Regulation of Tension in the Skinned Crayfish Muscle Fiber

I. *Contraction and relaxation in the absence of Ca ($pCa > 9$)*

J. P. REUBEN, P. W. BRANDT, M. BERMAN, and H. GRUNDFEST

From the Laboratory of Neurophysiology, Department of Neurology and Department of Anatomy, College of Physicians and Surgeons, Columbia University, New York 10032

ABSTRACT In isolated skinned crayfish muscle fibers bathed in solutions that were buffered to be virtually free of Ca^{2+} (pCa 8-10) the substrate for both contraction and relaxation is the MgNTP complex. Tension increased up to 50% of the maximum capability of the fiber as the substrate MgATP increased to an optimum (pMgATP = 5.5). Relaxation was induced by further increases in MgATP. Similar bell-shaped curves of tension vs. pMgNTP were obtained with UTP and ITP, but optimum pMgUTP was about 4.5 and optimum pMgITP was about 2.6. The relation between equilibrium tension and pMgNTP is described by an equation analogous to that for the kinetics of enzymes regulated by substrate inhibition.

INTRODUCTION

Most of the specific information on the biochemical events of contraction and relaxation of muscle is derived from studies on model systems, such as actin-myosin solutions, glycerinated muscle, and various moieties from minced or homogenized muscle (Needham, 1960; Carlson, 1963; Hasselbach, 1964; Weber, 1966; Perry, 1967; Ebashi and Endo, 1968; Mommaerts, 1969; Bendall, 1969). This paper presents data on the role of several nucleoside triphosphates in contraction and relaxation of the skinned crayfish muscle fiber preparation (Reuben, Brandt, and Grundfest, 1967), when no Ca is added to the solution and free ionized Ca is kept at a very low level by buffering with EGTA or EDTA.[1]

[1] The following abbreviations are employed: pCa, pMg, etc. signify the negative logarithm of the concentration of the given species. NTP = nucleoside triphosphate; adenosine = ATP; uridine = UTP; inosine = ITP. EDTA and EGTA are the two chelating agents, ethylenediaminetetraacetic acid and ethylene glycol-bis-(β-aminoethyl ether) N,N'-tetraacetic acid. When Mg or another moiety is used without additional notation it refers to the total concentration in the solution irrespective of physical state.

Our initial focus was to determine the relation between tension and pCa of the bathing medium (Brandt et al., 1970; Reuben et al., 1970). Some of our data were similar to those reported on the Natori (1954) skinned frog fiber preparation by Hellam and Podolsky (1969). However, the pCa vs. tension curve was strongly dependent on various characteristics of the bathing solutions with which the skinned fiber was challenged (Reuben et al., 1970). Among these, the concentrations of ATP and Mg were most critical. Therefore, as a basis for further study of contractile activation with buffered pCa systems we carried out the investigation described here,[2] on the effects of Mg and ATP or other nucleoside triphosphates (UTP and ITP) when these were applied in solutions in which pCa was maintained two or three orders above the normal threshold for excitation of contractions by Ca^{++}. Filo et al. (1965) and White (1970) obtained data on glycerinated muscle that are related to our findings.

While this study was in progress Weber (1969), Weber et al. (1969), and Dancker (1970) reported what seem to be the biochemical parallels of our physiological experiments. In the virtual absence of Ca the effects of MgATP on hydrolysis rates and syneresis in minced myofibrils and natural actomyosin are strikingly similar to our results which relate pMgATP to tension in the structurally intact myofibrils of the skinned fiber preparation. Similar or related biochemical findings were reported by Tonomura and Yoshimura (1960), Weber and Winicur (1961), S. Watanabe and his colleagues (Watanabe et al., 1964; Watanabe and Yasui, 1965), and by Levy and Ryan (1967).

METHODS

The Preparation Single fibers from the flexor of the carpopodite of walking legs were prepared as described earlier (Girardier et al., 1963). Animals were obtained from the Middle West (*Orconectes*) and California (*Procambarus*). There was no discernible difference in the results. The dissection was done under a binocular stereomicroscope with the muscle in the chamber that was to serve eventually in the experimental phase. The dissection was carried out with the fiber bathed in a standard crayfish saline (Reuben et al., 1964). After dissection, the chamber with the intact single fiber was placed on the stage of an inverted compound microscope (Nikon) which carried a strain gauge force transducer mounted on a manipulator attached to the stage. A small piece of chitin which was left at the site of insertion of the fiber onto the ischiopodite was fixed at one end of the chamber with a stainless steel clamp. The tendon at the other end of the fiber was inserted into a clamp attached to the strain gauge transducer and the resting length was adjusted by moving the transducer. The experiments were done for the most part after stretching the fiber approximately 25% above the slack length. Sarcomere lengths and fiber diameters were recorded.

The chamber was now flushed with the "skinning" solution (Reuben, Brandt, and

[2] A brief account of this work was given at the 1970 Meetings of the Society of General Physiologists (Brandt et al., 1971).

Grundfest, 1967) which contained (in mM/liter) 200 K propionate, 10 EGTA, 20 Tris, 1 ATP, 1 Mg. The pH was adjusted to 7.0 with a Radiometer pH meter (type PHM 4c). The rapid application of high K induced a maximal but transient tension (Reuben et al., 1964; Reuben, Brandt, Garcia, and Grundfest, 1967), which ranged between 3 and 8 kg/cm^2. After the fiber had been equilibrated in this solution for 10–20 min the surface near one end was scratched lightly with a sharp needle so as to cut the membrane. In most fibers the membrane, once cut, could be pulled back with the aid of a hooked needle, as a continuous sleeve (Fig. 1). The fibers were 4–5 mm long and about 90% of their surface was denuded of the membrane. The diameters of 25 fibers in one series ranged between 134 and 310 μ (average 205 $\pm$ 43 μ). The sarcomere lengths averaged 10.1 $\pm$ 0.6 μ. The diameters of the nine fibers of Fig. 6 ranged between 145 and 294 μ.

The chamber (Fig. 1) consists of two reservoirs, each of about 0.5 ml capacity, in which the fiber is held. The chamber walls are machined from Lucite, and a glass slide sealed into the chamber forms the floor. A suction outlet in one of the reservoirs permits rapid withdrawal of the solution. A pump system, containing about 5 ml of solution, in parallel with the chamber provides a continuous circulation of solution past the fiber at the rate of about 0.5 ml/sec.

When the solution was changed the circulating system was drained first and then new solution was introduced into the reservoir at the opposite end of the chamber. Approximately 5 ml of the new solution was flushed rapidly past the fiber and discarded. Then 10 ml were shunted into the circulating system to flush it and charge it. Measurements showed that the wash-in of a new solution into the chamber could be made in <0.5 sec.

In most of the experiments the temperature of the solution, which ranged between 22° and 24°C, was monitored continuously and registered on one channel of a polygraph. Tensions were recorded on two channels of the polygraph with different gains so that both small as well as large tensions could be measured accurately. The polygraph (Beckman dynograph, type R) had linear penwriters which could produce full scale deflection within <10 msec.

Solutions The various media to which the skinned fiber preparations were subjected will be described in connection with individual types of experiments. Only the principles on which we made these formulations will be stressed here. The experiments required the use of a wide range of concentrations of EGTA, EDTA, Mg, and ATP at high pCa levels. Since many of the components are polyvalent we chose to keep the ionic strength constant at the expense of constant osmolarity. The latter is not as important a parameter as is the ionic strength for the skinned fiber preparation (April and Brandt, 1970). When the ionic strength was 0.3 a change of no more than ± 0.05 could be tolerated for measurements of pCa vs. tension before the curves changed noticeably. Larger changes were therefore avoided by adding or deleting K propionate when the composition of the buffer system made this necessary.

Thus, the solutions were made according to the following criteria: The ionic strength was kept near 0.3, pH was buffered at 7.0 with Tris, Tris hydroxide, Tris maleate, or phosphate. Whenever pMg, pCa, or pMgNTP were to be greater than about 3 we used buffer systems. Solutions containing EDTA, ATP, and Mg can be

so devised as to buffer pMgATP and pMg from 4 to >9, by changing the concentration of MgEDTA in a solution containing a fixed amount of free EDTA.

The concentration of MgATP that was formed when a given concentration of MgEDTA was added in the presence of fixed amounts of ATP and free EDTA was calculated by programming a computer for an iterative procedure involving equations 1–3.

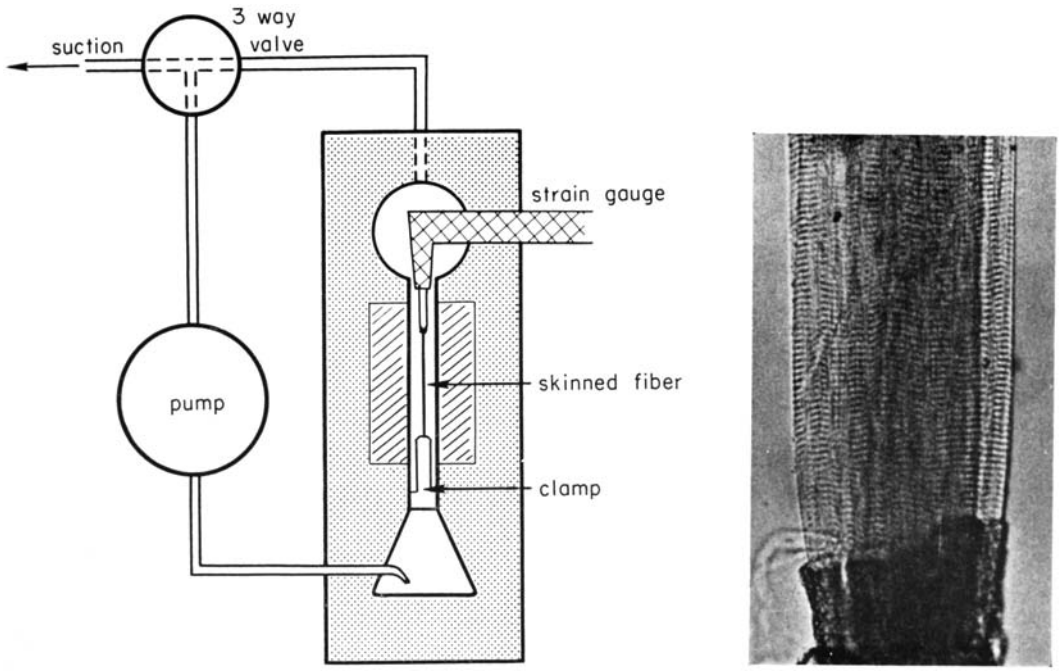

FIGURE 1. The skinned fiber preparation (right) and the experimental chamber (left). The membrane, pulled back from most of the fiber, is seen as a dark sleeve at the bottom of the micrograph. The myofibrils in the skinned portion maintain their integrity. The top view of the chamber shows the two reservoirs (each of about 0.5 ml capacity) connected by a narrow channel in which the fiber is held by a clamp at one end and a forceps connected to the strain gauge at the other. The outlet at the circular reservoir connects either to a suction outlet or to a pump system which circulates fluid from a 5 ml reservoir (not shown) into the triangular reservoir.

$$\text{Mg total} = \text{Mg}^{++} + \text{MgEDTA} + \text{MgATP} \tag{1}$$

$$\text{Mg}^{++} = \underset{(a)}{\frac{\text{MgEDTA}}{\text{EDTA} \cdot K_{a(\text{EDTA})}}} = \underset{(b)}{\frac{\text{MgATP}}{\text{ATP} \cdot K_{a(\text{ATP})}}} \tag{2}$$

Let

$$\text{MgEDTA} = x \text{ and } \text{MgATP} = y$$

Then

$$\frac{x}{(\text{EDTA total} - x)\,(\text{Mg total} - x - y)} = K_{a(\text{EDTA})} \qquad (3\,a)$$

$$\frac{y}{(\text{ATP total} - y)\,(\text{Mg total} - x - y)} = K_{a(\text{ATP})} \qquad (3\,b)$$

The program began with solving for $x_{(y=0)}$ in equation 3 *a*. (Mg total $- x$) was then inserted into equation 3 *b* which was solved for y. The procedures were repeated using updated x and y values to give successively closer approximations for x and y. Calculation was ended when successive values for x differed by no more than 10^{-9} M since this accuracy was judged sufficient for our experiments. Table I shows the values calculated for pMgATP, pMg, pMgEDTA in solutions containing 1 mM ATP and 10 mM EDTA in excess of the added MgEDTA. Similar tables were computed for all other experimental conditions.

When pMgNTP was to be <3.5 the EDTA buffer system was replaced with EGTA. EDTA has a high affinity for both Mg and Ca, while EGTA binds Ca predominantly and the added Mg is either ionized or associated almost exclusively with the NTP. The apparent association constants used in this study were calculated for pH 7.0 from the absolute constants, but were not corrected for ionic strength. The calculated values used and the sources of the absolute constants are given in Table II.

Contamination Levels of Mg When the pMg was not buffered with EDTA the level of Mg present as a contaminant was mainly that associated with the NTP's. The amounts of Mg and Ca present in the different components of the media were determined with an atomic absorption spectrophotometer (Jarrell-Ash Model 82-500, Jarrell-Ash Co., Waltham, Mass.) and are shown in Table III.

Characteristics of the Skinned Fiber The useful "lifetime" of the crayfish skinned fiber preparations made as described above is considerably longer than is that reported for frog muscle fibers skinned according to the Natori technique (Hellam and Podolsky, 1969). Most fibers responded to the same solution with tension reduced by only 10–20% after having been subjected to numerous other challenges for periods of 2–6 hr.

The maximal force of the skinned fiber was seldom larger than 50% of the tension produced in the same fiber when it was exposed to 220 mM K before skinning. The frog skinned fiber preparation also appears to respond with about half the maximum force developed by intact fibers. Hellam and Podolsky (1969) reported an average value of 1.4 kg/cm² for the skinned fiber preparation, or about one-half the value (2–4 kg/cm²) reported for intact single fibers (Hodgkin and Horowicz, 1960). The tensions reported in intact frog fibers by Lüttgau and Oetliker (1968) were larger still, 1.7–5.8 kg/cm² (average of 12 fibers, 3.5 kg/cm²).

Several factors are probably responsible for the smaller measured tensions of the skinned fibers. (*a*) About 10% of the crayfish fiber remained covered by the membrane. Since the myofibrils of this portion were not as accessible to the bathing solutions as those of the skinned region, the sarcomeres in this region became stretched when the fiber was developing tension. These relatively inactive sarcomeres represent

TABLE I

pMg ATP CALCULATED AS DESCRIBED IN TEXT

Total Mg	MgEDTA	pMgEDTA	MgATP	pMgATP	Mg^{++}	pMg^{++}
mM	*mM*		*mM*		*μM*	
0.01	0.009947	5.00	0.000048	7.31	0.005	8.30
0.02	0.019895	4.70	0.000096	7.01	0.009	8.04
0.03	0.029842	4.52	0.000145	6.83	0.013	7.88
0.04	0.039790	4.40	0.000193	6.71	0.017	7.76
0.05	0.049737	4.30	0.000241	6.61	0.022	7.65
0.06	0.059685	4.22	0.000289	6.53	0.026	7.58
0.07	0.069632	4.15	0.000338	6.47	0.030	7.52
0.08	0.079580	4.09	0.000386	6.41	0.034	7.46
0.09	0.089527	4.04	0.000434	6.36	0.039	7.40
0.10	0.099475	4.00	0.000482	6.31	0.043	7.36
0.20	0.198951	3.70	0.000964	6.01	0.085	7.07
0.30	0.298427	3.52	0.001445	5.84	0.128	6.89
0.40	0.397904	3.40	0.001926	5.71	0.170	6.76
0.50	0.497381	3.30	0.002407	5.61	0.212	6.67
0.60	0.596859	3.22	0.002887	5.53	0.254	6.59
0.70	0.696338	3.15	0.003365	5.47	0.297	6.52
0.80	0.795817	3.09	0.003844	5.41	0.339	6.46
0.90	0.895296	3.04	0.004323	5.36	0.381	6.41
1.00	0.994776	3.00	0.004800	5.31	0.424	6.37
2.00	1.989600	2.70	0.009553	5.01	0.847	6.07
3.00	2.984480	2.52	0.014251	4.84	1.269	5.89
4.00	3.979400	2.40	0.018909	4.72	1.691	5.77
5.00	4.974380	2.30	0.023508	4.62	2.112	5.67
6.00	5.969400	2.22	0.028066	4.55	2.534	5.59
7.00	6.964470	2.15	0.032576	4.48	2.954	5.52
8.00	7.959590	2.09	0.037036	4.43	3.374	5.47
9.00	8.954750	2.04	0.041456	4.38	3.794	5.42
10.00	9.949960	2.00	0.045827	4.33	4.213	5.37
20.00	19.904300	1.70	0.087308	4.05	8.392	5.07
30.00	29.862400	1.52	0.125061	3.90	12.539	4.90
40.00	39.823700	1.39	0.159636	3.79	16.664	4.77
50.00	49.787900	1.30	0.191343	3.71	20.757	4.68
60.00	59.754600	1.22	0.220575	3.65	24.825	4.60
70.00	69.723400	1.15	0.247715	3.60	28.885	4.53
80.00	79.694200	1.09	0.272879	3.56	32.921	4.48
90.00	89.666800	1.04	0.296270	3.52	36.930	4.43
100.00	99.640900	1.00	0.318167	3.49	40.933	4.38

a series compliance which is absent when the intact fiber is uniformly activated. The "extra" series compliance was evidenced by the fact that the sarcomeres in the immediately adjacent skinned region shortened considerably more than did the sarcomeres in the central portion of the fiber. (*b*) Even when the bathing solution is changed rapidly, the myofibrils in the depth of the fiber are activated after some diffusional delay.

Both these factors contribute to a nonuniform distribution of sarcomere lengths along the length of the myofibrils. Whatever may be the force developed by any one myofibril, it cannot be greater than that developed by the sarcomere whose length deviates most from the peak of the length-tension curve.

RESULTS

A. *ATP, Mg, and Contractile Activity in Ca-Free Media*

CONTRACTIONS ON WITHDRAWING MG AND ATP The starting point of these studies was the observation that the skinned fiber developed considerable ten-

TABLE II

APPARENT ASSOCIATION CONSTANTS AT pH 7.0
CALCULATED FROM THE ABSOLUTE CONSTANTS

	K_a EDTA	K_a EGTA	K_a ATP
Ca	2.4×10^{7}*	4.8×10^{6}*	5×10^{3}‡
Mg	2.3×10^{5}*	40*	11.4×10^{3}‡

* Portzehl et al. (1964).
‡ Nanninga (1961).

TABLE III

CONTAMINANT CONCENTRATIONS OF Mg AND Ca*

Salt	Mg	Ca	Remarks
	mM/M salt	*mM/M salt*	
ATP Na_2	0.5; 0.85	0.81; 1.5	1% La present
UTP Na_2	1.25; 1.5		" " "
ITP Na_2	5.75; 6.75		" " "
EGTA Na_2	0.014	0.085; 0.22	
EDTA Na_2	0.014		
K propionate	0.002	0.012	
$CaCO_3$	0.5		
$MgCl_2$		0.028	
Tris OH		0.014	

* Mg in $CaCO_3$ as given by manufacturer. All other values for Mg and Ca were determined by atomic absorption spectrophotometry. Where two values are given they indicate repeat measurements on different samples. The NTP's were obtained from Sigma Chemical Company.

sion when the standard skinning solution was replaced by a medium without Mg or ATP. The tension, though slowly rising, could attain an amplitude of about 50% of the maximum that is recorded in the skinned fiber when the contractions were evoked by adding Ca. The optimum tension in the Ca-free media was evoked when only trace amounts of both ATP and Mg were present in the bathing solution.

VARIATION OF MG IN LOW ATP Fig. 2 shows the tensions that were induced on replacing the relaxing solution (↑) with one containing 0.1 mM

ATP, 10 mM EGTA, and various low amounts of Mg. Free ionized Ca must have been less than about 10^{-9} M. An insignificant amount of Mg was bound as MgEGTA since the apparent association constant for Mg and EGTA is low (Table II). After some 3–4 min in the presence of 0.05 mM Mg (A) the fiber attained a tension of 280 mg. The onset of the response occurred about 20 sec after the change in solution. The fiber was again exposed to the relaxing solu-

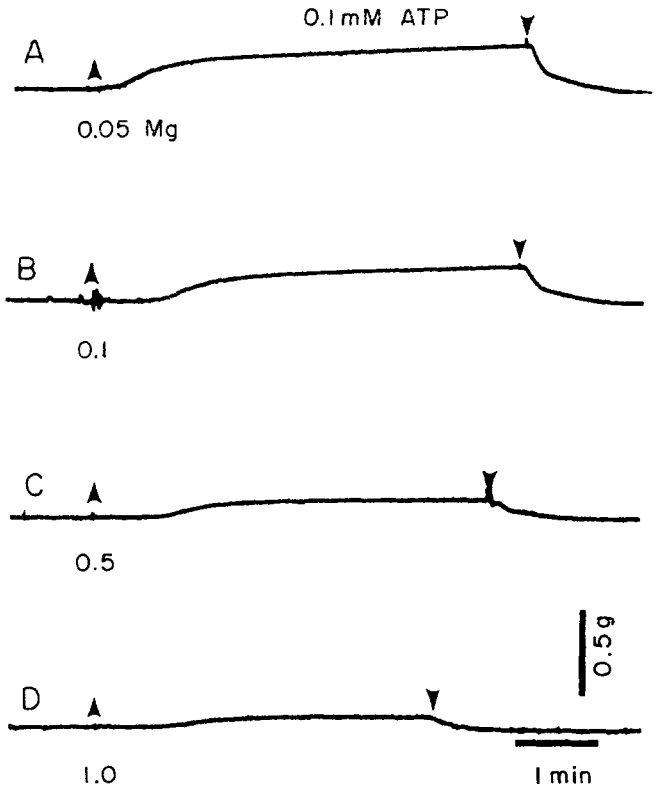

FIGURE 2. Tensions induced with the fiber bathed in a virtually Ca-free medium. The standard relaxing solution containing 10 mM EGTA, 1 mM ATP, and 1 mM Mg was replaced (↑) with the test solution which contained 10 mM EGTA, 0.1 mM ATP, and Mg as indicated. The relaxing solution was again applied at (↓). The concentrations of MgATP (in μM) were 23, A; 40, B; 82, C; and 91, D. Further description in text.

tion (↓) after which a new experimental solution, containing 0.1 mM Mg was applied (B). The evoked tension was smaller, 210 mg, and its onset was delayed by about 1 min. In the third cycle (C) when the Mg had been increased to 0.5 mM the tension was only 110 mg. When the Mg was elevated to 1 mM (D) an even smaller tension developed. These responses were also delayed by

about 1 min. Thus, not only was the tension reduced on increasing Mg, but also its onset was slowed.

INDEPENDENT VARIATION OF MG OR ATP The data of Fig. 2 might lead to the conclusion that ionized Mg participates in an inhibitory process which opposes the development of tension (Weber and Winicur, 1961; Hasselbach, 1964). This interpretation is invalidated, however, by the evidence of Fig. 3. The tensions recorded as open circles were obtained with ATP constant at 0.02 mM while the Mg was increased in the sequence of solutions, starting from an initial level of 0.02 mM. For the measurements recorded as solid circles the Mg concentration was kept constant at 0.02 mM and the ATP was increased progressively from an initial level of 0.02 mM. Thus, the two points on the extreme left represent identical conditions (Mg = ATP = 0.02 mM). The maximal tensions obtained were 280 and 300 mg, differing by only about 7%. The

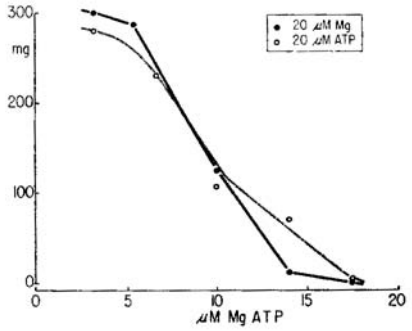

FIGURE 3

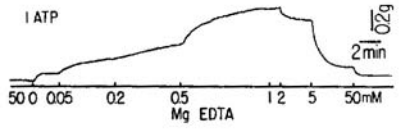

FIGURE 4

FIGURE 3. Tensions induced by varying Mg or ATP independently. Open circles show the tensions obtained with 0.02 mM ATP always present and with concentrations of Mg increasing from 0.02 to 1 mM. The abscissa is in micromolar MgATP. The solid circles show another sequence of measurements on the same fiber, but with Mg now maintained constant at 0.02 mM and ATP increasing from 0.02 to 1 mM. Thus, the two points on the extreme left represent the same condition: ATP = Mg = 0.02 mM. Further description in text.

FIGURE 4. Tensions induced by applying Mg buffered with EDTA. The solutions contained 1 mM ATP and 10 mM free EDTA as well as the concentrations of MgEDTA specified on the abscissa. Further description in text.

tension decreased when either Mg or ATP was increased. This finding implicates the concentration of the complex, MgATP, in the tension-regulating processes. In fact, when the data are plotted, as they are in Fig. 3, with the tension as a function of the MgATP concentration the two graphs coincide.

TENSION AS A FUNCTION OF MGATP The curves of Fig. 3 show that maximum tension can be elicited only when the total concentrations of Mg and

ATP are very low, 0.02 mM or less. However, the Mg present as a contaminant in ATP and the various constituents of the saline solution becomes an appreciable quantity when the Mg added to the solution is < 0.02 mM (Table III). To overcome this constraint, solutions buffered for Mg and MgATP were used. The test solutions were made so as to contain 10 mM *free* EDTA in place of the 10 mM EGTA. The Mg was added as MgEDTA, and appropriate steps were taken (see Methods) to keep the ionic strength of the solutions essentially unchanged; otherwise, the contractile response of the skinned fiber would have been drastically altered by changes in the ionic strength (April and Brandt, 1970) as is that of the intact fiber (April et al., 1968). Since the apparent association constant for CaEDTA is higher than that for CaEGTA (Table II) the solutions still contained an extremely low concentration (ca. 10^{-10} M) of ionized Ca. The concentration of MgATP that was formed when MgEDTA was added in the presence of 1 mM or more of ATP was calculated as described in the Methods section. Even if MgATP is rapidly hydrolyzed, this buffer system tends to keep the pMgATP constant.

Fig. 4 shows the tensions that were induced when MgATP was increased by the addition of MgEDTA. Prior to the 35 min sequence shown, the fiber had been subjected to various other challenges and the recording began with the fiber bathed in a solution containing (in mM) 50 MgEDTA, 1 ATP, and 10 free EDTA. This solution, which contains 190 μM MgATP (Table I), caused complete relaxation of the fiber as is seen at the end of the sequence when the same solution was again applied. At the mark "O" all the MgEDTA and ATP were withdrawn. A small tension (ca. 70 mg) resulted.

The small tension having remained steady for about 2 min, 1 mM ATP was again introduced with incremental stepwise additions of MgEDTA. With 0.05 or 0.2 mM MgEDTA present the tension rose very slowly to higher steady values but the tension and its rate of rise increased when MgEDTA was raised to 0.5 mM. The tension (ca. 630 mg) did not change with 1 mM MgEDTA. High concentrations (2 or 5 mM MgEDTA) caused stepwise relaxation. When 50 mM MgEDTA was again introduced at the end of the sequence the fiber relaxed to within 10 mg of the original resting tension.

Two similar experiments, but on another fiber, are shown in Fig. 5, plotted as tension vs. free Mg in A, and as tension vs. MgATP in B. In one sequence (open circles) 1 mM ATP was present; in the other it was 5 mM. The forms of the two curves in B are rather similar, with the peak tension occurring when MgATP was about 2.4 μM. In both sequences the tension increased on increasing MgATP from the lowest levels available, and decreased when MgATP was increased beyond 2.4 μM. Thus, the increments of MgATP cause both contraction and relaxation when the level of free Ca is of the order of 10^{-10} M.

The graphs of Fig. 5 show a striking similarity to the data of Weber et al. (1969) on the rate of ATP hydrolysis vs. Mg and MgATP in Ca-free minced

rabbit myofibril preparations. These authors concluded, and our data support that view, that it is the concentration of MgATP rather than free Mg or free ATP which controls hydrolysis (and tension) in the absence of a significant level of ionized Ca. Furthermore, in agreement with the data of Levy and Ryan (1967) and Weber et al. (1969) on hydrolysis rates, large tensions can occur in the skinned fiber preparation when the concentration of ionized Ca is several orders lower than the level required to remove the inhibitory effect of troponin. The latter is believed to prevent the occurrence of tension in the absence of ionized Ca (cf. Ebashi and Endo, 1968).

The relation between tension and the concentration of MgATP (Fig. 5) was studied extensively and Fig. 6 is a compilation of 12 experiments on 9

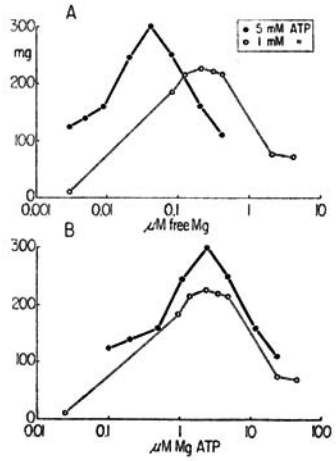

FIGURE 5. Two sets of curves plotting data similar to those of Fig. 4, but with ATP present at two concentrations (1 and 5 mM) in the two sequences of tests on one fiber. A, the abscissa shows the free Mg^{++}. In the presence of 5 mM ATP the curve shifted to the left (lower concentrations of Mg^{++}). B, when the same data were plotted in terms of the concentration of MgATP the peaks of the two curves coincided. Further description in text.

fibers bathed in solutions containing 4 different concentrations of ATP. All these experiments were done in the presence of 10 mM free EDTA. Similar data were also obtained when the free EDTA was 1 mM. The tensions were normalized in Fig. 6 and the abscissa is in units of pMgATP. The peak tensions in fibers exposed to 5 or 10 mM ATP were in some cases 10–20% larger than those measured in lower concentrations of ATP (cf. also Fig. 5). The difference may reflect a diffusional limitation for ATP which should be more significant when substrate is being hydrolyzed at a high rate, as it may be in the range near peak tension, or when the free ATP concentration of the bulk solution is low. However, the error must be minimal, since the peak tensions in Fig. 6 (points inside the rectangle) occurred within a narrow range of pMgATP (5.7 to 5.3) over a wide range of bulk ATP concentrations.

This conclusion is reenforced by data on three of the fibers of Fig. 6, each of which was exposed to two widely different concentrations of ATP. The second smallest fiber (diameter = 191 μ) is also that shown in Fig. 5. While the peak

tensions differed by about 30% (225 mg in 1 ATP and 300 mg in 5 ATP) optimum pMgATP was 5.61 and 5.62 (Fig. 5), despite the fivefold difference in ATP. Another fiber, one of the largest of the series (284 μ), was exposed to 1 and 10 mM ATP. The respective peak tensions were 350 and 370 mg, while the corresponding pMgATP's were 5.44 and 5.43, respectively. The largest fiber (294 μ) was exposed to a still greater difference in ATP concentration, 1:25. In 0.2 mM ATP the peak tension was 305 mg and pMgATP was 5.71. With 5 mM ATP present the corresponding values were 340 and 5.45.

The data summarized in Fig. 6 in conjunction with the biochemical data of

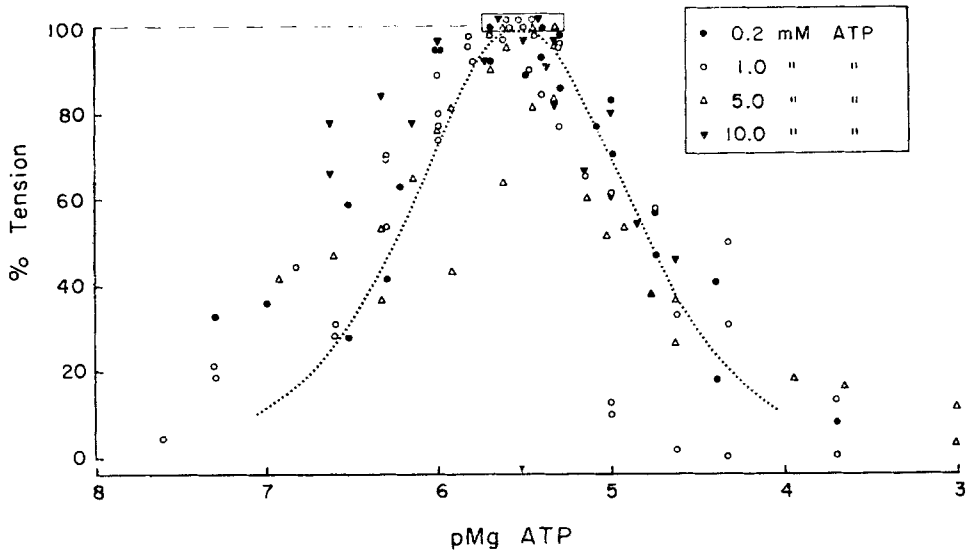

FIGURE 6. Data from 12 experiments like those shown in Fig. 5, with different concentrations of ATP in the test solutions. The abscissa shows pMgATP. The measured tensions were normalized relative to the peak values. The values of pMgATP at which the peaks were attained in the individual experiments are shown by the points within the rectangle. The curve (dotted line) was calculated as described in the text by assuming $K_1 = 1.5$ μM.

Weber et al. (1969) lead to the following conclusions: (*a*) The complex, MgATP, can act as a substrate for the development of tension and for hydrolysis by actomyosin. (*b*) An increase in MgATP causes an increase in both responses, up to an optimum. The optimum tension is about 50% of the maximum capacity of the skinned fiber. (*c*) Further increase in substrate MgATP causes a decrease in both the tension and hydrolysis rate.

In summary, when Ca is virtually absent (pCa > 9) MgATP can cause both activation and relaxation, depending upon its concentration. The resulting relation for tension vs. pMgATP is a bell-shaped curve, such as is frequently obtained in studies of the kinetics of enzymes which manifest substrate inhibition.

Tonomura and Yoshimura (1960), Levy and Ryan (1967), and Dancker (1970) obtained similar bell-shaped curves for superprecipitation or hydrolysis rates as functions of substrate concentration and proposed that MgATP is an inhibitor as well as a substrate. Watanabe et al. (1964) reported a diphasic action of MgATP in glycerinated rabbit fibers. White (1970) studied glycerinated insect flight muscle and rabbit psoas with EGTA present to maintain pCa at about 9. His starting condition was a "rigor solution," with either 5 mM $MgCl_2$ or 5 mM ATP present. As we have shown in Figs. 2 and 3, the concentration of MgATP may have been sufficiently high to induce near optimal tensions. An increase in the concentration of MgATP caused relaxation which he attributed to substrate inhibition. Thus, his data are limited to the supraoptimal portion of the bell-shaped curve. A curve that does include the suboptimal portion was obtained for glycerinated psoas muscle by Filo et al. (1965, Fig. 2) when they measured tension as a function of Mg concentration in the absence of added Ca (0.5 mM EGTA; 5.0 mM ATP).

For substrate inhibition in the skinned fiber preparation one may write the following schema:

$$\underset{\text{(substrate)}}{2\,\text{MgATP}} + \underset{\text{(enzyme)}}{\text{AM}} \overset{K_1}{\rightleftharpoons} \underset{\text{(tension)}}{\text{MgATP} - \text{AM}} + \text{MgATP} \overset{K_2}{\rightleftharpoons} \underset{\text{(relaxation)}}{\text{MgATP} - \text{AM} - \text{MgATP}}$$

According to this schema the enzyme (AM) has one site or mode of combination with the substrate MgATP which operates to convert chemical energy to mechanical energy for contraction. A second site, or mode of combination with the substrate, inhibits the operation of the energy-releasing site and leads to relaxation. If the dissociation constants for the two reactions are K_1 and K_2, respectively, the observed tension, (P), at equilibrium is:

$$P = \frac{P_o}{1 + \frac{K_1}{S} + \frac{S}{K_2}} \tag{4}$$

where P_o is the maximum tension that the skinned fiber can exert, and S is the substrate concentration.

Assume:

$$P \approx \text{AM} - \text{MgATP}$$
$$P_o \approx \text{AM}_{\text{total}} = \text{AM} + \text{AM} - \text{MgATP} + \text{AM} - 2\,\text{MgATP}$$

At equilibrium

$$\frac{dP}{dt} = 0.$$

Differentiating equation (4) with respect to the substrate concentration and equating dP/dS to zero define the substrate concentration for the optimum tension:

$$S_{opt} = (K_1 \cdot K_2)^{1/2} \tag{5}$$

The maximal tension (P_o) would be attained only when the inhibitory (or relaxing) effect of the substrate is absent. In the present experiments when pCa > 9.0 the peak tensions were about half of the maximum tensions that the skinned fiber could develop in solutions in which pCa < 6 (Fig. 7).

A series of theoretical curves was generated by selecting values for K_1. The data of Fig. 6 provided an estimate of S_{opt}, since the mean value for the 12 points at 100% relative tension was 3.05 μM MgATP (pMgATP = 5.5 ± 0.12 SD). Thus, a singular value of K_2 could be calculated for each chosen value of

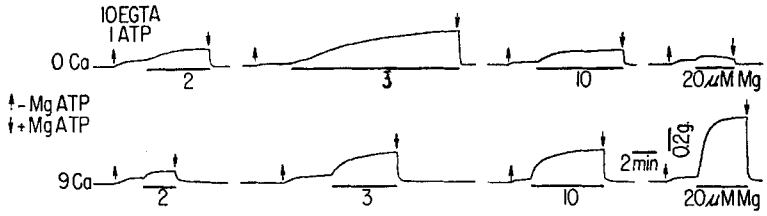

FIGURE 7. The effect of added Ca on the tension induced by MgATP. Arrows show the withdrawal (↑) and reapplication (↓) of the relaxing solution. The bars denote addition of Mg, in the concentrations (μM) specified, to solutions containing 10 mM EGTA and 1 mM ATP. Upper sequence, no added Ca. The largest tension was induced by 3 μM Mg. Further additions caused smaller tensions. Lower sequence, 9 mM Ca was added (free Ca^{++} about 2 μM). Increasing additions of Mg caused progressively larger tensions.

K_1. The scatter diagram of Fig. 6 is composed of points on a number of curves. A theoretical curve that appears to be fairly representative of the data is included as the dotted line.

TENSION IN THE PRESENCE OF ADDED Ca Experiments on the role of Ca in regulating tension output will be described in detail in another report. The data of Fig. 7, however, illustrate the effect of Ca, which is to permit the skinned muscle fiber to develop its maximal tension, P_o.

The fiber of this experiment was subjected to two sequences of challenges with increasing concentrations of Mg in the presence of 10 mM EGTA and 1 mM ATP. No Ca was added in one series (Fig. 7, upper), so that the value of pCa was >9, and the data resemble those of the experiments described earlier. At the beginning of each experiment (↑) the fiber was washed in a medium without ATP or Mg. The withdrawal of these substances induced a small tension, as already described. Then the fiber was challenged (bar under record) with 1 mM ATP and the indicated amount of Mg. At the end of each

record (↓) the fiber was relaxed by a solution containing 1 mM Mg and 1 mM ATP (750 μM MgATP) before it was subjected to a new challenge. The optimum tension (ca. 280 mg) was obtained in a solution containing 3 μM of Mg. When Mg was increased further the tension decreased.

The records of Fig. 7 (lower) show a similar series on this fiber in the presence of 9 mM of Ca. Since 10 mM EGTA was also present the concentration of ionized Ca was about 2 μM and this is usually sufficient to permit the skinned fiber to generate 90–95% of its maximum tension in solutions containing 750 μM MgATP (Reuben et al., 1970, and data to be published). When the Mg and ATP were removed at the beginning of each record there was the typical small rise in tension. It is noteworthy that this tension was similar to that in Fig. 7 (upper) with and without Ca. After a few minutes the fiber was exposed to 10 mM EGTA, 9 mM Ca, 1 mM ATP, and Mg. The series of records shows that the tension rose with each increase in Mg when Ca was present. A tension of 560 mg developed when Mg was increased to 20 μM. Further increase in Mg tended to increase tension only slightly.

Thus, ionized Ca tends to suppress relaxation (Weber and Winicur, 1961; Weber and Herz, 1963; Weber et al., 1964). This "disinhibition" (Levy and Ryan, 1967) of the MgATP inhibition of tension can in turn be suppressed by higher concentrations of MgATP (data to be published), as has also been observed with actomyosin preparations by Levy and Ryan (1967) and Dancker (1970). The peak tension induced by the optimum concentration of MgATP is approximately twofold larger in the presence of Ca (Fig. 7; cf. also Dancker, 1970, Figs. 5 and 7; and White, 1970, p. 589). This increase is predicted by equation (4) if K_2 increases in the presence of Ca. The rising phase of the theoretical curve (e.g., like that in Fig. 6) remains the same, but the rise continues with increasing concentrations of MgATP, as in Fig. 7. The optimum and the subsequent falling phase of the curve are obtained at still higher concentrations of MgATP. The rising phase of the curve obtained in glycerinated psoas muscle without added Ca (Filo et al., 1965) was changed only slightly when 10^{-5} M Ca was present. The tension then continued to rise with increasing concentration of Mg, as in our Fig. 7. Also, the maximum tension developed in the presence of Ca was about twice the peak tension in the absence of Ca.

B. Substrate-Enzyme Relations for Other MgNTP Complexes

Muscle fibers are capable of hydrolyzing other nucleoside triphosphates (cf. Hasselbach, 1964). The present work using UTP and ITP confirms the conclusion of Weber (1969) and Dancker (1970) that various MgNTP complexes do in fact act like MgATP, but at different concentrations of the substrate.

In the EDTA buffer system the highest obtainable concentration of MgUTP or MgITP is about 200 μM. Since it was desired to examine the relaxing effects

of MgUTP and MgITP as well as their capacity for contractile activation it was anticipated that higher concentrations than this might be required. In order to achieve high levels of MgUTP and MgITP we reverted to the experimental procedure in which EGTA was the chelator for Ca. The concentrations of MgUTP and MgITP were calculated assuming that their association constants were identical with that of MgATP (cf. Martell, 1964). A large difference is not expected, since Mg is believed to interact with the triphosphate moiety which is common to the various NTP's (Cohn and Hughes, 1962).

VARIATION OF MGUTP The high cost of uridine triphosphate (UTP) limited the number of experiments on this substance. The data of Fig. 8 are based on six experiments, five with 1 mM UTP present (solid circles) and one with 2.5 mM UTP (open circles). The data of all six experiments with UTP fell on a single bell-shaped curve. The peak tension was produced when the concentration of MgUTP was about 30 μM, or about 10 times that of MgATP at peak tension. As already discussed in connection with the data on MgATP, the extreme left branch of the curve could not be obtained without the EDTA buffer system. With that buffer, however, it would not have been possible to obtain all of the right branch. The data of Fig 8 are sufficient, however, to show that there is an optimum concentration of MgUTP as the substrate and that increasing concentrations of MgUTP induce relaxation.

VARIATION OF MGITP In Fig. 9 the test solution contained 10 mM EGTA

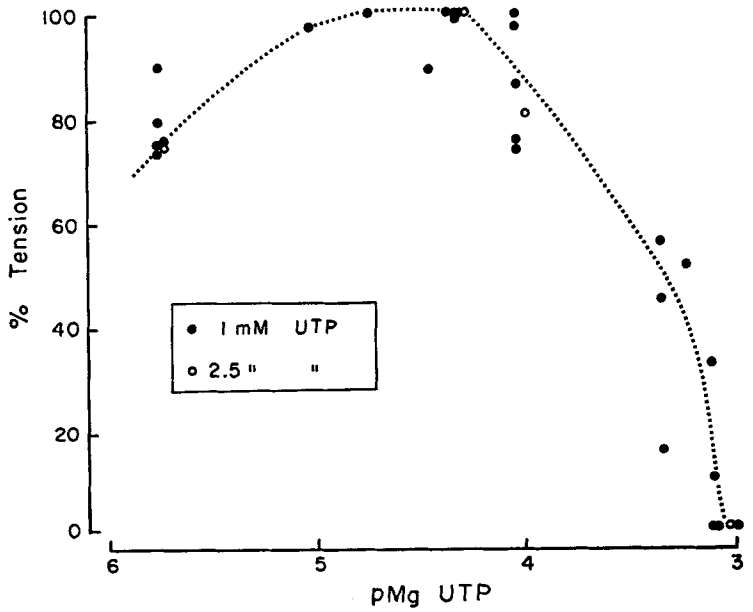

FIGURE 8. Tensions induced by MgUTP. Data for six experiments (five with 1 mM and one with 2.5 mM UTP present) are normalized with respect to peak tensions (as in Fig. 6). The abscissa is in units of pMgUTP. 10 mM EGTA was present in the test solutions.

and 1 mM Mg, in addition to various amounts of ITP. Withdrawal of Mg and ATP from the bathing solution induced a tension of about 45 mg. Addition of 0.5 mM ITP increased the tension to 130 mg and in the presence of 1 mM ITP the tension rose to 580 mg. A further rise to about 750 mg occurred when the ITP was increased to 5 mM. Relaxation had to be induced by substituting ATP for ITP.

If relaxation was to be induced by high MgITP it seemed that the concentration of the complex would need to be drastically increased above 1 mM, the limit set by the Mg. The records of Fig. 10 show that this was in fact the case. The four experiments were all done on one fiber. In each sequence ITP was increased in steps from 1 to 10 mM and Mg, which was constant for a given sequence, was varied from 0.1 mM (A) to 5 mM (D). In the presence of 0.1 mM Mg (A) the maximum tension attained, even on adding 10 mM ITP, was only slightly above the tension (ca. 70 mg) that was induced on withdrawal of Mg and ATP. Large increments in tension were produced when ITP was increased

FIGURE 9. Tension induced by increasing concentrations of ITP. The ATP in the relaxing solution was withdrawn at the large arrow (↑). The test solutions (small arrows) contained 10 mM EGTA and 1 mM Mg as well as the concentrations of ITP shown on the base line. Increasing ITP from 1 to 5 mM induced only a small additional tension. Relaxation was effected by reintroducing 1 mM ATP.

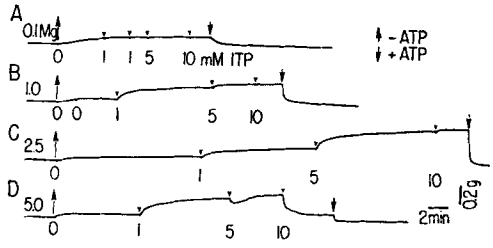

FIGURE 10. Responses to increasing concentrations of Mg as well as ITP. The ATP of the relaxing solution was withdrawn at the large upward arrow and reintroduced at the large downward arrow. Small arrows indicate the addition of ITP in the concentration specified on the base line of the tension registrations. When only 0.1 mM Mg was present (A) there was scarcely any tension increment above that produced on withdrawal of ATP. When Mg was raised to 1 mM (B) and 2.5 mM (C) the successive increments of ITP caused higher tensions, as described in the text. Relaxation was induced by 10 mM ITP when 5 mM Mg was present (D).

in the presence of 1.0 and 2.5 mM Mg (B and C). Noteworthy is the fact that in 1 mM Mg there was no increase in tension when ITP was raised from 5 to 10 mM (B), but an increment was observed in the presence of 2.5 mM Mg (C). This is expected if the substrate for activation is the MgITP complex. The concentration of the complex cannot be greater than that of one of the constituents, and the limiting factor in these experiments was the Mg. The maximum concentration of MgITP in B and C was insufficient to induce relaxation, but relaxation was obtained (D) when 5 mM of Mg was available to form the complex. When the level of ITP was raised to 5 mM there was a transient relaxation and a subsequent increase during which the tension oscillated somewhat. Oscillations were frequently observed when the chemomechanical response to MgNTP was close to the optimum and particularly on the supraoptimal branch of the curve. When the ITP was increased to 10 mM (Fig. 10 D) there was an obvious decrease in tension.

Data like those of Fig. 10, but with the ITP kept constant and the Mg varied, were also obtained. Three such curves on a single fiber are plotted in Fig. 11. When the ITP was 1 mM the concentration of MgITP (750 μM) was near its upper limit in the presence of 1 mM Mg. Increasing Mg beyond 1 mM had little effect (cf. also Fig. 10) nor could it have had. Thus, these measurements yield only the lower portion of the branch for increasing tension (solid circles). When 5 mM ITP was present (open circles) the curve extended to encompass about two-thirds of the branch for overoptimal substrate and decreasing tension. Complete relaxation was elicited in 10 mM ITP. The optimum of the tension-substrate curve for this fiber was at a concentration of 2.2 mM MgITP or nearly 1000 times greater than the optimum when MgATP was the substrate. The mean optimum concentration of substrate for all the 12 experiments with ITP present in 1, 5, and 10 mM concentrations was 2.35 mM MgITP, or pMgITP = 2.63 ($\pm$ 0.312 SD).

DISCUSSION

Skinned crayfish muscle fibers which are bathed in solutions containing millimolar quantities of Mg, ATP, and EGTA develop tensions when the ionized

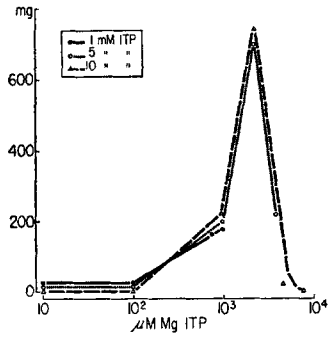

FIGURE 11. Tensions elicited by increasing concentrations of Mg with ITP present in constant concentration. Three sets of measurements on one fiber with 1, 5, and 10 mM ITP. The abscissa shows the concentration of MgITP. Peak tension was elicited when the MgITP was about 1000-fold greater than the concentration of MgATP (see Figs. 5 and 6). Further description in text.

Ca exceeds about 10^{-7} M (Reuben et al., 1970). This is a typical threshold value for many muscle preparations (Portzehl et al., 1964), including skinned frog fibers (Hellam and Podolsky, 1969) and forms the basis for ascribing to Ca a primary role in initiating contraction. Views regarding the precise role of Ca have undergone continuous change in the past decade (cf. Needham, 1960; Carlson, 1963; Hasselbach, 1964; Sandow, 1965, 1970; Weber, 1966; Ebashi and Endo, 1968; Bendall, 1969; Mommaerts, 1969). The current opinion is that under the above conditions Ca binds to troponin and, in so doing, removes an inhibitory effect of the troponin-tropomyosin system which, in the absence of Ca, prevents the interaction of actin and myosin and the hydrolysis of ATP.

The present experiments demonstrate that when pCa is maintained about three units above the threshold value both contraction and relaxation can be induced in skinned crayfish fibers by only varying the concentration of MgNTP. The peak tension is substantial, about 50% of the maximum capability of the skinned fiber. The tension occurs within a certain range of concentration of MgNTP, decreasing on either side of an optimal value. The latter depends upon the species of NTP and varies approximately in the ratio 1:10:1000 for the ATP, UTP, and ITP complexes with Mg.

Entirely different types of data, from studies on minced myofibrils of rabbit muscle, also in the virtual absence of Ca (Weber, 1969; Weber et al., 1969), are strikingly similar to our findings which measured tension in skinned muscle fibers of crayfish. Considering the difference in the experimental material and methods of study, the precise agreement is truly remarkable. Hydrolysis rates and syneresis of the myofibril preparations are quantitatively similar functions of pMgNTP as is tension in the crayfish fibers. The effectiveness of the substrates, MgATP, MgUTP, and MgITP, is in the same order in the rabbit, but the spread between MgATP and MgITP is 1:100. The optimum concentration of MgATP for hydrolysis in rabbit myofibrillar fragments is very close to the optimum concentration for tension in the crayfish fibers.[3] Data similar to those of Weber (1969) and Weber et al. (1969), but with the experimental conditions less well-defined, were reported by Tonomura and Yoshimura (1960), Watanabe and his colleagues (Watanabe et al., 1964; Watanabe and Yasui, 1965), Levy and Ryan (1967), and Dancker (1970).

White (1970) found that glycerinated muscles bathed in a relaxing solution containing 5 mM EGTA, 5 mM ATP, and 5 mM $MgCl_2$ contracted when transferred to a rigor solution that contained 5 mM $MgCl_2$, 5 mM EGTA, and no ATP. They relaxed reversibly when ATP was added or when MgATP was increased. White also confirmed earlier work by Jewell and Rüegg (1966) and

[3] Weber et al. (1969) used an apparent association constant (K_a) of 33,000 to calculate 10 μM as the optimum concentration of MgATP for hydrolysis (personal communication from Dr. Weber). Our calculation of 3 μM for optimum tension (Fig. 6) was based on a threefold smaller K_a (11.4×10^3, Table II).

vom Brocke (1966) that insect muscles contract in a rigor solution that contains no Mg or Ca but has 5 mM ATP and EDTA. Thus, the glycerinated muscles contract in the absence of Ca, provided that only trace amounts of either Mg or ATP are present (as in our Fig. 3).

Since similar less than maximal tensions are produced in the presence of suboptimal and supraoptimal levels of MgNTP, it might be supposed that the physical state of the muscle fiber is similar on the two sides of the optimum. We have measured (unpublished data) the compliance of the skinned fibers at two corresponding points of the bell-shaped relation by applying passive stretch. The tensions evoked by these stretches are closely similar. This finding is in accord with that of Levy and Ryan (1967) and Weber (1969) that superprecipitation is inhibited at both suboptimal and supraoptimal concentrations of MgNTP.

White (1970) regards the contraction of glycerinated muscle in the absence of either added ATP or Mg (rigor solutions) as "rigor." In view of the present results this concept of rigor needs further definition. The data of Fig. 7 are relevant in this connection. When Ca was present (lower row), increasing Mg to 10 or 20 μM caused an increase in tension. Yet it will be noted that the tensions induced by adding 2 or 3 μM Mg were essentially the same whether Ca was present or absent. With Ca present the smaller contractions form part of a continuum from no tension to the maximum output of the fiber, and these reversible responses are not considered to be due to rigor.

The data on MgUTP and MgITP (Figs. 8–11) establish that the nucleoside moiety is the important determinant of the range of MgNTP concentrations over which the exergonic chemical events are optimal. The curve relating tension and concentration of MgUTP (Fig. 8) is broader than that for MgATP (Figs. 5 and 6), while that for MgITP (Fig. 11) is narrower. In order to produce the effects seen in Figs. 8–11 larger amounts of the NTP's were added than was the case with ATP. This was particularly true for the experiments with ITP, since 5–10 mM were necessary to produce relaxation (Figs. 10 and 11).

It is possible that the ITP may have contained sufficient ATP as a contaminant to affect the shape of the curve seen in Fig. 11. This is unlikely, however. The presence of ATP as a contaminant should be more evident with 10 mM ITP than with 5 mM. The two curves in Fig. 11 might have been expected to deviate to some degree as Mg was increased, and in the presence of 10 mM ITP a secondary elevation on the rising branch should have developed, due to the accumulation of MgATP. This was not observed in any of the experiments which replicated the data of Fig. 11.

The literature on muscle mechanochemistry has expressed numerous and divergent views regarding the possible roles of free Mg or Ca ions, free ATP,

and the metal-ATP or NTP complexes in contraction and relaxation (Weber, 1959; Tonomura and Yoshimura, 1960; Weber and Winicur, 1961; Hasselbach, 1964; Watanabe et al., 1964; Watanabe and Yasui, 1965; Levy and Ryan, 1967; Weber, 1969). In general, the uncertainties and conflicts stem from the complexities of the mechanochemistry of muscle, the trace concentrations of Ca and MgATP which are essential, and the multiple equilibria involved. Weber (1969) and Weber et al. (1969) in presenting their experimental biochemical data stressed the role of MgATP much in the same way that we have done in the present study on the physiological responses of the muscle fiber. This presentation greatly simplifies the interpretation of the results. Both types of data agree that it is the MgNTP that is the substrate for the phosphatase of actomyosin. Both hydrolysis of the substrate and tension output can occur in the virtual absence of Ca. The role of Ca appears to be one of preventing the reduction in ATPase activity when the concentration of MgNTP is raised beyond an optimum value (cf. also Levy and Ryan, 1967).

Skinned fibers, which respond to MgNTP with contraction and relaxation, are also sensitive to Ca (Reuben et al., 1970; and data to be published). Only fibers that are relaxed in supraoptimal concentrations of MgNTP can be activated by decreasing pCa (Fig. 7; Brandt et al., 1970). Sensitivity to Ca is generally taken as evidence for the presence of a functional troponin-tropomyosin system and the latter in turn is believed to inhibit hydrolysis of MgATP when pCa is greater than 7.5. Thus, the troponin-tropomyosin system appears to be intact in the skinned crayfish muscle fiber.

The data of Ebashi and Endo (1968, Fig. 5 A) indicate that when pCa is 6.3 and MgATP is 500 μM superprecipitation occurs immediately in the absence of the tropomyosin system. In the presence of the tropomyosin systems superprecipitation is delayed up to 1 hr. This may be interpreted as indicating that the tropomyosin confers the property of inhibition by supraoptimal substrate on the actomyosin (Levy and Ryan, 1967; Weber et al., 1969; Dancker, 1970). However, Dancker (1970) found that high MgATP induced inhibition in actomyosin when tropomyosin was absent or was inactivated by tryptic digestion. In view of our results and those of Weber et al. (1969) it would be desirable to test for the presence of inhibition in tropomyosin-free systems at higher pCa's and larger ranges of substrate concentration.

The relation between pMgNTP and tension yields bell-shaped curves (Figs. 5, 6, 8, and 11) and can be described operationally by the kinetics of substrate inhibition (Fig. 6). Earlier workers have suggested various models of substrate inhibition (for references see Weber, 1959; Needham, 1960; Levy and Ryan, 1967). As far as we know, however, only Tonomura and Yoshimura (1960) and Levy and Ryan (1965, 1967) have proposed substrate inhibition based on the combination of MgNTP, as the substrate, with two or more functionally

distinct sites on the enzyme. On the basis of kinetic data on ATPase activity Weber (1959) had proposed, furthermore, that whether Mg and ATP caused contraction or relaxation depended upon the concentration of ionized Ca.

The simplest possible assumptions regarding substrate inhibition, which satisfy our data, involve the reaction of one MgNTP with the enzyme for activation and of a second MgNTP binding to the enzyme for relaxation. A more complex model which attempts to account for the role of Ca and for the movement of filaments has been presented by Levy and Ryan (1966, 1967). We assume for simplicity that the sites are located on myosin or actomyosin, but it is also possible that the reaction sites may be on a complex containing tropomyosin (cf. Dancker, 1970), and perhaps also troponin (cf. Weber et al., 1969).

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