Adenosine Triphosphate: Changes in Muscles Doing Negative Work

Abstract. *Frog sartorius muscles were isolated, treated with 1-fluoro-2,4-dinitrobenzene at 0°C, then stimulated tetanically at the length* in situ *and stretched with a Levin-Wyman ergometer during stimulation. The normal adenosine triphosphate breakdown during the tetanus was reduced by about half during the forced stretch. The tension was increased by about 70 percent, but resynthesis of adenosine triphosphate did not occur. Thus, on the basis of A. V. Hill's results, adenosine triphosphate is probably not the direct final energy source for muscular contraction, although it intimately participates in the process. The use of adenosine triphosphate during negative work was less than one-tenth that needed for positive work.*

Perhaps the most dramatic recent discovery concerning the biophysics of muscular activity is that "when a muscle is stretched during a maintained contraction, the whole of the work may disappear" (*1*). The findings of Hill, Abbott, Aubert, and Howarth (*1–4*), based on earlier work by Fenn in Hill's laboratory (*5*), "leave little doubt that the chemical reactions which normally occur during contraction can be reversed by a stretch under the influence of the mechanical work supplied" (*3*). Since the work absorbed in stretching a "contracting" muscle apparently "can reverse the chemical processes of activity" (*4*), experiments were carried out to find out if adenosine triphosphate (ATP) would be resynthesized under such conditions. Such a close energy coupling would be analogous to the production of ATP during electron transport in oxidative phosphorylation compared to the reversal of electron transport by ATP (*6*).

The methods were similar to those used previously (*7–12*). The muscles were treated with 1-fluoro-2,4-dinitrobenzene (FDNB) which can completely inhibit ATP–creatine phosphoryltransferase *in situ* in isolated frog muscle and prevent any regeneration of ATP except through the action of myokinase (ATP–adenosine monophosphate phosphoryltransferase) (*8–10*). This made possible the direct demonstration of a breakdown of ATP associated with work during single contractions of isolated intact muscle (*8–12*), which confirmed the widely held theory of the role of ATP in muscle contraction based on experiments on isolated and reconstituted actomyosin fibers, glycerinated muscle models, and isolated enzymes by Weber, Needham, Szent-Györgyi, Engelhardt, and many others [see review by Huxley (*13*)].

The sartorius muscles of female frogs (*Rana pipiens*) were dissected in pairs and allowed to rest at room temperature in a physiological bicarbonate-saline solution for 2 to 3 hours. The muscles were then maintained for 35 minutes at 0°C in a similar solution gassed with 5 percent CO_2 + 95 percent N_2, containing 0.38 millimoles of FDNB per liter. They were then mounted at rest length between an immobile support and a jeweler's chain and incubated for an additional 5 minutes at 0°C after which time they were rapidly frozen in a 1:1 mixture of Freons 12 and 13 (CF_2Cl_2 + CF_3Cl) at −172°C either without further treatment, or during tetanic isometric contraction, or stretch produced by a Levin-Wyman ergometer. One of the sartorius muscles served as a control for the other pair in each of the series of experiments. The forces involved were recorded from the output of a Grass tension transducer (FT 03) attached to the arm of the ergometer and connected to the muscle by means of the jeweler's chain. The distances moved and the rate of stretching were determined by prior adjustments of the ergometer. The times of stimulation, stretching, and freezing were controlled by a synchronous motor fitted with appropriate switches. The frozen muscle was ground to a fine powder at −196°C and extracted and assayed in duplicate (*8*).

In Table 1, in series B, C, and D, the experimental conditions for the stretching of the muscles were similar to and, in series E, identical with those used by Hill and Howarth (*3*, Fig. 6) for sartorius muscles of English toads (*Bufo bufo*). There was a linear breakdown of ATP with time (series A, C, and H) and there was no significant difference between the numerical results for the changes in ATP and inorganic

Table 1. Changes in adenosine triphosphate and inorganic phosphate in isometric tetanic contractions at the rest length *in situ* and in stretches during continued activation of frog sartorius muscles treated with FDNB a 0°C. Tetanic supramaximal electrical stimulation at 12 pulses per second was continuously applied during the stretch. The rate of stretching was 4 mm per 0.5 second in all cases. The average rest length *in situ* was 34 mm for muscles weighing approximately 100 mg. All stretched muscles were frozen just at the end of the stretch. When inorganic phosphate was measured, the muscles were powdered and the powder was extracted by stirring in 0.5*M* perchloric acid at 0°C for 2 minutes. A portion of the supernatant after centrifugation was immediately assayed for inorganic phosphate by the method of Wahler and Wollenberger (*20*). The remaining perchloric acid extract was stirred for 4 minutes at 35°C, neutralized, and assayed for ATP (*8, 9*). All values of differences are the means of the difference within each pair ± the standard errors of the means. The numbers in parentheses refer to the number of muscle pairs.

	Treatment of muscle pairs	ATP concn. (μmole/g)		Inorganic phosphate concn. (μmole/g)	
		Av. of means	Diff. in means ± S.E.	Av. of means	Diff. in means ± S.E.
A	Unstimulated control 0.56 second isometric tetanus	2.85 2.52	−0.33 ± 0.10(7)	2.30 2.63	+0.33 ± 0.14(6)
B	Unstimulated control 0.06 second isometric tetanus + 0.50 second stretch	2.62 2.54	−0.08 ± 0.14(8)	1.83 2.02	+0.19 ± 0.11(8)
C	Unstimulated control 0.7 second isometric tetanus	3.40 3.00	−0.40 ± 0.10(6)	2.05 2.53	+0.48 ± 0.13(8)
D	Unstimulated control 0.3 second isometric tetanus + 0.4 second stretch	2.96 2.69	−0.27 ± 0.13(8)	1.85 2.17	+0.32 ± 0.11(9)
E	1.0 second isometric tetanus 0.5 second isometric tetanus + 0.5 second stretch	2.09 2.18	+0.09 ± 0.10(11)	2.59 2.37	−0.22 ± 0.14(5)
F	0.5 second isometric tetanus 0.5 second isometric tetanus + 0.5 second stretch	2.48 2.38	−0.10 ± 0.13(16)	2.50 2.58	+0.08 ± 0.10(11)
G*	1.0 second isometric tetanus 0.5 second isometric tetanus + 0.5 second stretch	2.12 2.28	+0.16 ± 0.12(9)	2.61 2.44	−0.17 ± 0.08(9)
H	Unstimulated control 1.15 second isometric tetanus	2.99 2.17	−0.82 ± 0.08(6)	2.14 2.88	+0.74 ± 0.08(6)

* The muscles were mounted at 90 percent of their rest length *in situ*.

phosphate. Thus myokinase activity remained slight under these conditions with this muscle (*8*). Although the individual changes are small and the variance between the left and right muscles of each pair is relatively high, it is clear from taking together all the various conditions used (Table 1) that stretching an activated muscle caused a reduction in the breakdown of ATP ($P < .001$), even though the tension developed was about 70 percent greater than the isometric tension at rest length. Thus, the changes in series B and D were less than in A and C, respectively, and in series E and G the reduced breakdown of ATP left more ATP and less inorganic phosphate in the stretched muscles than in their pairs which had been stimulated isometrically for the same length of time. The value of P can be calculated both from the distribution of the signs of the observed changes or by combining the probabilities of the individual results by the method of Fisher (*14*), taking account of the signs of the differences.

The reduction in ATP breakdown was about half, and thus net resynthesis certainly did not occur; otherwise, on the basis of series A, C, and H, the changes in ATP and Pi contents, respectively, would have been +0.3 and −0.3 μmole/g in series F, zero in series B and D, and +0.66 and −0.66 μmole/g in series E and G.

This conclusion that the breakdown of ATP is reduced by stretching an activated muscle is in apparent contradiction to the recent results of Aubert and Maréchal (*15*) who found an increased breakdown at 0°C of phosphorylcreatine in iodoacetate-treated sartorius muscles of the frog which were stretched several times when activated. However they had used rapid stretches and obtained results quite similar to ours with slower rates of stretch comparable to that used by us (*16*).

Our results indicate that the net ATP (phosphorylcreatine) breakdown found previously (*8, 12, 17*) really is associated with work and not with a function of developed tension × time. The stretched muscles in Table 1 absorbed an average of 400 g-cm of work per gram. A comparison of the ATP requirements for positive work (*9*) and for this negative work shows that under these conditions the ATP usage during negative work was only about 1/13th that required during positive work. This is in good agreement with the finding that the extra oxygen needed in man for negative work can be less than 1/10th that needed for positive work (*18*).

These results are relevant to the conclusion of Hill and his colleagues concerning the reversal of the chemical processes of muscular activity during negative work (*1–4*). If their conclusion is correct and applicable to sartorius muscles previously treated with FDNB, and if ATP were the direct final energy source for muscle contraction, then it should have been resynthesized. This did not occur; so it seems that ATP is, after all, not the direct final energy source for muscular contraction. The finding of a reduction of ATP breakdown, but not a resynthesis in these experiments was predicted by a recent molecular theory of muscle contraction (*19*) which is based on the view that ATP is used to extend potential linkages between myosin and actin, which in the presence of calcium, form actual linkages, contract, and do work by the energy of hydrogen and hydrophobic bond formation to make α-helices, this process being repeated cyclically during muscular contraction. Stretching an activated muscle should thus break hydrogen bonds and reduce the breakdown of ATP which has now been observed.

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Amphibian Cell Culture: Permanent Cell Line from the Bullfrog (Rana catesbeiana)

Abstract. *A line of fibroblast cells has been established from tongue tissue of the bullfrog* (Rana catesbeiana). *The cells are near-triploid and were subcultured 57 times during the 2⅔ years of their existence. Some of their characteristics are described.*

Among vertebrate classes permanent cell lines have been established from mammals, birds, and teleosts. The sole report concerning repeatedly subcultured amphibian cells is that of Freed (*1*), who cultured frog cells through at least ten passages. Our report describes a permanant frog cell line which is over 2⅔ years old and is now in its 57th passage.

Line FT was initiated in July 1961 from bullfrog (*Rana catesbeiana*) cells obtained by a primary cultivation of a macroscopically normal-appearing tongue from an adult female. Tissue was dispersed by treatment with trypsin in the cold for 16 hours, and original cultures were established at 20°C in medium having a freezing point of −0.57 to −0.61°C. Methods and media were those (*2*) used for cold-blooded vertebrates. While these procedures are entirely appropriate for fresh-water teleosts, we subsequently found that our results with amphibian cells were better when solutions and media measured