*STUDIES ON THE FORMATION OF AN ENZYME-SUBSTRATE COMPLEX BETWEEN MYOSIN AND ADENOSINETRIPHOSPHATE**

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Knowledge regarding the formation of an enzyme-substrate complex between myosin and adenosinetriphosphate‡ has implications beyond its relation to formal enzyme kinetics. It has been concluded that during its transient combination with ATP, the myosin molecule undergoes some change, which in the case of actomyosin solutions expresses itself in a reduction of the viscosity and turbidity.[1–6] In actomyosin gels or in organized fiber systems, this same binding of ATP may be crucial in the energetics of contraction.[2, 3, 7] Indeed, the quantitative aspects of the turbidimetric change in actomyosin induced by stoichiometric amounts of ATP maintained by a rephosphorylating enzyme system[8] can be treated from the point of view of a reversible binding of ATP to myosin.[9] It is true, however, that the experimental study of such problems has remained restricted to indirect findings regarding the ATP binding, derived from the magnitude of the associated secondary effects, and that these may be complicated by the energetics of the dissociation of the actomyosin in addition to that of the ATP-myosin complex itself.[10]

In the present work, we have accomplished a direct demonstration of the binding of ATP by myosin, employing the light emitted by the firefly luciferase-luciferin system in response to ATP[11, 12] as a continuous measure of the concentration of free ATP in the system. This first publication deals with the general foundation of this methodology and with the determination of the stoichiometry of the ATP-myosin interaction, while the subsequent paper will deal with the determination of the various kinetic constants.[13]

Materials and Methods.—Myosin: Myosin was prepared from rabbit skeletal muscle with the standard procedures of this laboratory[14]; it was homogeneous in the ultracentrifuge. The protein was dissolved in 0.3 *M* Na_2SO_4, 0.01 *M* Tris buffer pH 7.0 and was preserved in ice with a drop of chloroform. The stock solution had a concentration of the order of 1 per cent, as determined by Kjeldahl analysis.§

Luciferase-luciferin: A partly purified extract was prepared from dried firefly lanterns‖ as described by McElroy and Strehler,[15] but the fractionations with ammonium sulfate were performed at pH 7.5 throughout, except for the final dissolution at pH 7.0. The extract was preserved in 1–2 ml samples in a deep freeze and each sample was used on the same day it was opened (repeated freezing and thawing leads to a loss in activity); after thawing, the sample was cleared by centrifugation and kept on ice until use.

Creatine phosphoryltransferase: This enzyme was purified according to Kuby, Noda, and Lardy[16, 17] or according to Padieu and Mommaerts[18] by a narrowly cut acetone precipitation. The latter product approached the crystalline preparations in its specific activity and in its boundary spreading in the ultracentrifuge. The product was lyophilized and preserved in the deep freeze.

Incubation mixture: The constituents of the reaction mixture depended on the nature of the experiments but, unless indicated otherwise, had the following composition. A medium was prepared by mixing 20 ml 0.2 *M* Na_2HAsO_4, 10 ml 0.2 *M* $MgSO_4$, and 50 ml 0.5 *M* Na_2SO_4, and adjusted to pH 7.0 or 7.6 with H_2SO_4. In this mixture, 1 to 5 (usually 2.5) mg CPase were dissolved per ml. In a 2-ml micrometer syringe with a 4-inch 18-gauge needle, 0.78 ml of medium containing CPase was aspirated, followed by 0.02 ml of 0.05 *M* CP, 0.08 ml of FF and 0.02 ml of ATP solution of a given concentration in this sequence. There was no reaction at this point, since the ATP remaining located in the needle was not mixed with the other reactants, notably the Mg. Then the contents were suddenly dispensed into the cuvette of the apparatus (see below) giving a rapid light flash, followed by a steady level of light production, declining only slowly in the course of many minutes. After a standard time, 0.10 ml of 0.3 *M* Na_2SO_4, 0.01 *M* Tris pH 7.0 was added (in lieu of myosin, see below) by rapid ejection from a 0.25 ml syringe, which reduced the light because of dilution. A calibration curve for the dependence of the light intensity upon the free ATP concentration was made, after Na_2SO_4 had been added. This curve was linear below a final ATP concentration of 5×10^{-6} *M*, while a progressive curvature occurred at higher ATP concentrations. Without CP and CPase or with only one of these components, no luminescence occurred in the absence of added ATP. With CP and CPase present, however, often a small amount of luminescence was found in the absence of added ATP so that the calibration curve did not go through the zero point. By extrapolating the linear part of the calibration curve to the negative side of the ordinate until it intersects the abscissa, the ATP present in the system under these conditions is obtained (maximally 0.6×10^{-6} *M*). This concentration is added to the concentration of the added ATP. We presume that the effect is caused by the presence of some ADP in the firefly enzyme.

In the experiments proper, 0.10 ml of a myosin solution in 0.3 *M* Na_2SO_4, 0.01 *M* Tris pH 7.0 was added by rapid ejection from a 0.25 ml syringe. The final concentration of the myosin in the total mixture was of the order of 0.1 per cent or 2.4×10^{-6} *M*, based upon a molecular weight of 420,000.[19, 20]

Recording of the light emission: The mixture described above was contained in a tall cuvette of $0.8 \times 0.8 \times 10.0$ cm inside dimensions, placed in a closed aluminum holder facing a photomultiplier cell, and the photocurrent was recorded as described in the Appendix. The micrometer syringes by means of which the additions were made fitted through a light proof seal in the cap of the cuvette holder. Mixing was rapid, occurring in about 0.25 second as judged by the time needed for the light to find its new, reduced, level upon the addition of Na_2SO_4 in the blank experiments.

Effect of the turbidity of the mixture upon the emitted light: If the light is homogeneously emitted by all parts of the solution in a container of horizontal cross section $l \times l$ cm, each vertical sheet of thickness dl facing the photocell will contribute the amount Idl to the light reaching the detector directly (Fig. 7). Due to the turbidity t of the medium, the total light so reaching the photocell will not be Il, but a lesser amount obtained as follows:

$$I'l = \int_0^l Ie^{-tl}\, dl = I(1 - e^{-tl})/t$$

while the attenuation, defined as $1 - (I'/I)$ becomes zero for $t = 0$. When myosin solutions, as customarily used in the experiments after centrifugation for 30 min at 30,000 rpm, were diluted into the reaction mixture, the resulting turbidity in the spectral range of the luminescence reaction was about 0.01, and hence the attenuation was calculated to be 0.5 per cent. (It would be 3.9 per cent for $t = 0.1$.) Since the distance between photocell and cuvette was 4 times the width of the latter, the pathlength of obliquely traveling light is still closely the same. Somewhat more effect will be exerted upon light traversing the entire cell after reflection by the walls, and the total attenuation may be 0.5 to 1.0 per cent in average cases. This has been neglected in the description of the measurements, since it is considered to be within the limits of error.

Accessory Experiments.—Kinetics of creatine phosphoryltransferase: Since the design of the principal experiments required a knowledge of the Michaelis constant and maximal velocity of CPase, acting as the rephosphorylating enzyme, under the conditions of the experiment, we have determined these quantities with the same general methodology, but for the omission of myosin and the use of 100 times lower CPase concentrations. The reaction mixture therefore consisted of 0.78 ml of medium with 0.025 mg CPase per ml, 0.02 ml of 0.05 *M* CP, 0.08 ml of FF and 0.02 ml of water. No light is emitted from this mixture. When 0.1 ml of ADP solution is added, luminescence starts and is evaluated in terms of the velocity of ATP formation at different concentrations (Fig. 1). When the same amount of ADP was

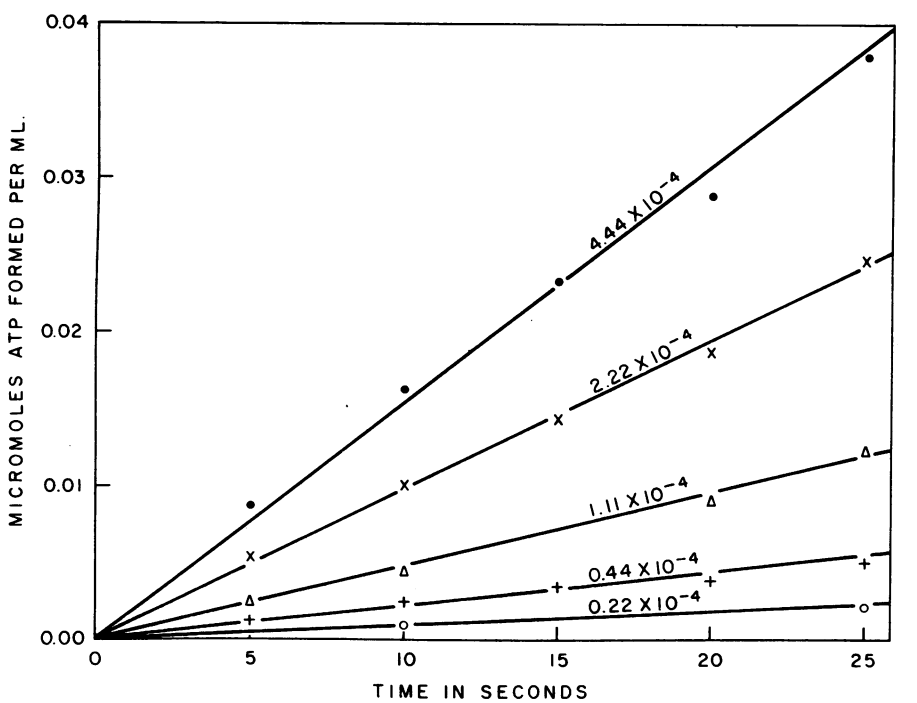

FIG. 1.—Determination of the velocity of ATP formation by creatine kinase, at different initial ADP concentrations indicated at the individual curves. Description of the experiment in the text; 0.025 mg CPase per ml of medium; 0.001 *M* CP; 0.22 to 4.44 $\times 10^{-4}$ *M* ADP.

added in a 0.02 ml volume, due to some imperfection in mixing, there was an initial flash, but when added as described, the velocity could be evaluated throughout the entire course. The ADP concentrations in the final mixture were of the order of 10^{-5} to 10^{-3} M; the CP concentration was 10^{-3}, as in the major experiments, so that the data obtained apply to the same conditions. In this manner we obtained 7×10^{-4} for the Michaelis constant, and 3.6×10^{-9} moles per ml per sec. for the maximal velocity (Fig. 2). The maximum velocity is therefore 100 times higher; i.e., 3.6×10^{-7} moles per ml per sec. at 2.5 mg of CPase per ml of medium (concentration in final mixture 1.95 mg/ml) as employed in the principal experimental series.

The velocity of ATP formation was not measurably diminished by the addition of ATP (final concentration 10^{-5} M) and it is estimated that, if ATP acts as a

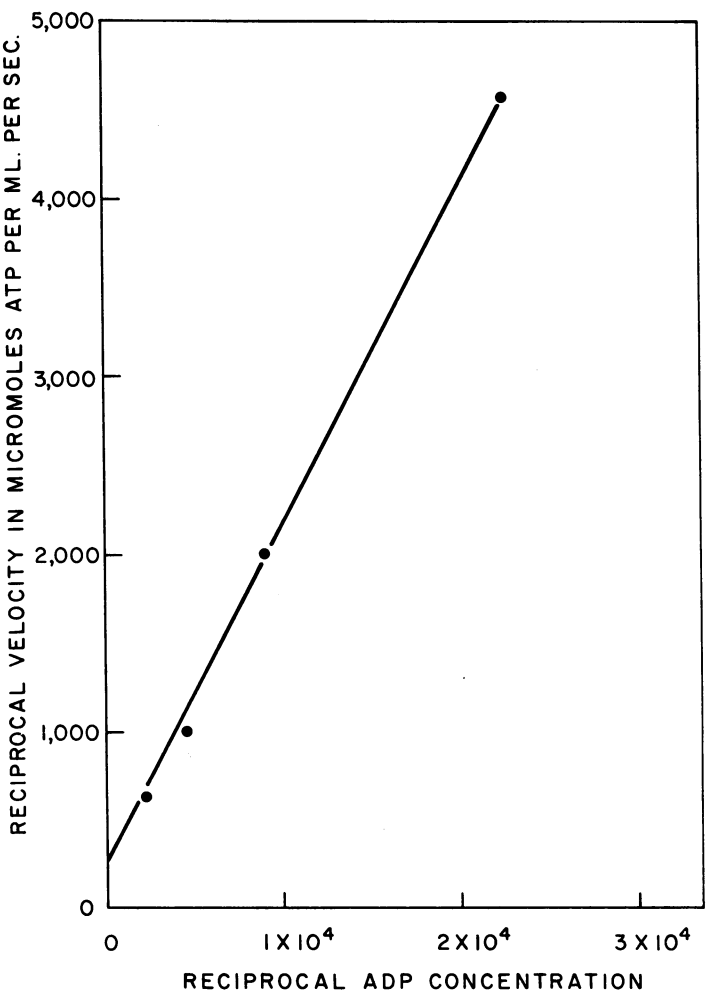

FIG. 2.—Reciprocate plot of the reaction velocities found from Fig. 1 for the determination of K_B and the maximal velocity ($k_B \times B$) of creatine kinase.

competitive inhibitor, the inhibitor constant K_I must be in considerable excess over 10^{-5} [when $1 + (10^{-5}/K_I) \simeq 1$, then $K_I \gg 10^{-5}$]. That, accordingly, the binding of ATP to CPase is negligible was shown experimentally by the demonstration that the luminescence in response to ATP is identical in the presence and absence of CPase (provided that the CPase solution is sufficiently clear so that its turbidity does not attenuate the light).

Kinetics of myosin-ATPase: As this will be discussed at length in the following paper, it suffices to state that the drop in light emission upon the addition of myosin in the absence of CP and CPase at different concentrations of ATP has been used to determine the Michaelis constant (1 to 2 $\times$ 10^{-6}) and the maximal velocity (6.0×10^{-11} moles per ml per second for 0.1 per cent myosin) in the environment described.

The Binary Enzyme System.—In the principal experiments of this work, we have studied the changes in concentration of free ATP during the approach to a steady state in the two-enzyme system:

$$\text{ATP} \xrightarrow{\text{myosin-ATPase}} \text{ADP} + \text{P} \qquad \text{(A)}$$

$$\text{ADP} + \text{PC} \xrightarrow{\text{CPase}} \text{ATP} + \text{C} \qquad \text{(B)}$$

wherein the ADP is continually rephosphorylated to ATP in reaction (B) to the extent that it is formed in reaction (A) (compare Mommaerts and Hanson[8]).¶

We shall make use of the simplification that in reaction B, CP is present in such excess (in proportion to the velocity of its breakdown, as limited by the low velocity of reaction A), that during the time of observation the CPase remains saturated with CP to a constant degree, so that only its dependence upon the ADP concentration needs to be considered.

The following notations will be used:

A, B: the total molar concentrations of myosin and CPase, respectively.

k_A, k_B: the breakdown constants, commonly called k_3 (or sometimes k_2), for these enzymes; hence, $k_A A$ and $k_B B$ represent the maximal velocities, commonly called V_m, for these enzymes.

K_A, K_B: the Michaelis constants, $(k_2 + k_3)/k_1$, or $(k_{-1} + k_2)k_1$ for these enzymes.

a, b: the concentrations of free ATP and free ADP, respectively.

$a_A{}^*, b_A{}^*$: the concentrations of ATP and ADP, respectively, bound to myosin.

$a_B{}^*, b_B{}^*$: the concentrations of ATP and ADP, respectively, bound to CPase.

$\bar{a}$: the total ATP concentration added.

Hence: $\bar{a} = a + b + a_A{}^* + b_A{}^* + a_B{}^* + b_B{}^*$.

Negligible quantities: Not all the components contributing to $\bar{a}$ in the last formulation are preponderant, and we shall show that the following can be neglected.

$b_A{}^*$. While ADP acts as a competitive inhibitor for myosin, it does so largely by virtue of its Ca-binding power; its actual affinity toward myosin is quite small.[22] From the basic equation for competitive inhibition,

$$K_I = \frac{(A - a_A{}^* - b_A{}^*)b}{b_A{}^*},$$

we obtain $b_A{}^* = (A - a_A{}^*)\, b/K_I$ when $b \ll K_I$, and estimate, with a likely value

of $K_I \approx 10K_A$,[22] that $b_A^* \simeq 0.1\,b$ as $A \approx K_A$ in our experiments, and is, therefore, a negligible second order quantity.

a_B^*. As has been indicated under the heading "kinetics of creatine phosphoryltransferase," this quantity is likewise negligible. Also, this conclusion can be arrived at by calculation. As $a_B^* = (B - b^*)\ a/K_I$ when $a << K_I$, $K_I > K_b$ ($= 7 \times 10^{-4}$), and $B = 24 \times 10^{-6}\ M$, (for 2 mg per ml; mol. wt. = 80,000 according to Kuby, Noda and Lardy[16]), $a_B^* < 0.03a$.

b_B^*. Since this is equal to $Bb/(K_B + b)$, and since $B = 24.10^{-6}$, and $K_B = 7.10^{-4}$, $b_B^* = 0.03\,b$, and is a negligible second order quantity.

The steady state: It results from the foregoing section that

$$\bar{a} = a + b + a_A^*$$

while the determination of a_A^* is the purpose of the investigation. This is a considerable part of a since $a_A^* = Aa/(K_A + a)$, and A is of the order of K_A. Since $\bar{a}$ is set by the experimenter and measured from the blank calibration curve, and a is indicated by the measurement, it remains to examine b.

The steady state level of b is found as follows:

$$-\frac{da}{dt} = \frac{k_A A a}{a + K_A} \qquad \text{and} \qquad -\frac{db}{dt} = \frac{k_B B b}{b + K_B}$$

and hence,

$$b = \frac{k_A A K_B a}{k_B B(a + K_A) - k_A A a}$$

Using the values obtained as described in the section *Accessory Experiments*, one obtains the quantities listed in Table 1.

TABLE 1

VALUES OF b AND OF THE ATP MAINTENANCE RATIO $a/(a + b)$ AS A FUNCTION OF a FOR 2.5 MG/ML CPASE IN THE SALT MEDIUM (FINAL CONCENTRATION CPASE 2.02 MG/ML) AND FOR 1 MG/ML MYOSIN FINAL CONCENTRATION AT PH 7.0; a = FREE ATP; b = FREE ADP BOTH EXPRESSED IN MOLE PER LITER.

	$K_A = 1.0 \times 10^{-6}$		$K_A = 2.0 \times 10^{-6}$	
a	b	$a/(a + b)$	b	$a/(a + b)$
0.2×10^{-6}	1.9×10^{-8}	0.912	1.05×10^{-8}	0.950
0.5	3.9	0.928	2.32	0.956
1.0	5.8	0.945	3.9	0.962
2.0	7.74	0.963	5.8	0.972
5.0	9.7	0.981	8.3	0.984
10.0	10.6	0.988	9.8	0.990
$>>K_A$	11.65	1.0	11.65	1.0

It is concluded from Table 1 that under the conditions of our experiments there is effective maintenance of ATP, but that b cannot entirely be ignored. That $a/(a + b)$ approaches unity at high values of a is not relevant, since under those conditions the absolute value of b is sizeable, and not necessarily negligible in comparison with a_A^*. We have preferred not to derive a general expression for these factors, but to calculate b for individual cases, and apply these data as a correction factor, by means of the equation $a_A^* + b = \bar{a} - a$ (see the section on *Binding curve of ATP*). In order to have the most favorable conditions, the experiments were

performed in a medium of Na-salts instead of K, and with Mg as also required by the FF. The actually occurring rates of splitting were very low indeed as in other studies in a Na-milieu in the absence of Ca,[23] but inasmuch as only a small stoichiometric excess of ATP over myosin is present, this would still disappear in a few seconds. Whether this activity is due to traces of Ca or to a true functioning of Mg-myosinate as enzyme, although with much lower activity than Ca-myosinate, is not known.

Experiments in the Steady State Condition.—Description of an experiment: As is shown in Figure 3, shortly after mixing the components there was established a fairly constant light emission with only a small and steady decline, and a stepwise diminution when, usually 30 seconds later, in a blank experiment, Na_2SO_4 solution was injected. These curves were reproducible, and were used to evaluate the background effects of the time decay of the FF luminescence, and the dilution factor in the experiments proper. The calibration curves, as noted before, were determined after this addition of Na_2SO_4 solution. With the addition of myosin, on the other hand, there was a greater reduction of the light emission (Fig. 3), indicative of the ATP binding by myosin.

Binding curve of ATP: From these experiments, expressing the resulting luminescence level I in terms of the remaining free ATP concentration a, and its diminution $-\Delta I$ in terms of $a_A{}^*$ by means of the calibration curve, plots were obtained (Fig. 4) for the bound ATP versus the free ATP concentration. Such experiments have been performed for various concentrations of myosin and ATP at pH 7.0 and 7.6 with identical results.

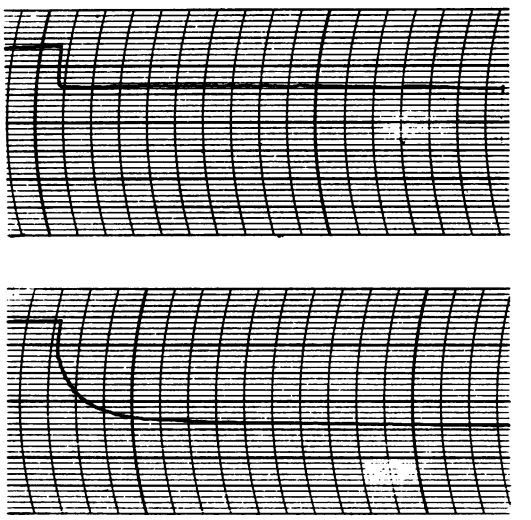

FIG. 3.—Examples of actual tracings recording the changes in luminescence in the complete reaction medium containing 6×10^{-6} M ATP. In both records, the baseline corresponds to zero light intensity, and the curve starts at the left at its original level. Timescale, 5 seconds per division. The upper tracing shows the effect of dilution following the injection of 0.1 ml 0.3 M Na_2SO_4, 0.01 M Tris buffer, and the slow spontaneous decline of the luminescence. The lower tracing shows the effect of injecting 0.1 ml of 0.1% myosin, the dimunition of light due to the binding of ATP, and the establishment of the steady state in the binary enzyme system.

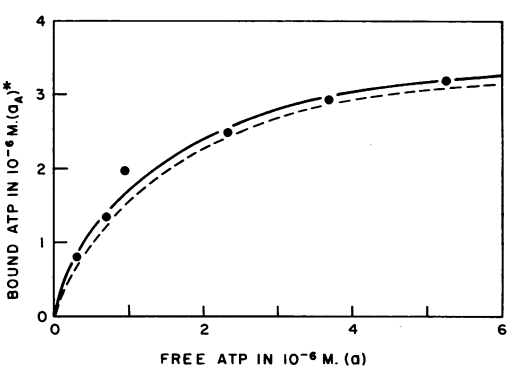

FIG. 4.—A binding curve for the ATP-myosin complex, showing the bound ATP as a function of the free ATP. The points are determined experimentally (myosin concentration 0.166% or 3.96×10^{-6} M) and the solid curve is drawn through them. The broken curve is obtained by subtraction of the respective values of b, evaluated by calculation as indicated in the text.

In Figure 4, the experimentally obtained binding curve has been corrected for incomplete maintenance of the free ATP concentration by calculating b according to Table I, taking into account the higher myosin concentration, 0.166 per cent, in this particular experiment. The final level of the ATP binding at saturation was determined accurately from a plot of a_A^*/a versus a_A^*.[24] The maximal value, n, of a_A^* found by extrapolation to the abscissa, corresponded to a combination of 1 mole of ATP with 1 mole of myosin, with an error of a few per cent (Fig. 5). From the same data, by extrapolation to the ordinate, K_A was found to be 1.6×10^{-6} in good agreement with the Michaelis constant derived from conventional kinetic measurements[13] as mentioned before.

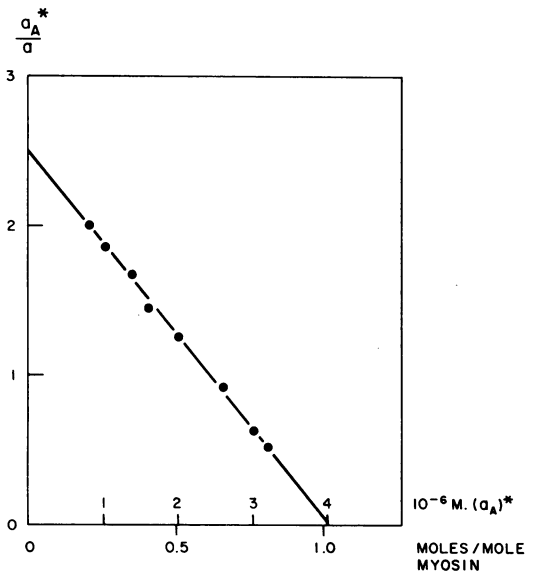

FIG. 5.—Plot of the function a_A^*/a versus a^*_A. The points were taken from the dotted curve in Figure 4, and therefore, corrected for the effect of the b factor, as indicated in the legend to Fig. 4. The line is straight, as required by theory, and it intercepts with the abscissa and the ordinate, respectively, yield the maximum binding value n, and n/K_A. In this experiment the myosin concentration was 3.96×10^{-6}, and n is found to be 4.02×10^{-6}.

Does myosin bind luciferin? The given presentations have all been based upon the assumption that the described effects are entirely due to ATP binding by myosin. We shall now consider whether, in addition, a binding of luciferin could also have been contributory. This was unlikely to be the sole cause, since in that case the results would, at constant myosin concentration, not have followed the described dependence on the ATP concentration but would, instead, have shown a constant fractional diminution of the luminescence for each ATP concentration. It was found desirable, however, to examine experimentally whether any part of the effects could have resulted from that contingency, by testing whether myosin can bind luciferin.

The luciferin used for that purpose was prepared[25] by extraction of dried firefly lanterns with water for 10 min at 80°C, acidification to pH 3.5, extraction into ethylacetate, vacuum evaporation of the solvent, and dissolution of the residue in water at pH 7.0. The product was characterized by its fluorescence at pH 6.5 and 11.9.[25] It was established that in our FF preparations the luminescent intensity was only weakly enhanced by adding luciferin to double or triple the original concentration, as determined by fluorescence measurement, so that sizeable degrees of binding would be required to give effects comparable to those observed.

The possible binding of luciferin was investigated by incubating luciferin or FF with myosin, precipitation of the latter by dilution, and removal by centrifugation. Luciferin was determined in the supernatant solution by its fluorescence, and was found to be undiminished. Also, a luciferin solution of a concentration as found in FF was added (0.4 ml) to 10 ml or 0.1 per cent myosin in the usual medium, and 5

ml of this was dialyzed against 5 ml of water for 48 hr. In that time, dialysis had come to equilibrium as judged from a similar experiment without myosin, and also in the myosin experiment the inside and outside luciferin concentrations, determined fluorometrically, were identical within the limits of error. No evidence was, therefore, obtained of any binding of luciferin by myosin. Hence, the phenomena under investigation were neither wholly, nor partly, due to such binding.

Discussion.—The results communicated in this paper provide a direct determination of the stoichiometric ratio in which ATP and myosin combine. Earlier estimations of this proportion by authors mentioned in the introduction were all of a preliminary nature and tended to give too low a value for the myosin equivalent, partly due to ATP hydrolysis (even in the experiments with rapid recording techniques by Tonomura *et al.*[6] which otherwise constituted a greatly improved approach to the problem), partly because there was no way to distinguish between free and total ATP. Previously[8, 9] on the basis of measurements in a binary enzyme system, we have found that 1 mole of ATP interacts with 504,000 grams of actomyosin, which presumably contained 400,000 to 450,000 grams of myosin, and recently Tonomura and Morita[26] came to a similar value by an extrapolation procedure. Now, the reaction with one mole of pure myosin has been directly observed.

The direct demonstration that 1 mole of ATP reacts with about 420,000 grams of myosin is in excellent accord with the currently accepted value of the molecular weight of this protein. This result was obtained by Mommaerts and Aldrich[19] from studies on the approach to equilibrium sedimentation and diffusion on pure myosin, while von Hippel *et al.*[20] obtained a similar value for the lightest component in an unfractionated myosin actomyosin mixture and for myosin.

The finding of one enzymatic site per myosin molecule agrees with Gergely's result that myosin binds 1 mole of pyrophosphate[27] (but see Tonomura and Morita[26] for a deviating report), and is in line with the situation in several other enzymes, such as chymotrypsin and trypsin (quoted by Dixon and Webb[28]), enolase[29] and acyldehydrogenase.[30] It also invites a comparison with the univalent interaction between ATP and G-actin.[31]

The direct demonstration of ATP binding by pure myosin gives rise to several further comments. In work on the ATP effect upon actomyosin, it had not been rigorously excluded that actin rather than myosin might have been the recipient protein.[32] This decision has now been brought, and is furthermore in accord with the finding,[33] that F-actin acts as a competitive inhibitor for myosin-ATPase. More explicitly than in the work on ATP effects upon actomyosin, the present measurements would also detect any binding of ATP by myosin on sites not enzymatically active or not involved in the interaction with actin. Therefore, our result is unfavorable to the assumption by Morales[7,34] that myosin is multivalent for ATP and, upon combination with a number of ATP molecules, acquires a considerable diffusely distributed negative charge, leading to extension or to contraction. This difficulty has, meanwhile, been recognized by Morales[35] as well. We cannot rigorously exclude that additional ATP-binding at nonenzymatic sites might occur at much higher concentrations, but this would then imply that those complexes would have a much higher dissociation constant.

Kinetic aspects of the myosin-ATP combination will be discussed in the subsequent paper.[13]

APPENDIX: THE RECORDING OF LUMINESCENCE IN THE ENZYME SYSTEM

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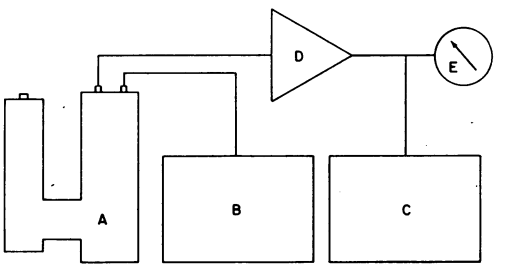
The photometer is of a type similar to commercially available instruments such as the Photovolt. It consists of a self-contained high voltage power supply for the 1P21 photomultiplier and a direct-coupled amplifier capable of driving a 0–200 μA meter and a chart recorder (Fig. 6). The gain of the amplifier is adjustable in accurate decade steps with a maximum sensitivity of 10^{-9} amp full scale which corresponds to approximately 10^{-12} lumens. Although the 1P21 was selected from a large number for maximum sensitivity and minimum noise, during operation at 25°C. the noise was still sufficient to require some reduction in the bandwidth of the amplifier. This was accomplished by placing a capacitor in shunt with the microammeter.



A – PHOTOMETER HEAD
B – 1,000 V D.C. REGULATED POWER SUPPLY
C – CHART RECORDER
D – D.C. AMPLIFIER
E – METER

FIG. 6.—Block diagram of photomultiplier photometer and recording equipment.

The photometer head (Fig. 7) was built largely from available parts resulting in a relatively inefficient optical system. This was improved to a degree by coating the interior of all parts except the cuvette chamber with Linde Sapphire A. Ideally the cuvette and the photomultiplier should occupy a chamber with reflecting walls, but the described arrangement (in which it is estimated that 15 to 20 per cent of the light reaches the photocell) had a sensitivity and signal-noise ratio sufficient for the present work.

Summary.—A methodology has been developed by which the concentration of ATP in a reaction system is continuously measured by recording the light emitted by the firefly luciferase-luciferin system. When in such a system, also containing phosphocreatine and creatine kinase, myosin is introduced, the decrease in luminescence is due to the binding of ATP by myosin, since the system could be so designed that all other factors were negligible.

The enzyme-substrate binding takes place in a one to one molar ratio.

* The substance of these investigations was presented at the Federation meeting at Atlantic City, April, 1959. This work was carried out with support of grants from the Life Insurance Medical Research Fund and from the National Heart Institute (Grant No. H-3067) of the National Institutes of Health.

† Scholar of the Sister Elizabeth Kenny Foundation.

‡ The following abbreviations are used throughout this paper: ATP (adenosinetriphosphate); ADP (adenosinediphosphate); CP (creatine phosphate); C (creatine); ATPase (adenosinetriphosphatase); CPase (creatinekinase or creatine phosphoryltransferase); FF (partly purified firefly extract, containing luciferase and luciferin); M (myosin).

§ We are indebted to Mr. Alfred Wallner for the majority of these preparations and Kjeldahl analyses.

‖ We are indebted to Dr. G. D. Novelli, Oak Ridge National Laboratories, for generous gifts of dried fireflies.

¶ Reaction B is an equilibrium reaction, but since at pH 7.0 it occurs predominantly as written,[21] the influence of its reversal has been ignored. There is experimental justification for this, since we never observed a reduction of the steady-state level of ATP when the reaction was continued for far longer times than the usual periods of observation.

[1] Mommaerts, W. F. H. M., *Studies Inst. Med. Chem. Szeged.* **1**, 37(1942).

[2] Mommaerts, W. F. H. M., *J. Gen. Physiol.*, **31**, 361 (1948).

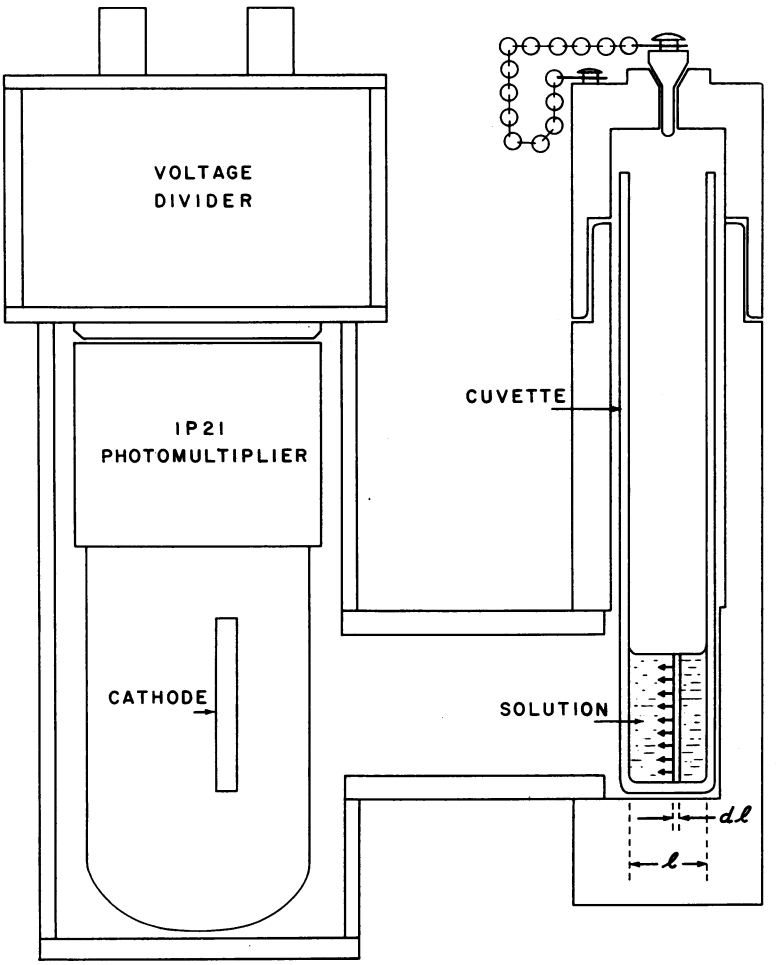


FIG. 7.—Cross-sectional view of the photometer head assembly.

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*KINETIC CONSTANTS OF THE INTERACTION BETWEEN MYOSIN AND ADENOSINETRIPHOSPHATE**

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In the preceding paper,[1] we have indicated how in a multienzyme system consisting of firefly luciferase and luciferin, creatine phosphoryltransferase, phosphoryl creatine and adenosinetriphosphate,‡ it is possible to determine the binding of ATP which ensues upon the addition of myosin to the system. It was demonstrated that one molecule of myosin binds one molecule of ATP. In the present work, we have applied the same methodology to the study of additional kinetic properties of the system, namely the formation constant k_1 of the enzyme-substrate complex, and, in the absence of a rephosphorylating enzyme, the Michaelis constant K_A and the decomposition constant k_3. The determination of K_A and k_3 is always possible by conventional enzyme kinetic approaches; the direct determination of K_A from a binding curve, as mentioned in the previous article, and the determination of k_1 as presented here, are possible only under special circumstances such as those provided by our methodology. The results will, among others, permit an interpretation of the nature of K_A, which in turn has a bearing upon some aspects of the theory of muscle contraction.

Methods.—All the methods employed were the same as those described in the preceding paper.[1] When the various constants were to be interrelated, they were