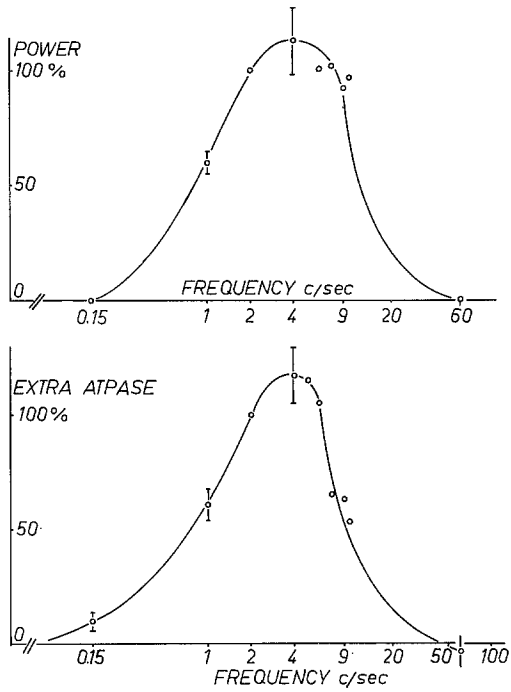


Fig. 4. *Frequency dependence of oscillatory power and extra ATPase at low [Ca^{++}].* The oscillation induced extra ATPase activity (below) and the power output (above) obtained at various frequencies in experiments of the type shown in Fig. 3 are expressed as a percentage of the extra ATPase and the power output obtained at the reference frequency (2 c/sec). The curve shows the means and the S.E. of the mean (of 6 experiments at each frequency). For experimental conditions see Fig. 3

solution or they were sinusoidally stretched and released about that length with a frequency ranging from 0.2 to 60 c/sec, and with a reference frequency of 2 c/sec. Driven oscillation at 2 cycles but not at 0.15 c/sec produced power output and increased the ATPase activity above the activity level during isometric contraction (Fig. 3a). The activity difference is the oscillation induced extra ATPase activity, which at an oscillation frequency of 2 c/sec is larger than at 1 c/sec (Fig. 3b) and smaller than at 4 c/sec (Fig. 3c). At 60 c/sec driven

oscillation produces no power output and no oscillation induced extra ATPase (Fig. 3d). The apparent parallelism in the frequency dependence of power output and extra ATPase activity is more clearly seen in Fig. 4 than in Fig. 3. Here, the extra ATPase activities and the power output obtained at the various frequencies were expressed in per cent of the extra ATPase activity and the power output obtained at the reference frequency of 2 c/sec.

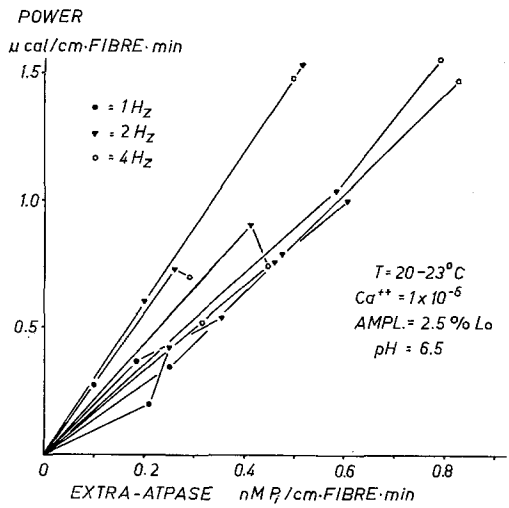


Fig. 5. *Oscillatory extra ATPase of glycerinated fibres in proportion to power output.* Each straigth line shows the relationship of power output and oscillation induced extra ATPase (means) obtained in experiments of the type shown in Fig. 3b and 3c. Oscillation frequencies: • 1 c/sec, ▾ 2 c/sec, ○ 4 c/sec. Experimental conditions as in Fig. 3. Ratio of power/extra ATPase = mechano-chemical coefficient ("efficiency")

In conclusion, we demonstrated the presence of a frequency dependent ATPase activity which is to a certain extent coupled to the output of oscillatory power, as shown by the linear relationship of a plot of power output versus ATPase activity (Fig. 5). When the frequency of driven oscillation is increased from 1 to 2 c/sec or from 2 to 4 c/sec at an amplitude of 2% of the muscle length, the linear ATPase power relationship has a slope indicating a mechano-chemical coefficient of about 2 kcal/mole of ATP split. The decrease of ATPase activity and power output occurring even in presence of myokinase and high (12—15 mM) concentrations of Mg-ATP after increasing the frequency of driven oscillation above about 10 c/sec is a consistent observation (Fig. 4). It is

certainly not due to a diffusional rate limitation of the ATP supply. For if it were, a further increase of ATPase activity by raising the free calcium ion concentration ought to increase the ATP free core in the fibre and it should thus decrease rather than increase the oscillatory work per cycle (see below).

3. The Frequency Dependence of Power Output, ATPase Activity and Efficiency during Driven Oscillation at High Concentrations of Ca^{++}

At high free calcium concentration causing full ATPase activation (about 10^{-5} M at pH 6.5, see Schädler [22]) and with only 5 mM Mg-ATP in the medium we were at first unable to study the oscillatory frequency dependence because the fibres entered the "high tension state" (see [8]). Since this state is due to rate limiting ATP supply, it could be avoided by increasing the concentration of Mg-ATP to 12 mM or by adding myokinase. Then, the frequency optimum for oscillatory power and extra ATPase activity was found to be about 10 c/sec (Fig. 6) which is about twice as large as in the low calcium experiments described above (see section 2). This frequency shift of the optimum is just the opposite of that occurring when the fibres enter the "high-tension state" after raising the free calcium (see Fig. 5 in Jewell and Rüegg) and it may be related to the oberservation that the delayed rise of tension after quick stretch (Rüegg and Stumpf [20]) is much faster at high free calcium concentration than it is at low concentration. The maximum power output and ATPase activity obtained at 10 c/sec driven oscillation were 4.0 μ cal/min and 2.2 nmoles/min in 1 cm fibre. Above this optimal frequency both extra ATPase activity and power output decrease again (Fig. 6). At 60 c/sec, work done on the fibres by stretching and releasing them is aborbed (negative work) and the rate of ATP splitting is then only about 2/3 of the rate under static conditions (negative extra ATPase). The ATPase "inhibiting" effect is associated with a drop in mean tension and it is reversible and repeatable (Fig. 7).

Fig. 6 and Table 2 show also that the chemo-mechanical coefficient (the "efficiency") is about 3—5 kcal/mole ATP split at an oscillation frequency of 10 c/sec, but only 1 kcal/mole ATP at a frequency of 2 cycles or 15 c/sec. Obviously the efficiency is optimal at that frequency where the power output is greatest. The low efficiencies at 2 c/sec cannot be due to a diffusional artifact causing an ATP-free core in the fibre bundle since the rate of ATP splitting is much lower than at 10 c/sec oscillation. Similarly, there appears to be no ATP-free core absorbing work at a driven oscillation of 15 c/sec for, if there were, an increase in temperature to 30 °C leading to a further increase in ATPase activity should

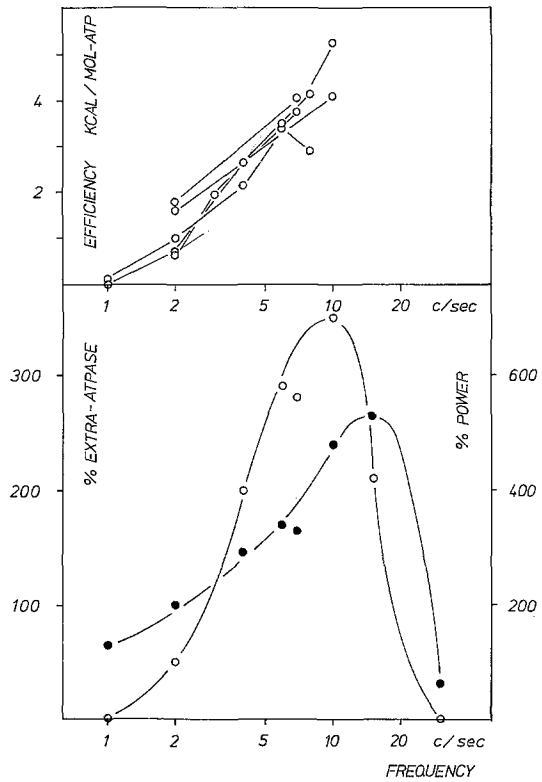


Fig. 6. *Frequency dependence of oscillatory power* (o——o), *extra ATPase* (•——•) *and "efficiency" at high [Ca^{++}]*. above: "efficiency" (ratio of power/extra ATPase) in relation to frequency in "typical" experiments. See also Table 2. Below: With glycerinated DLM, experiments of the type shown in Fig. 3 were carried out in activating solution containing 10^{-5} M Ca^{++}, 12 mM ATP and myokinase (temperature about 23 °C, pH 6.5) at a reference frequency of 2 c/sec and at a test frequency ranging between 1 and 30 c/sec, at an amplitude of about 2.5 % length change peak to peak. The power outputs and extra ATPase activities at various frequencies were expressed as % of the power output and extra ATPase activity at the reference frequency of 2 c/sec; the mean values were plotted as a function of the frequency. They were 240 ± 40 % (ATPase) and 700 ± 135 % (power) at 10 c/sec and 31 ± 22 % (ATPase) and zero power at 30 c/sec.

In 7 experiments the following absolute values were obtained at 10 c/sec:

nmol/min × cm fibre:	0.8	0.63	0.57	0.57	0.5	0.4	0.49
μcal/min × cm fibre:	4.0	2.6	3.0	1.9	1.3	0.96	0.76

increase the size of the ATP-free core and it should thus decrease rather than increase the oscillatory work per cycle. However, the oscillatory work greatly increases (Fig. 8) provided, of course, that the ATP supply is

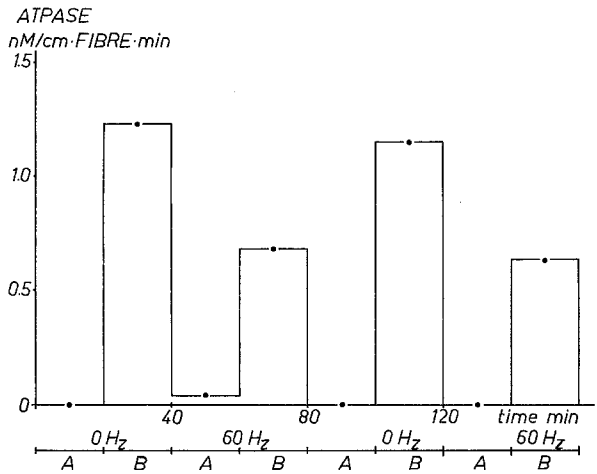


Fig. 7. *"Reduction" of Ca^{++} (10^{-5} M) induced ATPase activity at over-optimal oscillation frequency.* The preparation was incubated for successive periods (20 min duration each) into activating solution containing $\sim 10^{-5}$ M Ca^{++} (each time at B) or into relaxing solution (containing 4 mM EGTA each time at A). Periods during which the pre-stretched preparation was sinusoidally stretched and released about the reference length at 60 c/sec alternated with periods in which it was kept isometrically at the reference length. Note that the ATPase in the relaxing medium is unaffected by the high frequency oscillation, but the calcium dependent contractile ATPase is reduced by about 40% under conditions where the work done *on* the sinusoidally stretched fibres is absorbed. Amplitude of oscillation $\sim 4\%$ of the length, pH 6.5, $T = 20.5°C$

Table 2. *Efficiency decrease above frequency optimum in "high calcium" solution. Incubation in activating solution containing 12 mM Mg—ATP, myokinase and 10^{-5} M free Ca^{++}, pH 6.5, $T = 26°C$. Amplitude 2% length change of the glycerinated fibre bundles*

Experiment	Extra ATPase[a] %	Power[a] %	Efficiency[a] %
I	110	70	64
II	111	63	55

[a] Values at 15 c/sec oscillation expressed in % of the values at the frequency optimum (10 c/sec).

guaranteed by using a fairly large ATP-Mg concentration (15 mM) and myokinase as reconstituting system (see Methods).

The effect of the temperature increase is probably due to the observed decrease in the time delay between quick stretch and delayed tension since this would shift the frequency optimum for the phase angle between

tension and length changes and for the oscillatory work to greater frequencies (Fig. 8). Indeed at 30—35 °C the optimal work/cycle occurs at 15 c/sec and the optimal power output at about 20 c/sec.

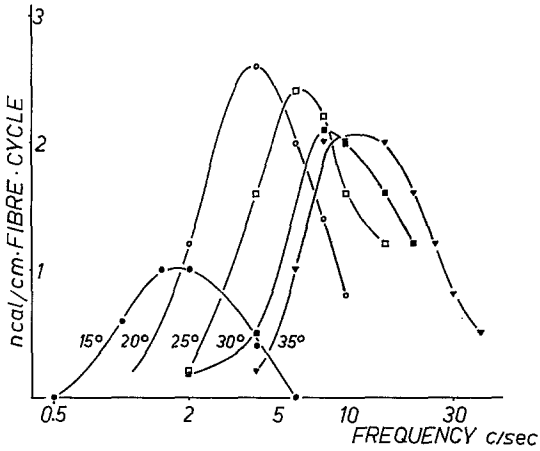


Fig. 8. *Oscillatory frequency optimum: temperature dependence.* The dependence of the work per oscillatory cycle on the frequency of driven oscillation at various temperatures of the activating solution containing 15 mM Mg-ATP and myokinase, pH 6.5, 10^{-5} M Ca^{++}. Amplitude about 2 % length change peak to peak. The power optima are even higher (see text)

4. The Effect of Calcium Ions on Power Output, ATPase Activity and Efficiency during Driven Oscillation

Comparing the frequency dependence of power output and ATPase activity at low and high calcium ion concentrations (cf. sections 2 and 3) it may be deduced that power output and ATPase activity should increase with increasing free calcium concentration at 10 c/sec oscillation but not necessarily at 2 c/sec. In order to study these calcium effects in more detail *each* fibre bundle was exposed to different calcium concentrations at either low or high frequency of driven oscillation. At calcium concentrations of less than 10^{-8} M Ca^{++} driven oscillation produces neither mechanical power nor extra ATPase (see also [19]). Increasing the concentration to about 10^{-6} M at pH 6.5 enhances both the work per cycle and the extra ATPase activity, but any further increase reduces the phase angle between tension and length changes and thus the power output at an oscillation frequency of 2 c/sec, and the chemo-mechanical coupling coefficient decreases from about 1—2 kcal/mole extra ATP split to about 0.5—1 kcal (Table 3).

Table 3. *The effect on increasing the free calcium concentration on power output and oscillation-induced extra ATPase during driven oscillation at 2 c/sec. Incubation medium as in Table 2; oscillation amplitude in glycerinated fibres 2 % length change*

$[Ca^{++}]$ (molar)	4×10^{-7}	1×10^{-6}	1×10^{-5}
Extra ATPase[a] (n mol/min × cm fibre)	0.35	0.26	0.26
Power[a] (μcal/min × cm fibre)	0.30	0.32	0.20

[a] Means of 3 experiments; in these and in 4 other experiments (in which the fibres were immersed into myokinase free activating solution) the mechano-chemical coefficients ("efficiencies") obtained at 10^{-5} M Ca^{++} were only 61 ± 12 % of the efficiencies at 10^{-6} M Ca^{++}; in 1 cm length of fibre the rates of total ATP-splitting (9 exp.) were: 15; 14.5; 13.7; 10.2; 10.0; 9.3; 7.6; 6.7; 5.2 p moles per oscillatory cycle at 10^{-5} M Ca^{++}.

Table 4. *The effect of increasing the free calcium concentration on power output and oscillation-induced extra ATPase during driven oscillation at 10 c/sec. Incubation medium contained 12 mM Mg-ATP, myokinase, pH 6,5; T = 23—25° C. Oscillation amplitude = 2 % length change peak to peak*

$[Ca^{++}]$ (molar)	4×10^{-7}	1×10^{-6}	4×10^{-6}	$> 10^{-5}$
Extra ATPase[a] (nmol/min × cm fibre)	0.11	0.40	0.60	0.55
Power[a] (μcal/min × cm fibre)	0.06	1.06	1.80	1.40

[a] Means of 3 fibre bundles (glycerinated DLM).

The decrease in power output and "efficiency" produced by the rise in calcium concentration might be attributed to lack of ATP caused by the calcium activation of ATPase activity, if the ATP supply were rate limiting. This is however not the case in the presence of 12 mM Mg-ATP and myokinase, since a further increase in ATPase activity produced by increasing the oscillation frequency to 10 c/sec even increases the oscillatory work per cycle and the "efficiency". At this frequency the extra ATPase activity depends on the free calcium concentration in a similar manner to the power output and to the "basic ATPase" (see [22]) or the actomyosin ATPase [3, 15]. In all cases the activity dependence on the pCa shows half maximal activation of the calcium sensitive enzyme or of the extra ATPase at $< 10^{-6}$ M (at pH 6.5) and full activation at 10^{-5} M (Table 4). However, at 2 c/sec oscillation the ATPase activation-effect of raising the calcium concentration from 10^{-6} M to 10^{-5} M is much less consistent. This is shown in Table 3 which also shows that the total amount of ATP split by 1 cm fibre in one oscillatory cycle may be as high as 15 pmoles, at an oscillation of 2 c/sec and 10^{-5} M Ca^{2+} (Table 3).

5. The Mean Tension during Driven Oscillation

In order to oscillate, the fibres must in most cases be slightly stretched (to about 102 % L_0, cf. [8]). With 10—15 mM Mg-ATP and myokinase present, the mean tension barely changes when the preparation is driven to oscillate in the range of 2 to 10 c/sec but at 60 c/sec the mean passive stretch tension drops to about one half (Table 5). If the ATP supply is rate limiting e.g. at low concentrations of Mg-ATP (about 5 mM), the mean tension often rises a few seconds after the onset of oscillation. Thus the oscillatory tension minimum is then similar to the former static tension at the mean length of the fibre (as described by TREGEAR [24]). The rise in tension might be attributed to the possible accumulation of ADP in the fibre at high rate of ATP splitting. Since even small concentrations of ADP may increase the contractile tension [1,5], the lack of an oscillation-induced rise in the mean tension in most of the experiments may indicate a very low ADP level inside the fibre.

Table 5

Mean fibre tension before—during—and after driven oscillation. Mean tension of glycerinated fibre bundle in dynes. Medium: Activating solution pH 6.5, $T = 25°$ C

Frequency	5 c/sec		60 c/sec	
$[Ca^{++}]$ (molar)	10^{-6} M	10^{-6} M[a]	10^{-6} M	10^{-5} M
Mean tension before or after oscillation	27	21	29	40
Mean tension during oscillation	22	17	9	23

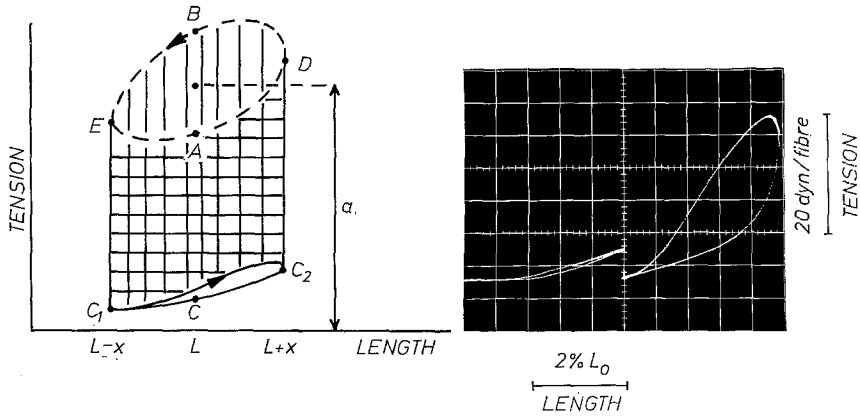
[a] With 10 mM Mg-ATP and myokinase. Driven oscillation amplitude = 2 % length change.

The tension measured in driven oscillation during the stretch half-cycle may be partly contractile tension in addition to passive tension, since it depends on the free calcium concentration (Fig. 9b). Thus the contractile force developed during the release (shortening half-cycle) in an activating medium may be equal to the difference of total tension (during release) and the passive tension (during stretch) as measured in the relaxing medium rather than in the activating solution (explanation see Fig. 9a). Maximum contractile tensions were about 30 dynes per fibre (equal to 0.8 kg/cm² fibre cross section or 1.7 kg/cm² myofibrillar cross section) during oscillation at 2 c/sec and at an amplitude of ~3 % fibre length.

Discussion

1. Power Output

In previous experiments [8] the driven oscillation frequency for optimal power output of glycerol extracted fibres of *Lethocerus maximus*



a b

Fig. 9. *Phase-plane diagram of an oscillatory cycle.* Closed loop in relaxing solution, open loop (broken line) in activating solution showing tension length diagrams similar to those shown in Fig. 2. Fig. 9a: Schematic diagram. Fig. 9b: Lissajous loops from an actual experiment in relaxing solution and in activating solution (containing 10 mM Mg-ATP, myokinase, 10^{-5} M Ca^{++}, pH 6.5, $T = 25°C$; Zero tension corresponds to the lower (lefthand) edge of the 2 Lissajous figures, in relaxing solution (left) and in activating solution (right). Fig. 9a. The fibre bundle is stretched and released about a reference length L with an amplitude of $2\ x$. Note that the mean tension (a) is greater in activating solution than in relaxing solution. Thus the total contractile tension at reference length (i.e. C—B) is considerably greater than the "delayed" or "quadrature" tension (i.e. A—B). Consequently the work of the contractile machinery during the shortening might be equal to that indicated by the vertically shaded area rather than to the net work (area enclosed in the Lissajous figure EADB). This would be the case if the work indicated by the horizontally shaded area were absorbed rather than "elastically" stored in the stretch cycle. However, the actual difference between the (hypothetical) total myofibrillar work (vertically shaded) and the net work (total work minus horizontally shaded work) may in reality (see Fig. 9b) be much smaller than suggested by the diagram. For further explanation see text p. 17. Also shown: "in-phase" tension difference = E—D. Elastic modulus = ED tension × fibre length/$2xa$. Viscous modulus = AB tension × fibre length/$2xa$. a cross-sectional area

suspended in ATP salt solution of low Ca^{++} concentrations (about 10^{-7} M) was only 2—4 c/sec and thus considerably lower than in live muscle at the same temperature (cf. [8]). Increasing the free calcium concentration decreased rather than increased the frequency optimum [8] since it induced the "high-tension state" [21] now known to be a diffusional artifact of rate limiting ATP supply (see p. 4). But, avoiding this artifact by supplying sufficient ATP and increasing the free Ca^{++} to 10^{-5} M, we are now able to increase the optimal frequency to 10 c/sec and even further to 20—25 c/sec at physiological amplitude (2—3 % L_0) if we also increase the temperature to 30—35°C. This temperature

corresponds to the probable body temperature of the tropical water-bugs and beetles which is about 6 °C above the ambient temperature [10]; the optimal frequency corresponds to the wing-beat frequency of flying Lethocerus maximus [2]. The maximum power output is then about 10^5 erg/sec in 1 g muscle as compared with 3×10^5 erg/g sec in living beetle muscle (cf. [8]).

The maximum oscillatory power output obtained at free calcium concentrations far above those needed to produce maximal ATPase activation is especially noteworthy. For it shows that the oscillatory movement is probably not linked to length dependent oscillatory changes in the calcium affinity of fibres [8] as implicated by certain calcium binding studies [4].

It seems possible that the myofibrillar work performed during the oscillatory shortening cycles is considerable greater than the net work as measured by the size of the Lissajous loop obtained during a stretch and release cycle (see Methods). For the net work of the fibre which is done during a stretch and release cycle on the forcing apparatus corresponds to the actual myofibrillar work only on the conditions that (1) the viscous work losses are negligible and that (2) the work done *on* the fibres during the stretch half-cycle is elastically or "chemically" stored so that it can be released again during the shortening half-cycle of an oscillation. If this were not so, i.e. if the work done by stretching were degraded into heat, the contractile work done by the myofibrils in any oscillation cycle would be considerably greater than the net work indicated by the area enclosed in the tension length diagramm; it would then correspond more closely to the shaded area in Fig. 9a. Since the actual size of the myofibrillar work is uncertain, it is quite remarkable that, nevertheless, the net oscillatory work is so clearly related to the extent of ATP splitting with an "efficiency" of up to 2—4 kcal work per mole ATP split.

2. The Chemo-Mechanical Coefficient Relating ATPase Activity and Power

The apparent "efficiency" or the chemo-mechanical coefficient is the ratio of the rate or work output and the rate of oscillation induced extra ATP splitting. This ratio is nearly constant over a wide range of driven oscillation frequencies (this paper, Fig. 5) and amplitudes of oscillation (Rüegg and Stumpf [19]). A close coupling of power output and oscillation induced extra ATPase activity is also suggested by the fact that the optimal power output occurs at the frequency at which the oscillation induced extra ATPase is optimal and that both power output and ATPase decline in a parallel fashion and eventually vanish

below and above the frequency optimum. It would seem therefore that—as in skeletal muscle—the rate of doing work apparently controls the rate at which energy is released to the contractile system (see FENN effect [7] and biochemical FENN effect; e.g. [13]).

On the other hand, KUSHMERICK [9] found recently that in skeletal muscle the chemo-mechanical coefficient (up to 9 kcal/mole of ATP split) is only constant within a certain shortening speed range and it decreases above and below the optimal range. At 20°C in glycerol extracted insect flight muscle the "efficiency" also decreases, especially at high calcium concentration, when the driven oscillation frequency becomes greater than 10 c/sec or smaller than 3 c/sec. The decrease in "efficiency" is associated with a decrease in the phase angle of sinusoidal tension and length changes. Such a relationship seems plausible since the peak tension probably reflects myofibrillar contraction associated with ATP splitting and since the net work gained from this contraction would seem to depend on the phase relation to the length changes. For instance, consider an extreme case: No work would be gained if the phase angle was 0, i.e. if the contractile event associated with ATP splitting coincided exactly with the moment of the greatest extension. It is also obvious that at low frequency the phase angle and the efficiency should decrease when the delay between stretch and "stretch induced rise in tension" decreases as a consequence of an increase in the free calcium concentration. Since the work generated at the actin myosin crossbridges is (depending on the phase angle of length and tension changes) only partly transformed into net work (and in particular, since some of the myofibrillar contractile work might be degraded during restretching) the comparatively low chemo-mechanical coefficient (2—4,000 cal/mole of ATP split) obtained in our experiments is not surprising.

This corresponds to an "efficiency" of the chemo-mechanical energy conversion of up to 50 % if we take the free energy available from ATP splitting as ~8 kcal/mole. This assumption seems reasonable in view of the finding (p. 5) that the myokinase "buffered" ADP/ATP ratio is about 1:10 under our equilibrium conditions.

3. Oscillatory Mechanical Performance and Rate of Actin Myosin Interaction

The simplest hypothesis to account for the mechano-chemical energy coupling of power output and ATPase activity supposes that a cross-bridge cycle (and actin-myosin interaction event) splits one molecule (or a small number) ATP and produces a fixed amount of work. Thus the rate of doing work and the rate of ATP splitting would be propor-

tional to each other, since both would depend on the rate of actin-myosin interaction. The frequency dependence of power output and ATPase activity would therefore reflect a frequency dependence of actin-myosin interaction rates in a sliding filament system. If an increase in the rate of filament sliding increases the rate at which actin sites are sliding past and react with exposed myosin sites, the power output and the ATPase activity should increase with increasing frequency or amplitude of oscillation as they, in fact, do. In this way "oscillation-induced extra-ATPase" is produced. If the oscillation frequency exeeds an optimal value, there is, presumably, not sufficient time for the interaction event to take place, so that both extra-ATPase activity and power output decrease or even vanish. The increase in the frequency optimum for power output and ATPase activity by raising the temperature may be assumed to be produced by a decrease in the time requirement for an interaction event, i.e. by a shortening of the fundamental cross-bridge cycle. Indeed both, a rise in calcium concentration and a rise in temperature are found to increase the rate of rise in tension induced by a quick stretch-activation. It is still unknown whether crossbridges move in synchrony with the oscillation cycle or whether they move repeatedly and asynchronously in each cycle [16, 18, 23]. As has already been pointed out elsewhere [19] the latter possibility seems more likely, in particular since we now find that up to 15 pmoles of ATP (see Table 3) may be split in an oscillation cycle of 0.5 sec duration by 1 cm muscle fibre containing about 3 pmoles of crossbridges (see [17] but cf. [6]), and we assume that not more than one or two ATP molecules are split during one crossbridge cycle [16].

To summarize then, oscillatory movements of glycerinated insect flight muscle exceeding a certain minimal frequency induce a "dynamic" increase in the actin myosin interaction rate associated with oscillatory power output and with an increased rate of ATP splitting (oscillation induced extra-ATPase) by the actin-activated ATPase. Significantly, in the ATP driven isolated contractile machinery, the rate of energy release is optimal at the optimal oscillation frequency of live muscle (20/sec; the wingbeat frequency of Lethocerus) and the power output is then also comparable to that of live muscle. This is also a clear demonstration that the ATP driven contractile machinery is—after its functional isolation by glycerol extraction of the muscle fibres—neither slower nor less powerful than live muscle.

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