with the antibiotic complex will be attacked by the enzyme and removed, so that the final apparent extent of antibiotic inhibition will be reduced. Vancomycin and ristocetin inhibited the *Streptomyces* carboxypeptidase action upon *B. rettgeri* peptide dimer (Figure 1) and upon *C. poinsettiae* disaccharide peptide dimer (Figure 2) but not upon the *B. subtilis* heptapeptide diamide in which latter case the terminal carboxyl group is amidated. Previous experiments (M. Nieto and H. R. Perkins, in preparation) had shown that a bulky side chain at the C-terminal position of the peptide greatly decreased combination with vancomycin but combination with ristocetin was relatively unaffected. The *C. poinsettiae* disaccharide peptide dimer showed good combination with ristocetin and somewhat less with vancomycin, as indicated by difference spectroscopy and paper electrophoresis of the complexes at pH 7. Thus the inhibition of carboxypeptidase action by the antibiotics was consistent with their combination with the substrate in question. According to the results reported by Bogdanovsky *et al.*, (1969), the action of the *E. coli* carboxypeptidase upon the *E. coli* peptide dimer (Table I, **8** in Ghuysen *et al.*, 1970) was not inhibited by ristocetin. However, ristocetin was used at such low concentrations (25–200 μM for a 250 μM peptide solution) that further experiments are needed to verify the conclusion.

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Transient State Phosphate Production in the Hydrolysis of Nucleoside Triphosphates by Myosin*

Richard W. Lymn and Edwin W. Taylor

ABSTRACT: The course of presteady-state phosphate liberation in the hydrolysis of nucleoside triphosphates by myosin was studied using a chemical-quench flow apparatus. Phosphate was liberated at rates greatly exceeding the steady-state rates for MgATP, MgITP, and CaATP as substrates at 20°, pH 8.0, 0.5 M KCl. Similar results were found for MgATP as substrate at 0°, and for hydrolysis of MgATP at 20° by heavy meromyosin in 0.05 M KCl. The time course of the transient state hydrolysis could be fitted by a single rate constant under all conditions. The rate constant varied with substrate concentration. For MgATP and CaATP at 20°, the rate constant approached a limiting value of about 50–100 sec^{-1} for substrate concentrations greater than 10^{-4} M. The size of the presteady-state burst varied with substrate for both MgATP and CaATP, extrapolating to 1.8 moles of $P_i/5 \times 10^5$ g of myosin. In the presence of EDTA, the mechanism of hydrolysis was completely different, exhibiting a lag before the state-state rate was reached.

The hydrolysis of nucleoside triphosphates by myosin and actomyosin has been the subject of a large number of studies, but several important features of the mechanism, notably the nature of the early burst and of activation by actin, are poorly understood. Recently, attempts have been made to clarify the problem, by examining the presteady-state behavior (Finlayson and Taylor, 1969; Finlayson *et al.*, 1969; a series of papers by Tonomura and collaborators, most recently Kinoshita *et al.*, 1969a,b; Imamura *et al.*, 1968).

We have previously observed an early rapid proton liberation phase by using a stopped-flow apparatus and monitoring protons by means of an indicator dye. Tonomura reported both a rapid absorption and liberation of protons (Tokiwa and Tonomura, 1965). Since the early phase was obtained in presence of Ca or Mg ion, while the stoichiometric early phosphate burst had been found only with Mg ion, it was not clear whether the proton step could be equated with

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hydrolysis. Although at pH 8 one proton is produced per ATP hydrolyzed, protons released during the presteady-state phase need not have come from bond hydrolysis.

We therefore undertook to measure the early phosphate liberation phase on a time scale comparable with the proton measurements. The early burst effect indicates that at least in the presence of Mg ion, the rate constant of hydrolysis, rather than steady-state rate of ATP hydrolysis by myosin, has not been measured. Since a method for the optical monitoring of phosphate was not available, an apparatus was developed to initiate and stop an enzyme reaction after a controlled period of time, with reaction times ranging from 10 to 500 msec. The initial mixing of enzyme and substrate and the quenching of the reaction by acid was achieved by using a syringe block and multiple jet mixers similar to those employed in the stop-flow apparatus.

Myosin at pH 8.0 and 20° yielded "early bursts" of phosphate liberation for the substrates MgATP,[1] MgITP, and CaATP. The size of the burst for MgATP and CaATP varied with ATP concentration, and extrapolated to 1.8 moles of P_i/500,000 g of myosin. Heavy meromyosin also gave a burst for MgATP at both high and low ionic strengths. In presence of EDTA with ATP as substrate, myosin showed no evidence of an early burst, but exhibited a distinct lag before reaching the steady-state rate. Analysis of the early burst showed that the actual hydrolysis step for ATP is similar with Mg or Ca as the activating ion, and corresponded to a rate constant of the order of 50–100 sec^{-1} at 20°.

Materials and Methods

Protein. Rabbit myosin was prepared by the modified Holtzer and Lowey (1959) method described previously (Finlayson and Taylor, 1969), with the first precipitation by dilution, the subsequent two by dialysis. Heavy meromyosin was made according to Young *et al.* (1965) with a digestion time of 5 min at 20°. After ammonium sulfate precipitation, the heavy meromyosin was dialyzed extensively against buffer, but was not chromatographed.

The concentrations of myosin and heavy meromyosin were determined using the difference in absorbance at 291 and 350 mμ in 0.5 N NaOH (0.735, 1 cm, 1 mg/ml). The determination was calibrated for myosin both gravimetrically and by micro-Kjeldahl analysis using a nitrogen content of 16.7%. For the calculations made in this paper the molecular weights of myosin and heavy meromyosin are assumed to be 5×10^5 and 3.2×10^5 daltons, respectively.

Reagents. All solutions were made in demineralized distilled water, using analytical grade reagents. To minimize possible inhibition, the $CaCl_2$ chosen had less than 25-ppm magnesium contamination.

ATP and ITP were purchased as the disodium salts from Pabst Laboratory Biochemicals. [^{32}P]Nucleotides (ATP and ITP) labeled in the γ position were synthesized by a modification of the method of Glynn and Chappell (1964). The reaction mixture, in a total volume of 1 ml, was applied directly to a 1 × 20 cm column of DEAE-Sephadex A-50 and eluted with a linear salt gradient from 0.05 to 0.25 M KCl–0.05 M Tris buffer (pH 8.3). Effluent was monitored by an Isco ultraviolet analyzer. The nucleoside triphosphate was eluted in less than 4–5 hr and was stored at −20°.

Solutions to be used in the rapid flow device were degassed with a water aspirator, at 3°, for at least 2 hr, and subsequently manipulated in a N_2 atmosphere.

Phosphate Determination. Labeled P_i was separated from the acid-stopped assay mixture by using a modification of the triethylamine precipitation of phosphomolybdate described by Sugino and Miyoshi (1964). The enzyme reaction was stopped by mixing with HCl or perchloric acid to a final concentration of 0.4 N. The protein precipitate was removed by centrifugation and unlabeled carrier (ATP, ADP, and P_i) was added, followed by 10 mg of Norit A. The mixture was agitated intermittently for 5 min (Vortex mixer) to facilitate adsorption of nucleotides and then filtered and washed twice with 1.5 ml of 0.1 N perchloric acid employing a Millipore filter (0.45 μ pore size). To the filtrate, whose volume was approximately 8 ml, was added 0.4 ml of freshly mixed molybdate–triethylamine solution (two parts of 10% by weight sodium molybdate to one part 0.2 M triethylamine) to precipitate the phosphate. The solution was filtered on a Millipore filter and washed three times with 2-ml aliquots of the wash solution (2.5% ammonium molybdate, 0.025 M triethylamine, 0.2 N perchloric acid plus sufficient phosphate to saturate the solution, *ca.* 5×10^{-5} M). The filter disk was cemented to a planchet and counted in a Nuclear-Chicago C-110B gas-flow counter or a Beckman-Sharp low-beta counter.

Recovery of radioactive phosphate by this procedure was essentially quantitative. The background count, *i.e.*, the radioactivity precipitated from the stock solution of γ-labeled [^{32}P]ATP was 0.5% for freshly made nucleotide. It should be noted that the primary limitation in measuring the rate of ATP hydrolysis during the transient phase is the size of the zero time blank compared with early time points.

Chemical-Quench Flow Apparatus. The instrument is a modified continuous-flow machine, with two mixers, one to start the reaction by mixing enzyme and substrate, and one to quench it by mixing the reaction solution with acid. By changing both the length of tubing between the two mixers and the flow rate of the solution, the time of reaction can be varied.

Figure 1 is a schematic diagram of the apparatus. The protein and substrate solutions are maintained separately in the first two chambers of the Lucite block and 1 N acid is in the third. The block is thermostated by circulating water from a Forma bath. When the electric clutch is engaged, the drive block forces the solutions through the mixers. The protein and substrate mix first, react while moving through the length of tubing, then mix with the acid, and pass through the assay valve. The electromagnetic assay valve shunts the fluid either into a waste container or an assay receiver. When the drive stroke commences, the valve is in waste position. This ensures that liquid remaining in the tubes from the last stroke is not sampled. It also enables the stroke to reach a uniform velocity before a sample is taken. At a given position of the drive stroke, the valve is actuated by a microswitch triggered by passage of the drive block and switches to the assay position. Just before the stroke is ended, the drive block triggers a second

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[1] Abbreviations used are: MgATP, CaATP, MgITP, the metal complexes of the nucleoside triphosphates; EDTA-ATP is ATP, with all contaminating Me^{2+} chelated by EDTA.

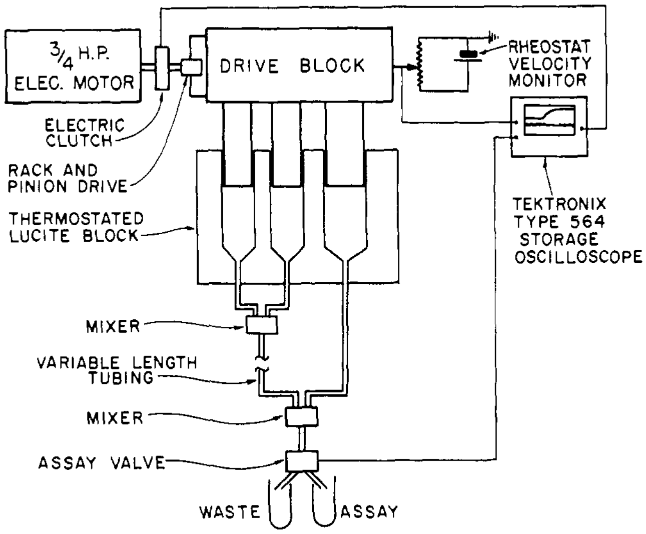

FIGURE 1: Schematic diagram of the chemical-quench flow apparatus.

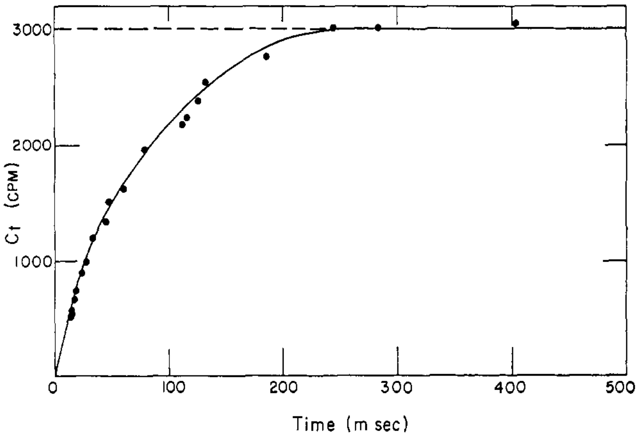

FIGURE 2: Phosphate liberation with MgATP as substrate. 0.5 M KCl–0.1 M Tris–0.01 M $MgCl_2$, pH 8.0, 20°, 32 μM ATP, and 2 mg/ml of myosin. The dotted line is an extrapolation of the steady state to zero time. The burst is 1.2 moles of P_i/mole of myosin.

microswitch and the valve returns to "waste." These switching events are recorded on one channel of a dual-beam memory oscilloscope (Tektronix 564, Type 3A3 amplifier). An aliquot of the solution collected in the "assay" position was taken for phosphate analysis.

The oscilloscope time base is triggered by the starting switch, and the position of the drive block is recorded by means of a voltage divider whose central terminal is attached to the drive block (Finlayson and Taylor, 1969). The voltage varied linearly with time over most of the range of the drive stroke, indicating a constant flow rate. From the size of the tubing connecting the mixers and the flow rate obtained from the voltage trace, the reaction time can be calculated. On this device the range of reaction times is from 10 to 500 msec.

Because of sensitivity of the protein to metal ions, all parts of the apparatus which contained the solutions were made of either Lucite or Teflon, except that the connecting tubing, between the mixers, was made from stainless steel to ensure constant diameter. The mixers were made from the design of Gibson and Milnes (1964). A more complete description of the apparatus is available.

To evaluate the apparatus, the hydroxide-catalyzed hydrolysis of 2,4-dinitrophenyl acetate was measured at 23° for three concentrations of NaOH. The rate constant calculated from the date was 59 M^{-1} sec^{-1}, in very good agreement with the rate constant of 52 M^{-1} sec^{-1} obtained for the same sample on the stop-flow machine.

To determine the effect of flow on myosin activity, myosin solutions were driven through the longest tubing used in the experiments, then assayed for ATPase activity. There was no appreciable reduction of activity in passage through the instrument.

Treatment of Data. The phosphate radioactivity at zero time was determined by mixing an aliquot of myosin with acid before addition of [^{32}P]ATP and this value was first subtracted from the radioactivity obtained for each sample. The difference was plotted *vs.* age of sample, as in Figure 2, which is an early burst experiment with MgATP as substrate. In order to evaluate the transient rate constant, the steady-state rate was extrapolated to zero time, and the differences between this line (CPM_{SS}) and the experimental points (CPM_t) were plotted *vs.* time on semilog paper, as in Figure 3. Occasionally, particularly at low substrate concentrations, the steady state was not reached within 0.5 sec, and the transient rate had to be estimated by drawing a line through the early linear phase of the burst, and converting this into a rate constant. This procedure applied to data of Figure 2 gives a rate constant of 13.8 sec^{-1} as compared with the value of 13.6 sec^{-1} obtained from the slope of the line in Figure 3.

Steady-state rates were also measured independently for all experiments by mixing reagents in test tubes and assaying over a time range of 3–5 min.

Results

P_i Liberation with MgATP as Substrate. The production of P_i from MgATP showed an early transient rate considerably

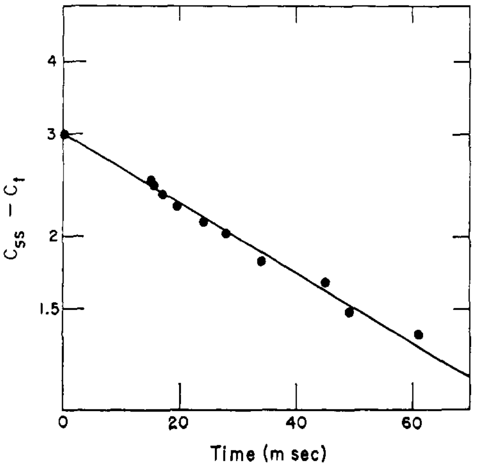

FIGURE 3: Semilogarithmic plot of the difference between the actual experimental points and the extrapolated steady state in Figure 2.

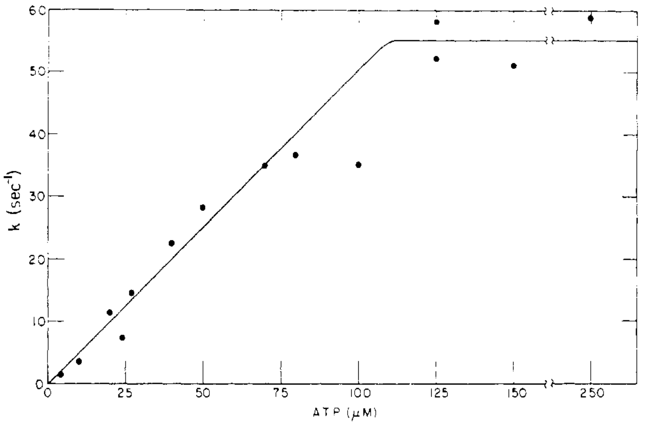

FIGURE 4: The variation of the presteady-state rate constant of P_i liberation *vs.* MgATP concentration. All solutions 0.5 M KCl–0.1 M Tris–0.01 M $MgCl_2$, pH 8.0, 20°. Myosin, 1.5–3 mg/ml.

higher than the steady-state rate. An experiment at 20°, 0.5 M KCl (pH 8) is shown in Figure 2. The virtually horizontal section of the curve is the steady state, $k = 0.02$ sec^{-1}. By extrapolating this rate to zero time, one measures the magnitude of the presteady-state "burst." In this case it amounts to 1.2 moles of P_i/mole of myosin.

Since the general solution of the rate equation, for constant substrate concentration, is a sum of exponential terms, the amount of hydrolysis minus the contribution from the extrapolated steady-state rate was plotted semilogarithmically, as in Figure 3. The points are adequately described by a single apparent rate constant of 13.6 sec^{-1}.

The variation of the rate constant with substrate concentration is shown in Figure 4 (pH 8.0, 20°). It reached a plateau of about 55 sec^{-1} at about 10^{-4} M ATP. Between 5×10^{-6} M ATP and 10^{-4} M ATP the rate constant increased linearly with concentration and from the slope one obtains an apparent second-order rate constant of 5×10^5 M^{-1} sec^{-1}. A small number of experiments at pH 7.0 and 8.5 showed little or no variation from the results at pH 8.0.

Some of the data, particularly at higher concentrations, could be better fitted by two presteady-state rate constants, but because of experimental errors we do not in general feel justified in fitting the data to more than one constant. To be able to distinguish between two rate constants of the same order, one has to have accurate information beyond where the approach to the steady state is seven-eights complete, and in this range the scatter begins to become comparable to the variation with time.

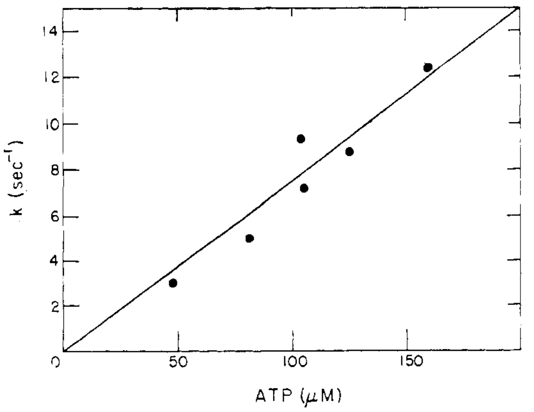

FIGURE 5: Variation of presteady-state rate constant of P_i liberation at 0°. 0.5 M KCl, 0.1 M Tris, and 0.01 M $MgCl_2$, pH 8.0. Myosin, 1.5–2 mg/ml.

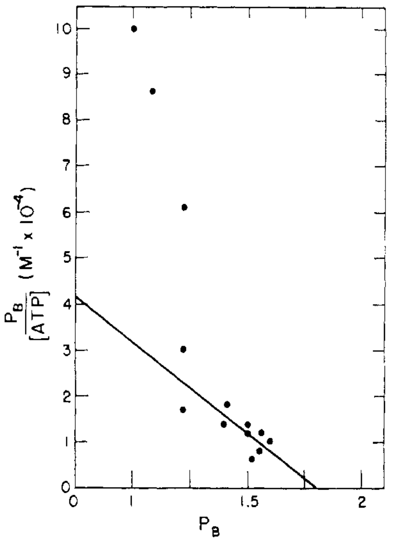

FIGURE 6: A Scatchard plot of the variation of size of P_i burst (P_B) with ATP concentration. 0.5 M KCl–0.1 M Tris–0.01 M $MgCl_2$ (pH 8.0) and 1.5–2 mg of myosin/ml.

At zero degrees, pH 8.0, a transient early phase was also observed. The rate contant varied with ATP concentration as shown in Figure 5, and gave no indication of reaching a maximum at concentrations up to 1.6×10^{-4} M. The rate constant obtained from the slope of a least-squares fit was 7.2×10^4 M^{-1} sec^{-1}.

The size of the phosphate burst varied with ATP concentration. A Scatchard plot of the data (Figure 6) is apparently nonlinear. Using the points at high ATP concentrations one obtains the line drawn in the figure which corresponds to an apparent association constant of 3×10^4 M^{-1} and extrapolates to a maximum burst size of 1.8 moles of P_i/500,000 g of myosin. The points for lower ATP concentrations correspond to a much larger association constant which is certainly greater than 2×10^5 M^{-1} but the data do not allow an accurate value to be calculated.

MgITP as Substrate. With MgITP as substrate, a transient early phase was found similar to, but slower than, that with MgATP. No leveling off of rate constant with substrate concentration was obtained in the range studied. A least-squares fit of the data yielded a rate constant of 6.2×10^4 M^{-1} sec^{-1} at 20°, pH 8, 0.5 M KCl.

CaATP as Substrate. At 20°, pH 8.0, an early burst was also obtained with CaATP. The results of one experiment are shown in Figure 7. The straight line corresponds to the steady state of 3.4 sec^{-1}. The intercept is 1.2 moles of P_i/mole of myosin. Plotting the difference between the steady-state rate and experimental points on a semilog graph gives a straight line as shown in Figure 8. In this example the rate constant is 54 sec^{-1}.

The effect on the transient rate constant of varying the sub-

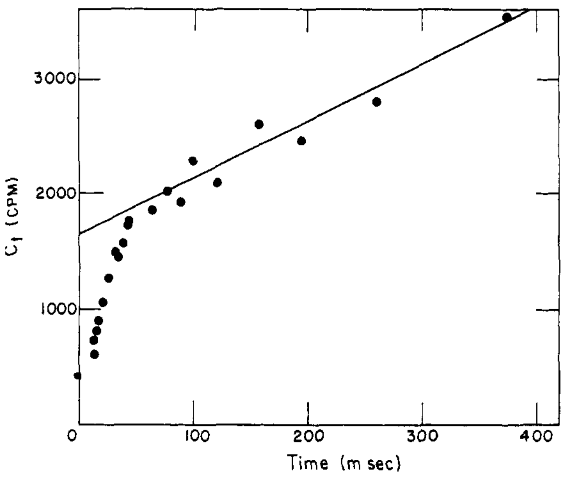

FIGURE 7: Phosphate liberation with CaATP as substrate. The straight line represents the steady-state rate, extrapolated back to zero time. 0.5 M KCl–0.1 M Tris–0.01 M $CaCl_2$ (pH 8.0), 20°, 98 μM ATP, and 2.2 mg of myosin/ml.

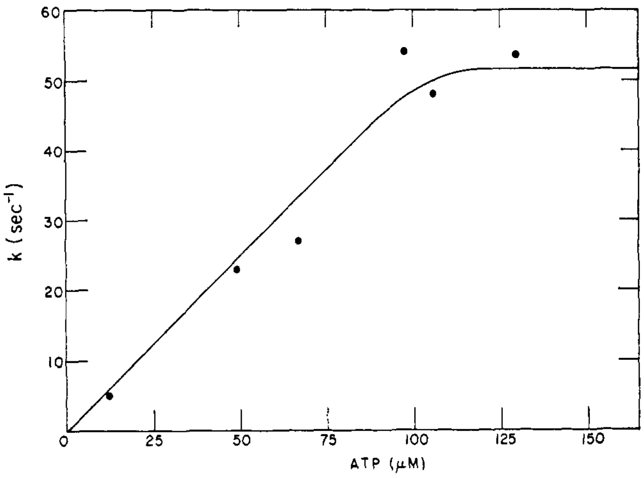

FIGURE 9: Variation of presteady-state rate constant with CaATP concentration. 20°, pH 8.0.

strate concentration is shown in Figure 9. As with MgATP, a plateau is reached at about 10^{-4} M ATP. The rate constant at the plateau is 52 sec^{-1}, while the second-order rate constant for lower concentrations is 5.5×10^5 M^{-1} sec^{-1}.

As with MgATP, the size of the burst varied with substrate concentration. Based on a small number of experiments the burst size extrapolated to 1.8 moles of P_i/mole of myosin with an apparent binding constant at relatively large ATP concentrations of 2×10^4 M^{-1}.

Heavy Meromyosin-MgATP. In order to study the system at a more nearly physiological ionic strength, P_i liberation was measured using heavy meromyosin ATPase at 0.05 M KCl–0.01 M $MgCl_2$, 20°, pH 8.0. The results were similar to those with myosin and are shown in the upper curve of Figure 10. Up to substrate concentrations of 30 μM, the transient rate constant varied with a second-order rate constant of 2.4×10^6 M^{-1} sec^{-1}, which is higher than that for myosin at 0.5 M KCl. To determine whether this increase was due to the ionic strength, experiments were performed with heavy meromyosin at 0.5 M KCl. These are shown in the lower curve of Figure 10. The slope is essentially the same as for myosin under the same conditions. Intermediate ionic strengths and a constant ATP concentration gave intermediate values for the rate constant. In 0.05 M KCl, the slope of the plot of rate constant *vs.* concentration begins to decrease above 30 μM. The maximum rate had not been reached at 50 μM ATP, consequently the actual first-order rate constant for hydrolysis is greater than 125 sec^{-1}. Experimental errors at the high rate did not allow the value to be determined accurately.

ATP Hydrolysis in Presence of EDTA. In these experiments

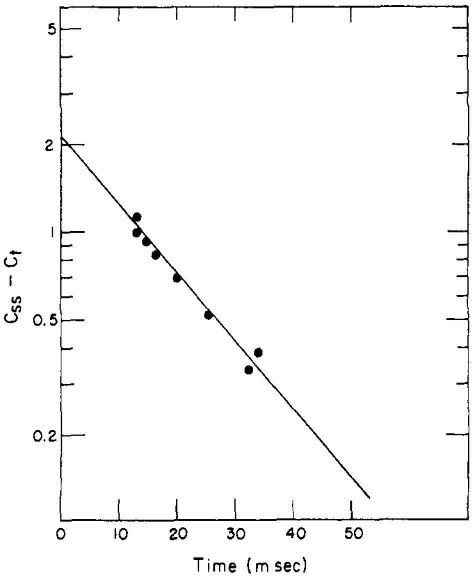

FIGURE 8: A semilog plot of the difference between the experimental points and the extrapolated steady-state line in Figure 7.

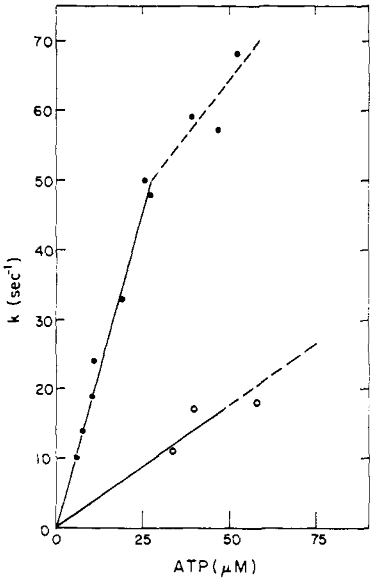

FIGURE 10: Variation of presteady-state rate constant with MgATP concentration for heavy meromyosin at low and high ionic strength. (-●-) 0.05 M KCl, 0.05 M Tris, and 0.01 M $MgCl_2$; (-○-) 0.5 M KCl, 0.1 M Tris, and 0.01 M $MgCl_2$. All experiments pH 8.0, 20°.

TABLE I: Phosphate Liberation.[a]

Protein	Substrate	Temp (°C)	$\Delta k/\Delta(S)$ (M^{-1} sec^{-1})	k_{max} (sec^{-1})
Myosin	MgATP	20	5×10^5	55
	CaATP	20	5.5×10^5	52
	MgITP	20	6.2×10^4	None
	MgATP	0	6.2×10^4	None
	EDTA-ATP	20	Different mechanism	
Heavy meromyosin	MgATP	20	2.4×10^6	>100

[a] All experiments at pH 8.0. With myosin, the solutions were 0.5 M KCl, 0.1 M Tris, and 0.01 M either $MgCl_2$, $CaCl_2$, or EDTA. The experiments with heavy meromyosin were 0.05 M KCl, 0.05 M Tris, and 0.01 M $MgCl_2$. "None" means no maximum rate was observed in the substrate concentration range studied.

both myosin and ATP solutions contained 10^{-2} M EDTA and are presumed to be essentially free from divalent metal ions. The hydrolysis of ATP by myosin did not yield an early burst. Instead there was a distinct lag, *i.e.*, the presteady-state rate was lower than the steady state, which is the type of behavior normally encountered in enzyme reactions. This is shown for one experiment in Figure 11. By extrapolating from the steady-state phase, one obtains a measure of the lag, τ. For simple Michaelis–Menten behavior $\tau = 1/k_1((S)_0 + K_m)$, where k_1 is the rate constant for substrate binding and K_m is the Michaelis constant. A plot of $1/(S_0)$ *vs.* $1/(S_0)\tau$ was linear and from the ordinate intercept, k_1 was calculated to be 1.4×10^5 M^{-1} sec^{-1}. The abscissa intercept gives K_m equal to 10^{-4} M. A Lineweaver–Burk plot of the steady-state rates for ATP concentrations from 2.5×10^{-5} to 10^{-4} M gave a K_m of 1.1×10^{-4} M and a value of 10 sec^{-1} for the rate constant for hydrolysis. The kinetic data are summarized in Table I.

Steady-State Rate of Hydrolysis. Rate constants describing the transient state are related to the Michaelis constant determined in the steady state. The value of $\bar{K}$, the reciprocal of the Michaelis constant, has been measured many times (recently by Schliselfeld and Bárány, 1968; Kiely and Martonosi, 1968) but concentration ranges employed were usually less than a factor of 100 and rarely extended below 10^{-5} M. Measurements of rate of hydrolysis of MgATP by myosin in 0.5 M KCl were made over the range from 3×10^{-9} to 3×10^{-3} M using the radioactivity assay. At the lowest concentrations the ratio of ATP to myosin was a factor of 4 or 5 and 10–20-ml samples were used for each point. The rate was determined from the slope of a plot of four time points. For the lower concentrations the samples were counted using a low-background counter (Beckman-Sharp). In order to cover a wide concentration range it was necessary to combine data from different experiments, which led to a relatively large experimental error. The results in three different myosin preparations (approximately 50 experiments) were combined to obtain the Scatchard plot shown in Figure 12. The results do not appear to fit a single straight line. At low concentrations ($<5 \times 10^{-7}$ M) the least-squares fit, which is the straight solid line and its dashed extension in the figure, corresponds to $\bar{K} = 3 \times 10^7$ M^{-1}. At higher concentrations the data appear to be fitted by a smaller value of $\bar{K}$, which is the order of 10^6 M^{-1} and agrees with the most recent values in the literature. However the experimental errors are relatively large, and the apparent curvature in the Scatchard plot might be an artifact.

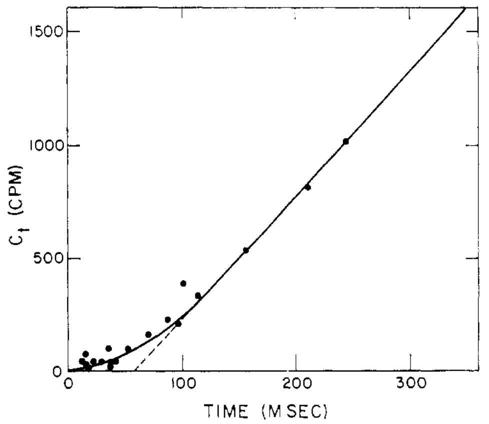

FIGURE 11: Phosphate liberation with ATP as substrate in presence of EDTA. The straight line is the steady-state rate. 0.5 M KCl–0.1 M Tris–0.01 M EDTA (pH 8.0), 25 μM ATP, and 2 mg of myosin/ml.

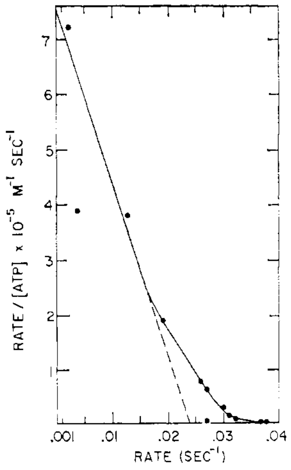

FIGURE 12: A Scatchard plot of the steady-state rate of MgATP hydrolysis. 0.5 M KCl–0.05 M Tris–0.005 M $MgCl_2$ (pH 8.0), 20°. The straight line is the least-squares fit for concentrations less than 5×10^{-7} M. Each point in the average of three or four determinations.

The least-squares fit to all of the data gives 2×10^7 M^{-1} for $\bar{K}$ and the value will be used to discuss the relation between transient and steady-state behavior.

Discussion

The transient phase in the hydrolysis of ATP or ITP by myosin has been examined employing a mixing apparatus which allows phosphate production to be measured for reaction times as short as 10–15 msec. An early phase of rapid hydrolysis, "the early burst" was found not only in the presence of Mg ion but for Ca ion as well. The rate constants describing the early phase were essentially the same for either divalent ion, the reaction differing only in the much larger steady-state rate in the presence of Ca. The magnitude of the early burst was similar in the presence of either divalent ion, namely, 1.5–2.0 moles/500,000 g of myosin. The failure to detect a stoichiometric Ca early burst previously has probably been due to the larger steady-state rate. It is necessary to assay the reaction at times in the range less than 400 msec in order that phosphate liberated in the early phase be an appreciable fraction of the total. For example, with a steady-state rate constant of 3 sec^{-1} for CaATP there will be 45 moles of P$_i$/mole of myosin at 15 sec and an extrapolation to zero time could not be made with sufficient accuracy to detect 1–2 additional moles of phosphate.

The early phosphate liberation phase after correction for the steady-state rate could be fitted reasonably well by a single exponential, *i.e.*, $(P_i)_t = (P_i)_\infty (1 - e^{-\lambda t})$, where $(P_i)_\infty$ is the magnitude of the early burst. Measurement of λ as a function of substrate concentration indicated that λ increases linearly with substrate at low concentrations and approaches a plateau at high concentrations. Thus the behavior is consistent with the model

$$\mathrm{M} + \mathrm{S} \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} \mathrm{MS} \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} \mathrm{MPr} \xrightarrow{k_3} \mathrm{M} + \mathrm{Pr} \qquad (1)$$

where S and Pr refer to substrate and products (ADP and P$_i$), respectively. Solution of the rate equations for this model shows that during the transient state phosphate production is the sum of two exponentials. One term is a short lag corresponding to the buildup of the MS complex and would not be readily observed because k_2 is so large. There is only one significant exponential term and at high S, $\lambda = k_2$. At low S, there are two possibilities, namely: if $k_{-1} \lessapprox k_2$, $\lambda = k_1(\mathrm{S})$; if $k_{-1} \gg k_2$, $\lambda = K_1k_2(\mathrm{S})$, where $K_1 = k_1k_{-1}$. The second case arises because for this choice of constants MS is essentially in equilibrium with M and S. Thus from the value of λ at high S we obtain k_2, the rate constantof bond hydrolysis while the data at low S yields the rate constant for substrate binding k_1 or the quanitity K_1k_2. Because k_2 is the order of 50 sec^{-1}, k_{-1} would have to be greater than 100 sec^{-1} for the second choice to be correct. Since K_1k_2 is 4×10^5 to 5×10^5 M^{-1} sec^{-1}, we have $K_1 = 10^4$ M^{-1} and $k_1 > 10^6$ M^{-1} sec^{-1}. Thus the second possibility cannot be rejected although it leads to a rather low value for the ATP binding constant, K_1. It is concluded that $k_1 \geq 5 \times 10^5$ M^{-1} sec^{-1}. A similar value for the apparent rate constant for substrate binding in the presence of Ca or Mg was recently reported by Morita (1969) employing the ATP-induced difference spectrum.

The mechanism given in eq 1 will lead to steady-state kinetics of the Michaelis–Menten type and the Michaelis constant must be related to the rate constants determined in the transient state. Denoting the reciprocal of the Michaelis constant by $\bar{K}$ we have

$$\bar{K} = k_1k_2/[k_{-1}k_{-2} + k_3(k_{-1} + k_2)] \qquad (2)$$

(The approximation was made that $k_2 + k_{-2} + k_3 \simeq k_2$ which is certainly valid in the case under Discussion.) To drive a simple limiting expression for $\bar{K}$ it is necessary to consider the same possibilities which arose in the discussion of the apparent rate constant for substrate binding which we will denote as k^a. (1) If $k_{-1} \ll k_2$; $\bar{K} = k_1/k_3$ if $k_{-1}k_{-2} \ll k_2k_3$; and $\bar{K} = K_1K_2$ if $k_{-1}k_{-2} \gg k_2k_3$ where $K_2 = k_2/k_{-2}$. For this choice of k_{-1}, $k_1^a = k_1$ and the measured values for MgATP are $k_1 = 5 \times 10^5$ M^{-1} sec^{-1}, $k_2 = 50$ sec^{-1}, $\bar{K} = 2 \times 10^7$ M^{-1}, $k_3 = 0.02$–0.04 sec^{-1}. In the first case $k_1/\bar{K} = k_3 = 0.025$ sec^{-1} in agreement with the directly measured value. In the second case $k_1/\bar{K} = k_{-1}k_{-2}/k_2$, thus the $k_{-1}k_{-2} \simeq 1.25$ sec^{-2}. However k_2k_3 is also approximately 1.25 sec^{-2} which contradicts the assumption that $k_{-1}k_{-2} \gg k_2k_3$ and this case can be rejected. (2) If $k_{-1} \gg k_2$: $\bar{K} = K_1K_2$ if $k_{-2} \gg k_3$; and $\bar{K} = K_1k_2/k_3$ if $k_{-2} \ll k_3$. Since $k_1^a = K_1k_2$ then in the first case $k_1^a/\bar{K} = k_{-2} = 0.025$ sec^{-1} which contradicts the assumption that $k_{-2} \gg k_3$. In the second case $k_1^a/\bar{K} = k_3$ 0.025 sec^{-1} in agreement with the experimental value.

Therefore it is concluded that $\bar{K} \cong k_1^a/k_3$ although the ambiguity remains as to whether the measurable quantity k_1^a is the rate constant for substrate binding k_1 or the quantity K_1k_2. In any case $\bar{K}$ is not the equilibrium constant for substrate binding.

It should be emphasized that the rate constants in the transient state for Mg or Ca activation are nearly the same and thus the substrate binding and hydrolysis steps are similar reactions in the presence of either ion. Since $k_2 \gg k_3$ in the presence of either Ca or Mg, the rate-limiting step which determines the steady-state rate must be the dissociation of the enzyme–product complex or some other slow step which follows bond hydrolysis. Evidence will be presented in the accompanying paper that product dissociation is indeed the rate-limiting step and this phase of the reaction will not be discussed further.

The stoichiometric early burst follows from the fact that $k_2 \gg k_3$. The experiments reported here do not have direct bearing on the nature of the MPr complex.

The rate constant of hydrolysis (order of 50–100 sec^{-1}) is large and under physiological conditions, namely, 0.1 M KCl and 10^{-2} M Mg^{2+}, this rate is sufficient to account for the energy output of muscle. Finally, the steady-state rate of hydrolysis of ATP by heavy meromyosin when fully activated by actin (Eisenberg and Moos, 1968) does not exceed the value of 50 sec^{-1} for the hydrolysis step. Our findings therefore suggest that actin activation effects a step which occurs after bond hydrolysis, which is presumably the product dissociation step. (We shall develop this interpretation further in a subsequent report.)

The behavior in the presence of EDTA is in sharp contrast to the reaction with Mg or Ca ion as activator. The transient state kinetics for EDTA activation clearly show an early lag and thus no early burst. This type of kinetics is the usual case

TABLE II: Proton Liberation.[a]

Substrate	Temp (°C)	$\Delta\lambda_1$ (M^{-1} sec^{-1})/Δ(S)	λ_1 max (sec^{-1})	$\Delta\lambda_2$ (M^{-1} sec^{-1})/Δ(S)	λ_2 max (sec^{-1})
MgATP	18.5	2×10^6	130	2.5×10^5	20
MgATP	0	----	----	3.8×10^4	None
CaATP	0	None	60	5×10^4	20

[a] All experiments were performed at pH 8 in 0.5 M KCl, 1–2 × 10^{-2} M MgCl, or 1.5 × 10^{-2} M CaCl. Subscript one is applied to the faster process when two rate constants are required to represent the data. Dashes indicated that a single rate constant was sufficient to describe the results. "None" indicates that no concentration dependence was detected, or that a maximum value was not attained in the concentration range investigated. $\Delta\lambda_1/\Delta$(S) is the slope of λ_1 *vs.* substrate curve at low substrate concentrations. Finlayson and Taylor (1969).

for a simple enzyme reaction

$$\mathrm{M} + \mathrm{S} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{MS} \xrightarrow{k_2} \mathrm{M} + \mathrm{Pr} \qquad (3)$$

Since MS accumulates during the transient state, a lag is expected and from the dependence of the lag on substrate concentration one obtains k_1 and $\bar{K}$ (the reciprocal Michaelis constant). The value of k_1 is 1.4×10^5 M^{-1} sec^{-1} and the substrate is presumably K (ATP^{-3}). The magnitude of k_1 is therefore five or ten times less than the value for MgATP^{-2} or CaATP^{-2} as substrate. The value of $\bar{K}$ of approximately 10^4 M^{-1} agrees with the value obtained independently from the steady-state data by a Lineweaver–Burk plot, and is considerably smaller than values for MeATP^{-2}. Since k_2 is approximately 10 sec^{-1}, $k_1/k_2 \approx 10^4$ M^{-1} which is equal to $\bar{K}$, thus $\bar{K}$ has the same interpretation in term of rate constants as for MeATP^{-2}.

Even though the steady-state rates with EDTA and Ca as activators are high compared with Mg, the reactions are quite different in character. In spite of the high rate for Ca, the existence of an early burst indicates that the rate-limiting step is still a slow step following hydrolysis, while for EDTA, *i.e.*, in the absence of divalent metal ions, the bond hydrolysis step is probably rate limiting.

One purpose in this study was to compare the rate constants for the proton early burst with the phosphate early burst. The data are summarized in Tables I and II. In attempting to compare rate constants obtained by different methods, on different myosin preparations, the errors in each set of determinations lead to an uncertainty of at least 50%. Measurements on MgATP at 0° are somewhat easier to make and the agreement is relatively good. At 20° the rate constants obtained from phosphate measurements are roughly the averages of the two values found in the proton experiments, thus we cannot equate the phosphate rates with one proton step in preference to the other. In the case of phosphate measurements, the time course is constructed point by point and the accessible time range is somewhat shorter than for proton measurements. It is therefore more difficult to detect two steps if they are present. It is concluded that rate constants for proton and phosphate liberation steps may arise from the same steps of bond hydrolysis.

If the possible intermediate states are considered, only the values of the second-order rate constants need agree since they measure the rate of substrate binding. Because the reaction was stopped with acid the phosphate measured on the transient state may be, in part, a labile intermediate and the liberation of a proton need not accompany its formation. For example, if hydrolysis proceeds through a metaphosphate step, as has often been postulated (Bruice and Benkovic, 1966; Sartorelli *et al.*, 1966) then

$$\mathrm{E{\cdot}PO_3} \overset{\pm\mathrm{H_2O}}{\rightleftharpoons} \mathrm{E{\cdot}H_2PO_3^-} \rightleftharpoons \mathrm{E{\cdot}HPO_4^{--}} + \mathrm{H^+}$$

In the steady state it is probable that the complex is bound phosphate (Sartorelli *et al.*, 1966) but in the transient state what we have referred to as phosphate could be metaphosphate, and the first-order rate constant measured for the proton step could be equal to or less than that for the phosphate step. A similar argument would apply to a covalent intermediate such as acyl phosphate as long as it is largely converted into bound phosphate in the steady state.

The mechanism (eq 1) appears to account for at least the main aspects of the kinetics but there are a number of features which suggest that it may be oversimplified. The magnitude of the early burst did not yield a linear Scatchard plot (Figure 6) as would be expected from a single class of site and the deviations appear to be far too large to attribute to experimental error. It is easily shown that eq 1 leads to the relation

$$P_{\mathrm{B}} = \frac{(\mathrm{MPr})}{(\mathrm{M_0})} = \frac{n(\mathrm{S})}{(\mathrm{S}) + 1/\bar{K}}$$

where P_{B} is the magnitude of the burst, n the number of catalytic sites, and $\bar{K}$ is the reciprocal of the Michaelis constant. At high S, $\bar{K} \sim 3 \times 10^4$ M^{-1} and the plot extrapolates to 1.8 moles/mole indicating that both sites are catalytically active. At low S the value of $\bar{K}$ is much larger but experiments were not done at low enough substrate concentration to compare with $\bar{K}$ determined in the low concentration range in the steady state.

The steady-state measurements also appeared to be better fitted to more than one value of $\bar{K}$ but experimental errors are sufficiently large that a conclusion cannot be drawn with confidence. In any case the average value of $\bar{K}$ at low S (2×10^{-7} M^{-1}) does not agree at all with $\bar{K}$ determined from the early burst at high S nor with rather low value of $\bar{K}$ reported by others for steady-state rate measurements at high S (Nihei and

Tonomura, 1959; Kiely and Martonosi, 1968). Previously reported rates of proton liberation (Finlayson and Taylor, 1969) were also fitted by two different substrate binding and hydrolysis constants.

There is growing evidence that myosin consists of two large polypeptide chains (Lowey *et al.*, 1969) plus two or three small subunits and that the chains may be identical (Weeds and Hartley, 1968). It has two catalytic sites or sites which can bind nucleotides or pyrophosphate (Lowey and Luck, 1969; Schliselfeld and Bárány, 1968; Nauss *et al.*, 1969; Nihei and Kay, 1968). The results summarized above suggest that in some sense the two sites are not identical. In studies of proton liberation during the transient phase the magnitude for the slower step was 2.5 ± 1.5 compared with the faster step which would be difficult to explain if there were two different and independent sites, since the reaction at the faster site should give the larger quantity of proton release. In the absence of evidence that the polypeptide chains are different the simplest explanation is an interaction between sites such that the rate of binding or hydrolysis of substrate is reduced if both sites are occupied. A proper test of this hypothesis will require accurate binding and rate measurements over a wide concentration range for heavy meromyosin and subfragment one.

It is necessary to briefly compare the results presented here with those of Tonomura and collaborators who have evolved a complex kinetic scheme. We will deal only with those aspects which bear on the early phosphate and proton phase. Tonomura originally reported a stoichiometric absorption of a proton (Tokiwa and Tonomura, 1965) but in later work (Onishi *et al.*, 1968; Tonomura *et al.*, 1969) it was concluded that this step was too fast to measure and is retained in the scheme only on the basis of a stoichiometric argument, namely, that unless a proton was absorbed two protons would have been produced in an experiment in which 1 mole of ATP was added to 1 mole of myosin. The rates of proton liberation and phosphate production were similar and increased with increasing ATP concentrations. Comparison of our results with Tonomura's is difficult because of his failure to distinguish between the apparent rate constant λ and the actual first- and second-order rate constants which can be evaluated only from measurements over a range of substrate concentrations large enough to include both a linear region and a plateau. For technical reasons Tonomura's measurements are confined to low ATP concentrations and therefore measure k_1 (of eq 1). From his data we calculate a rate constant of 2×10^5 to $5 \times 10^5\ \mathrm{M}^{-1}\,\mathrm{sec}^{-1}$ for H^+ or phosphate production. Thus the experimental results from the two studies are in reasonable agreement although his interpretation is far more complex.

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