V. 2. THE ROLE OF SH GROUPS IN THE INTERACTION OF MYOSIN WITH PHOSPHATE COMPOUNDS AND WITH ACTIN *

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I. Introduction‡

It is generally accepted that SH groups play an important role in the ATPase activity of myosin, in the interaction between actin and myosin, and in the interaction of nucleotides and other compounds with actomyosin (*1*). It has been postulated by Bailey and Perry (*2*) that the binding of

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‡ The following abbreviations will be used in this paper: ATP, adenosinetriphosphate; ADP, adenosinediphosphate; AMP, adenosinemonophosphate; IDP, inosinediphosphate; PP, inorganic pyrophosphate; PCMB, *p*-chloromercuribenzoate; NEM, *N*-ethylmaleimide; EDTA, ethylenediaminetetraacetate; DNP, 2,4-dinitrophenol; CSH, cysteine; AM, actomyosin; O.D., optical density; P_i^{32}, inorganic orthophosphate labeled with P^{32}; PP^{32}, inorganic pyrophosphate labeled with P^{32}; P_i, inorganic orthophosphate; c.p.m., counts per minute.

ATP to myosin involves the direct participation of an SH group. No direct evidence, however, has been forthcoming for this point of view. Bradley and Kielley (*3*) have recently shown that the blocking of some SH groups in myosin leads to an activation of the ATPase in the presence of Ca^{++}. Studies with ATP itself are complicated by the fact that it is both bound to and hydrolyzed by myosin. In view of the fact that PP affects the AM system in many respects similarly to ATP and is also known to influence the ATPase activity of myosin (*4–6*) it seemed of interest to study its binding to myosin, in the presence and absence of SH reagents, and to correlate these findings with the effect of PP and nucleotides on the physical state of AM. Studies of this type may open up further possibilities for obtaining information on the nature of the active center in myosin.

II. Experimental

1. Materials

a. Proteins

Myosin (crystalline) was prepared essentially according to Szent-Györgyi (*7*) as described earlier (*8*). Actin was extracted according to Feuer *et al.* (*9*) and F-actin purified by ultracentrifugation according to Mommaerts (*10*). F-actin was depolymerized by dialysis against an ascorbic acid-ATP solution (40 μg. per ml. of each) and repolymerized in 0.1 *M* KCl and 0.001 *M* $MgCl_2$, after removal of insoluble material by centrifugation. It was found important to insure thorough internal mixing during the dialysis in order to achieve rapid depolymerization and avoid denaturation in a drawn out process. The procedure was repeated once more.

Dog heart myosin was extracted for 30 minutes with a solution containing 0.3 *M* KCl, 0.2 *M* potassium phosphate buffer, pH 6, and .02 *M* potassium PP, with the pH adjusted to 6.2. The rest of the procedure is essentially identical with the one used for the preparation of skeletal myosin. Tryptic and chymotryptic meromyosins (*8, 11*) were prepared as described earlier with the modification of using 3-minute digestion times for the isolation of the heavy fractions (*12*). Natural AM (myosin-B) was prepared by extraction with the Weber-Edsall solution for 16 hours, with occasional stirring, at 2°. Two precipitation steps followed; the precipitates were in each case dissolved in 0.6 *M* KCl, pH 7, and the solutions filtered through eight layers of cheese cloth. The redissolved second precipitate was centrifuged for one hour at 35,000 × g in the presence of 10^{-3} *M* PP. The addition of PP reduces the viscosity and facilitates the removal of particulate impurities still present. The supernatant is limpid

and shows opalescence. In order to remove the PP two precipitations follow at 0.1 and 0.3 *M* KCl, respectively.

b. Commercially Obtained Substances

Crystalline Na-ATP, the Na salt of ADP, and AMP (free acid) were obtained from Pabst Laboratories. The ADP preparation contained less than 0.1% ATP. Adenosine was obtained from Nutritional Biochemicals Corp., IDP and crystalline PCMB from Sigma Chem. Corp. and Salyrgan from Winthrop-Stearns, Inc.

c. Isotopic PP

PP^{32} was prepared by heating 100 μmoles of K_2HPO_4 in the presence of essentially carrier free P^{32}, total activity 2-5 mc., obtained from the Union Carbide Nuclear Co., in a platinum crucible, for five minutes in an O_2-flame. The red hot crucible was placed immediately afterward into a desiccator containing $CaCl_2$. The dry material was taken up in 5 ml. of water and chromatographed on Dowex 1-formate (*13*). Two radioactive peaks were obtained, the first containing P_i^{32}, the second containing the PP^{32} (about 1% of the total activity appeared as P_i). P_i determinations were carried out on the effluent fractions both directly and after seven minute hydrolysis in 1 *N* HCl. The PP^{32} containing fraction was lyophilized and the ammonium formate removed by heating with an infrared lamp as described by Hurlbert *et al.* (*13*).

2. METHODS

a. PP Equilibrium Dialysis

2.5 ml. of the protein solution were placed in an $^8/_{32}$ in. dialysis bag, previously thoroughly washed with deionized, glass-distilled water. Preliminary experiments showed that there was no binding of PP to the tubing, and it was thus found unnecessary to use specially treated (boiled in hot water) tubing. In a few pilot experiments it was also found that the use of boiled tubing did in no way affect the experimental results. The filled dialysis bags containing the protein were placed in test tubes containing PP and PP^{32}. The tubes were closed with rubber stoppers, and placed in a test tube rack, which was attached to a rocking apparatus in the cold room at 2°. The results of preliminary experiments showed that after 24 hours of dialysis no further concentration changes occurred. In most of the experiments here reported the dialysis time was 24 hours. The volume of the outer liquid was 5 ml. At the end of the dialysis period aliquots were taken both from the liquid inside and the liquid outside the dialysis bag, and the radioactivity was determined.

b. Counting of PP^{32} and Calculation of Bound PP

The amounts of bound PP were determined on the basis of differences between the counts in the inside and outside fluids.* Samples taken from both were placed in stainless steel cups, dried, and counted with an end window Geiger tube.

Let: V_o be the volume of the liquid outside the dialysis bag in liters,
V_i be the volume of the liquid inside the dialysis bag in liters,
N^* be the specific activity of PP^{32}, c.p.m. per mole,
C_o be the outside concentration of PP, M/liter,
C_i be the inside concentration of PP, M/liter,
N_o be the number of counts due to PP^{32} outside, in c.p.m.,
N_i be the number of counts due to PP^{32} inside, in c.p.m.,
r be the number of moles of PP bound to one mole of protein,
P be the total number of moles of protein.

At equilibrium

$$C_o = C_i - r(P), \quad \text{and}$$

$$r = \frac{C_i - C_o}{(P)}. \quad \text{Substituting}$$

$$C_o = \frac{N_o}{V_o N^*}, \quad \text{and}$$

$$C_i = \frac{N_i}{V_i N^*}, \quad \text{one obtains}$$

$$r = \frac{1}{(P)N^*}\left(\frac{N_i}{V_i} - \frac{N_o}{V_o}\right).$$

c. Light Scattering Measurements

Light scattering measurements were carried out in a Brice-Speiser type apparatus (Phoenix Instrument Co.). The samples were placed in a cylindrical cell of circular cross-section and the measurements were taken at 90° with a Brown Recorder attached to the photomultiplier circuit.

d. Miscellaneous Determinations

Protein concentrations were determined with a standard micro Kjeldahl procedure and the factor 6.2 was used for converting N values into protein. pH was determined in a Beckman model G-instrument with a glass elec-

*In view of the high concentration of KCl present, and considering the net charge on myosin under the experimental conditions, the Donnan effect would have been less than 0.1%.

trode. The spectrophotometric measurements were carried out on a Beckman model DU instrument and on a Cary recording spectrophotometer.

3. Results

a. PP Binding to Various Proteins

(1) *Myosin.* In order to obtain information on the combining weight of the protein and on the affinity constant of the combination, experiments were carried out on several myosin preparations employing a fairly wide range of PP concentrations. The results could be best presented by the following equation (*14*):

$$\frac{r}{(A)} = Kn - Kr,$$

where r is the number of moles of PP bound per mole of protein, the molecular weight having provisionally been assumed to be 5×10^5; K is the binding constant defined as

$$K = \frac{(\text{protein site}-\text{PP})}{(\text{protein site})\,(\text{PP})},$$

n the maximum number of moles of PP bound per the assumed molecular weight, and (A) is the free PP concentration in moles per liter. It is assumed in the above equation that, if n does not equal 1, there is no interaction between sites. The intercept on the abscissa equals n, that on the ordinate, nK. A series of such experiments is shown in Fig. 1. The affinity constant for the myosin PP binding is 1.01×10^6, n is 0.88. From this experiment, assuming the true value of n to be 1, the minimum molecular weight would be 5.69×10^5. It was found, however, that the value of n, and thus the apparent molecular weight, varies somewhat with the age of the myosin preparation. With preparations only a few days old as low a value for the apparent molecular weight as 4.26×10^5 was obtained, in good agreement with recent determinations by other methods (*4, 15–17*). The highest combining weight found by the PP binding method was 6.7×10^5 gm., on a preparation 3 weeks old.

We have also carried out some experiments with cardiac myosin and the molecular weight suggested by the maximum binding is of the order of 4.5×10^5. The affinity constant seemed to be somewhat lower than that for skeletal myosin.

(2) *Meromyosins.* The meromyosins, that is the two fractions obtainable from the tryptic and chymotryptic digest of myosin were also investigated for PP binding. The light fraction, known to be devoid of ATPase activity, was found to bind no measurable amount of PP. PP binding to

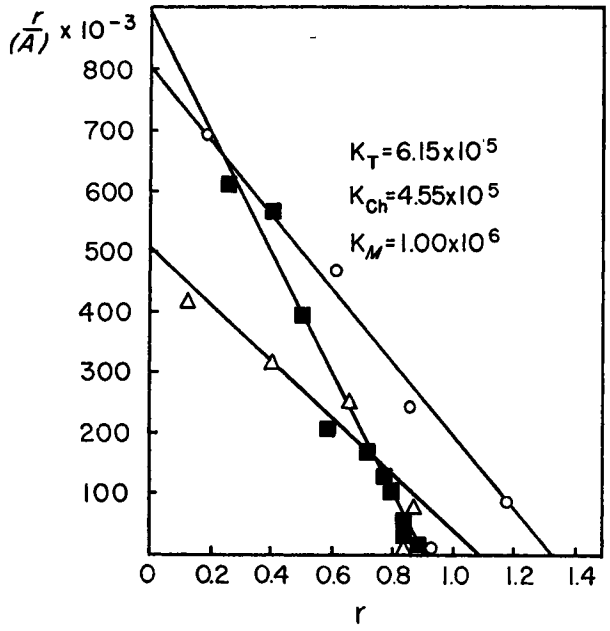

FIG. 1. Binding of PP by skeletal myosin and tryptic and chymotryptic heavy fractions. Ordinate: Moles of bound PP per assumed molecular weight of protein per free PP concentration at the end of the experiment. Abscissa: moles of bound PP per assumed molecular weight of protein. The assumed molecular weight of myosin is 500,000; that of the heavy fractions, 300,000. Composition of solution: 0.6 *M* KCl, 0.015 *M* phosphate buffer, pH 7, 0.001 *M* $MgCl_2$. Amount of protein in dialysis bag: myosin, 17.5 mg.; trypsin heavy fraction, 15.5 mg.; chymotrypsin heavy fraction, 24.2 mg. KEY: ○—○, trypsin heavy fraction; △—△, chymotrypsin heavy fraction; ■—■, skeletal myosin. For details see text.

the heavy fractions from the two digests is shown in Fig. 1. The affinity constants appear to be of the same order as the one for myosin, and molecular weights calculated from such experiments yield values around 3×10^5, in fair agreement with recent determinations by other physico-chemical methods (*12*).* There is some variation among the preparations and a careful correlation of the apparent molecular weight and the time of digestion will be necessary before we can draw definite conclusions from this type of experiment.

F-actin, both skeletal and cardiac, was found to be completely devoid of measurable PP binding.

(3) *The Actomyosin System.* Figure 2 shows a series of experiments with myosin and reconstituted AM (cardiac). It will be seen that actin

* S. Lowey, personal communication.

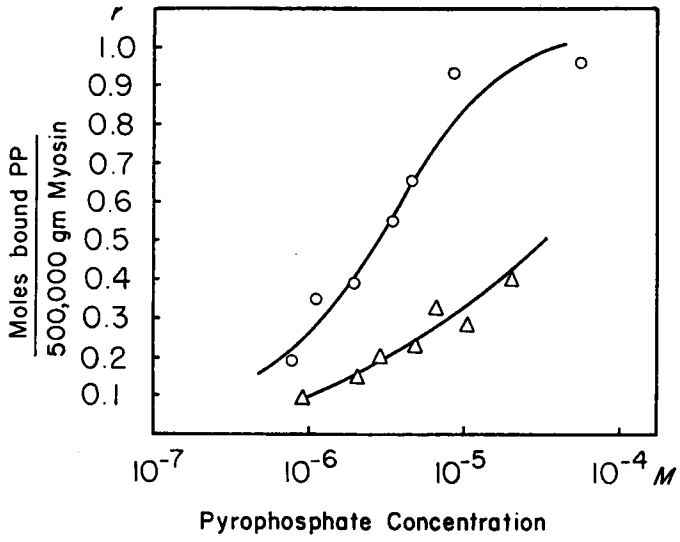

FIG. 2. The effect of dog heart F-actin on PP binding to dog heart myosin. Equilibrium dialysis for 36 hours. In the myosin experiment the dialysis bag contained 14.70 mg. of protein. In the experiment with actin the amount of myosin used was 11.6 mg., the amount of actin 2.54 mg. Both the inner and the outer solution contained 0.6 *M* KCl, 0.015 *M* PO_4, pH 7, 0.001 *M* $MgCl_2$. The initial PP concentration of the outside solution varied from 7.95×10^{-6} *M* to 9.23×10^{-5} *M*. Abscissa: concentration of free PP; Ordinate: moles of PP bound per 5×10^5 g of myosin. KEY: ○—○, heart myosin; △—△, heart myosin and heart actin. For details see text.

has a significant effect on the binding of PP and leads to a considerable reduction of the amount bound. It has not been possible to utilize concentrations high enough to reach the amount bound in the case of myosin alone because at higher concentrations the difference between the inner and outer solutions becomes rather small.

(4) *Ion Effects.* In view of the known effects of the various ions on the ATPase activity of myosin and on the interaction between myosin and F-actin, it appeared of interest to undertake a limited investigation of such effects on the PP binding by myosin (Fig. 3). Ca^{++} has only very slight inhibitory effects, if at all significant, and Mg^{++} increases the PP binding.

Potassium ions are known to influence the calcium activated ATPase of myosin and of the heavy fractions (*8*). A comparison of the K^+ effect on the PP binding to the trypsin heavy fraction with the effect on ATPase shows no correlation (Fig. 4). Thus, the likelihood arises that the potassium effect is due to an influence on the splitting rather than on the binding process.

(5) *Effect of Sulfhydryl Reagents.* As shown in Figs. 5 and 6, both PCMB and salyrgan exert an inhibitory effect on the binding of PP both to myosin and to the proteolytic heavy fractions. This inhibitory effect begins at a mercurial concentration of about $5 \times 10^{-4} M$ and reaches a virtual abolition of the binding in a saturated PCMB solution. If one expresses the amount of mercurial present in the system as μmoles per mg. of protein, one finds that the concentrations at which substantial inhibi-

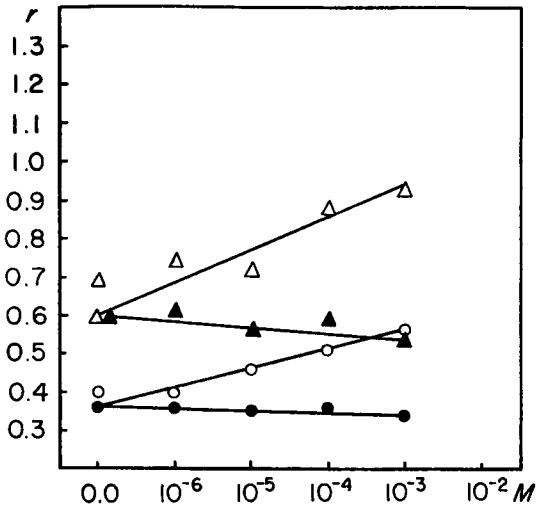

FIG. 3. Ca^{++} and Mg^{++} effect on PP binding by myosin. Equilibrium dialysis for 24 hours. Amount of protein used in experiments with lower PP concentration 17.5 mg., in experiments with higher PP concentration 47 mg. Composition of solution, 0.6 M KCl, 0.015 M PO_4 buffer, pH 7. Ca^{++} and Mg^{++} concentration as indicated in the figure. Ordinate: number of moles of PP bound per 5×10^5 gm. of myosin. Abscissa: Ca^{++} or Mg^{++} concentration, M. KEY: ○—○, initial (PP) $= 2.66 \times 10^{-6} M$, Mg^{++} as indicated on abscissa; △—△, initial (PP) $= 2.66 \times 10^{-5} M$, Mg^{++} as indicated on abcissa; ●—●, initial (PP) $= 2.66 \times 10^{-6} M$, Ca^{++} as indicated on abcissa; ▲—▲, initial (PP) $= 2.66 \times 10^{-5} M$, Ca^{++} as indicated on abcissa. Initial PP concentration is calculated on the basis of the total volume.

tion occurs are 0.1 to 0.2 μmoles per mg. These values are of the same order of magnitude as those found by Kielley and Bradley (*3*) for inhibition of myosin ATPase activity. Changes in the PP concentration had no apparent effect on the inhibition by mercurials. This point was further checked by spectrophotometric experiments. $10^{-2} M$ PP did not affect the reaction of PCMB with the SH groups of cysteine or myosin, detectable at 250 mμ (*18*). This experiment appears to exclude an effective displace-

ment of the mercurial by PP from the SH sites and also makes it rather unlikely that the inhibitory effect of mercurials on PP binding is based on a complex formation between PP and the inhibitor.* $K_3[Fe(CN)_6]$, iodoacetic acid, and NEM, all at a concentration of 10^{-3} *M*, were without effect on the PP binding. Similarly without effect was 0.1 *M* glycine, a

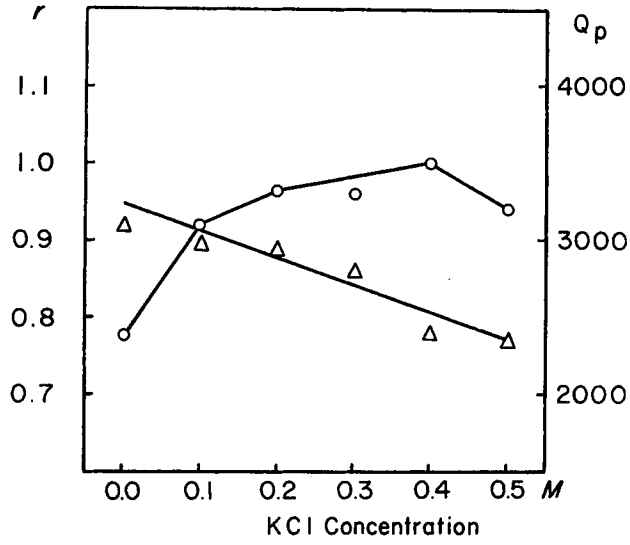

FIG. 4. Effect of KCl on PP binding and Ca^{++} activated ATPase activity of trypsin heavy fraction. Left hand side ordinate: number of moles of bound PP per 3×10^5 gm. protein. Binding experiments: equilibrium dialysis for 30 hours. Composition of solution: 0.015 *M* PO_4 buffer, pH 7, 10^{-3} *M* $MgCl_2$, KCl concentration as stated in figure. Dialysis bag contained 15.5 mg. trypsin heavy fraction. Initial PP concentration in outer solution, 1.02×10^{-5}. ATPase experiments: P_i liberation was measured at 38° during a 5 minute period. Total volume 2 ml., containing 0.01 *M* $CaCl_2$, 0.1 *M* glycine buffer pH 9, 10^{-3} *M* ATP, 15 mg. protein, KCl concentration as stated in figure. Right hand side ordinate: activities expressed as Q_P. KEY: ○—○, PP binding; △—△, ATPase. For details see text.

reagent which has been shown to interfere with the determination of SH groups in proteins (*19*).

(6) *Nucleotide and Nucleoside Effects.* In view of the similarities between the effect of PP and of ATP on the AM system (*4, 5*), it appeared of interest to carry out a limited investigation of the effect of some adenine

* There is no change in the spectrum of PCMB or salyrgan on addition of PP. In view of the studies to be described below the effect of ATP, ADP, and adenosine, on the spectra of the two mercurials and on the reaction of PCMB with cysteine and myosin was also checked. All these experiments were negative.

and hypoxanthine derivatives on the PP binding. Figure 7 shows that, of the compounds studied, ATP, ADP, and IDP exert a considerable inhibition while AMP and adenosine are without effect. The effects of ATP and ADP appear to be identical; this is to be expected in view of the fact that ATP would be broken down by myosin to ADP. Within the range of PP concentrations studied, from $7.6 \times 10^{-6} M$ to $2.4 \times 10^{-5} M$, no increase in the binding was observed in the presence of the effectively inhibiting

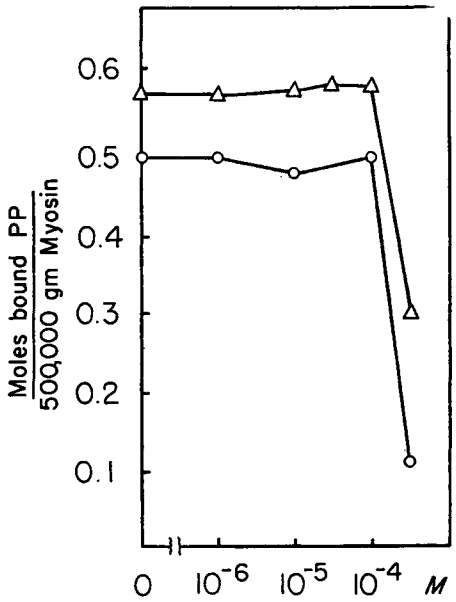

FIG. 5. Effect of mercurials on PP binding by myosin. Equilibrium dialysis. Ordinate: number of moles of bound PP per 5×10^5 gm. of myosin. 0.6 M KCl, 0.015 M PO_4 buffer, pH 7, $10^{-3} M$ $MgCl_2$. In the case of the PCMB experiments the amount of myosin in the dialysis bag was 47.0 mg., initial PP concentration outside $1.15 \times 10^{-5} M$, in the salyrgan experiment amount of myosin 17.5 mg., initial PP concentration outside $5.75 \times 10^{-6} M$. KEY: ○—○, salyrgan; △—△, PCMB. For details see text.

nucleotides. It was not feasible to use substantially higher PP concentrations, since the percentage binding would have decreased to the point where the experimental errors became greater than the expected results. In some experiments with the heavy fraction isolated from a tryptic digest of myosin, $10^{-3} M$ ATP or ADP reduced the PP binding, at a PP concentration of $2.4 \times 10^{-5} M$, by about 60%.

(7) *Miscellaneous Reagents Affecting PP Binding.* EDTA and DNP also inhibit PP binding at the concentrations shown in Fig. 8.

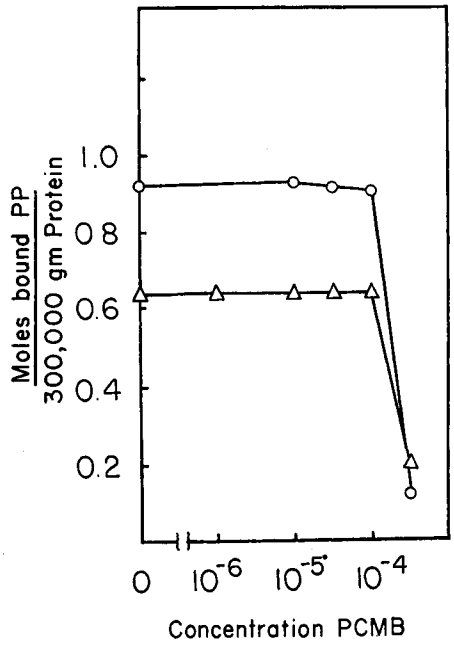

FIG. 6. Effect of PCMB on the PP binding to heavy fraction from tryptic and chymotryptic digests. Equilibrium dialysis for 24 hours. Composition of solution: 0.6 *M* KCl, 0.015 *M* PO_4 buffer, pH 7, 10^{-3} *M* $MgCl_2$. Initial PP concentration in outer solution 1.02×10^{-6} *M*. Amount of trypsin heavy fraction in dialysis bag was 15.5 mg., that of chymotrypsin heavy fraction was 24.2 mg. KEY: ○—○, trypsin heavy fraction; △—△, chymotrypsin heavy fraction. For details see text.

b. Light Scattering Studies

(1) *PP and Adenine Derivatives.* The decrease of the intensity of light scattered by actomyosin solutions on addition of ATP has been shown to be consistent with the dissociation of AM into actin and myosin (*20*). In view of the similarities between the PP and ATP effect (*4, 5*) it will be assumed in the following that reagents that cause a change in the intensity of the scattered light at 90° are causing dissociation of AM, and the change of the intensity of the scattered light is related, although not in a linear form (*4*), to the degree of dissociation. The interrelations between the ATP, ADP, and PP effects are illustrated in Fig. 9. ADP in concentrations higher than 10^{-3} *M* has a small but significant dissociating effect on AM at room temperature. Owing to a very small amount of ATP present in the ADP preparations at higher ADP concentrations there is a transient ATP effect, leading to a complete dissociation followed, as the ATP is removed by the ATPase activity of myosin, by a partial reversal in the tur-

bidity change. ADP seems to have a pronounced effect upon the rate of this reversal as a comparison between the reversal after addition of 5×10^{-6} M ATP, and the reversal after the effect of the presumably small amount of ATP in the presence of ADP shows. In the presence of ADP, cooling has a very marked effect, and the amount of ADP which causes a rather small change at room temperature will produce complete dissociation at 2°. Two points need be mentioned here: we wanted to exclude the possi-

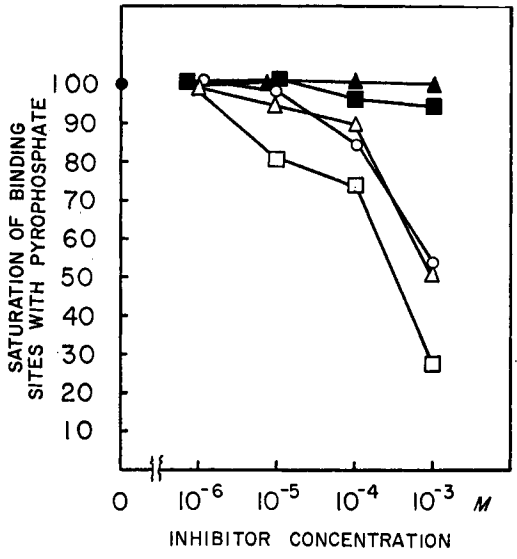

FIG. 7. The effect of adenine derivatives and IDP on PP binding by myosin. Equilibrium dialysis. Ordinate: binding expressed in per cent of that with PP only. Amount of myosin in dialysis bag 47.0 mg., in the experiments with IDP 23.5 mg., initial PP concentration in outside liquid 1.15×10^{-5} M, 0.6 M KCl, 0.015 M PO_4 buffer, pH 7, 10^{-3} M $MgCl_2$. Other additions as indicated. KEY: ○, ATP; △, ADP; □, IDP; ■, AMP; ▲, adenosine. For details see text.

bility that the ADP effect is due to a small amount of myokinase present in the myosin preparation that could have produced a low steady state concentration of ATP. We think that this is ruled out, or made extremely unlikely, by the following facts: addition of 10^{-2} M AMP has no effect on the turbidity in the presence of ADP; nor has addition of hexokinase and glucose although they quickly reverse light scattering changes caused by 2×10^{-5} M ATP. Addition of PP in the presence of 10^{-2} M ADP is ineffective, as indicated by Fig. 9. This fact is consistent with the inhibition

of PP binding by ADP. It is somewhat difficult to compare the two effects in the same concentration range, since the concentrations used in the PP-binding experiments are too low for light scattering studies.

(2) *Salyrgan and PCMB* Effects. It has been known from the work of various investigators that salyrgan and other mercurials cause a viscosity drop in an actomyosin solution. Similar changes can be observed in the light scattering system which we interpret as the dissociation into actin

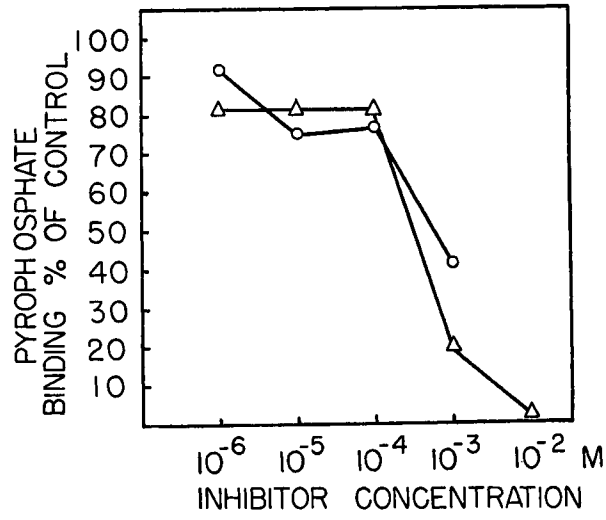

FIG. 8. The effect of DNP and EDTA on the PP binding to myosin. Equilibrium dialysis, 24 hours. Abscissa: inhibitor concentration; ordinate: PP binding expressed as percentage of binding in the absence of inhibitors. In the DNP experiment the amount of myosin in the dialysis bag was 47.0 mg., the initial PP concentration in the outside liquid was 1.02×10^{-5} *M*. Both the inside and outside solution contained 0.6 *M* KCl, 0.015 *M* PO_4 buffer, pH 7, 10^{-3} *M* $MgCl_2$. In the EDTA experiments the dialysis bag contained 47.0 mg. of myosin, the initial PP concentration of the outer liquid was 1.02×10^{-5} *M*, other components as in the previous experiment, except that no Mg^{++} was present. KEY: ○—○, DNP; △—△, EDTA. For details see text.

and myosin (Fig. 10). In the absence of ATP, salyrgan and PCMB cause a fairly slow dissociation of actomyosin, in comparison with the ATP effect. If ATP and salyrgan are added simultaneosuly, the ATP effect develops as though salyrgan had not been present, but the lower turbidity level persists indefinitely, presumably owing to the known inhibition of myosin ATPase by mercury compounds. Addition of salyrgan during the rising phase of the light scattering curve, when the ATP concentration in

the system is extremely low, stops the further rise of the scattered light intensity and causes a slow dissociation, taking place at about the same rate as would prevail if salyrgan alone were added to AM. If ATP is added about one minute or more after the addition of salyrgan, then, depending on the concentration of the ATP added, either no change occurs in the rate of decrease or rates intermediate between that observed in the presence of salyrgan alone and that found with ATP alone are seen.

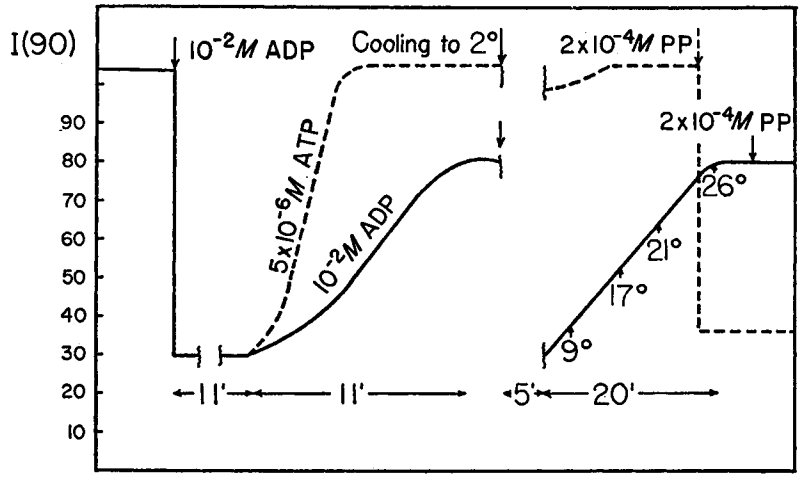

FIG. 9. Effect of ADP on the actomyosin PP system. Light scattering experiments. Ordinate: scattering intensity at 90°, in arbitrary units. Volume of solution 10 ml., containing 3.82 mg. of myosin and 1.65 mg. of actin, 0.6 *M* KCl, 0.015 *M* PO_4 buffer, pH 7, 10^{-3} *M* $MgCl_2$. Other additions as indicated. The first arrow shows the addition of 10^{-2} *M* ADP. As discussed in the text the ADP solution contains a small amount of ATP. The broken line shows the effect of 5×10^{-6} *M* ATP. At the second arrow the solution was taken to 0°, and kept at that temperature for 5 minutes. It was then allowed to come to room temperature in the apparatus, while the temperature and light scattering were being measured. The solution having reached the original temperature 2×10^{-4} *M* PP was added.

The rate of decrease is, however, very slow even on addition of ATP at 10^{-3} *M*, a concentration 1,000 times higher than the one leading to an instantaneous drop. PCMB in concentrations of the order of 10^{-5} *M* has the same effect as salyrgan.

In contrast to the inhibition of the ATP effect by salyrgan, the effect of PP and ADP (the latter to be observed only in the cold) on the turbidity of AM remains practically unchanged in concentrations of 2×10^{-4} *M* and 10^{-3} *M*, respectively.

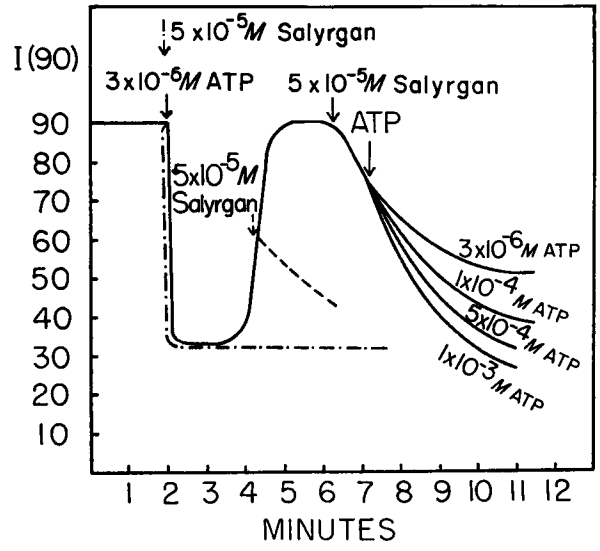

FIG. 10. The effect of salyrgan in the ATP-AM system. Light scattering experiments. The total volume was 10 ml. and the solution contained 4.5 mg. of natural AM, 0.6 *M* KCl, 0.015 *M* PO_4 buffer, pH 7, 10^{-3} *M* $MgCl_2$. Other additions as indicated in the figure. Ordinate: scattering intensity at 90°, in arbitrary units. The first solid arrow indicates the addition of ATP, 3×10^{-6} *M*. KEY: —•—•—, indicates simultaneous addition of salyrgan with ATP; — — — — —, indicates addition of salyrgan in the ascending phase of the ATP effect. The family of curves following the solid arrow indicates the addition of ATP in the amounts shown for each curve about one minute after the addition of salyrgan.

III. Discussion

The study of the binding of PP to myosin can be a source of information on the mechanism of the binding of adenosine nucleotides to myosin only if the binding of both types of compounds involves the same sites or site. Our experiments suggest that this, in fact, is the case, for higher concentrations of ADP are able to depress the binding of PP and do also inhibit the action of PP on the light scattering of AM solutions. Furthermore, the fact that ADP inhibits the ATPase activity of myosin (*21*), renders it more probable that a common center is involved in the binding of these compounds and in the enzymatic activity. If this view is correct then the discrepancy between the effect of K^+, DNP, and EDTA on the binding of PP and their effect on the ATPase activity of myosin (*8*, *22–24*), should be attributed to their being not so much involved in the binding process, as to their being modifiers of the specific rate of hydrolysis of the phosphate group. An obvious experiment to clinch the identity or at least the ex-

tremely close relationship of the binding sites for these various compounds would be to study the effects of PP on the binding of labeled ADP to myosin. The inhibition of PP binding occurs at the same mercurial to myosin ratio as found by Kielley and Bradley (*3*) to inhibit ATPase activity. Assuming that the mechanism is the same in the case of PP and ATP it would appear that the ATPase inhibition by mercurials is based on the inhibition of the combination of the substrate with the enzyme. PP binding appears to be restricted to that part of the myosin molecule which after proteolytic digestion appears as the so-called heavy meromyosin fraction. The remainder of the molecule seems to be devoid of any binding ability.

Bailey and Perry have suggested that the combination of myosin and actin depends on the participation of SH groups (*2*), and more recently Perry has proposed (*25*) that the link between myosin and actin is mediated through an ADP-Mg-SH bridge. The experiments here reported lend further support to such a concept and appear to furnish some evidence for the identity of the binding site for PP and other substances with the one for actin, since actin effectively inhibited the binding of PP to myosin.

The results of the light scattering experiments allow further tentative conclusions to be drawn about the more detailed properties of the active site. Since the dissociating action of the mercurials on actomyosin is rather slow, it is possible to investigate the availability of the dissociating site to other substances while the turbidity is still near the initial value. It is thus found that under these conditions ATP is rendered largely ineffective, while PP even in a lower concentration is able to produce rapid dissociation. This suggests that the mercurial is bound to the actomyosin even before a change in turbidity occurs and, secondly, that there exists another site which, in the presence of the mercurial bound to the first site, is accessible only to a smaller molecule, such as PP, but not to ATP. This picture would further suggest that the slow dissociation caused by the mercurials is due to a rate-limiting step whereby the mercurial reaches the second postulated site. We would say on the basis of the actin effect on PP binding that actin is bound to myosin at this second site. If PCMB or salyrgan are added to myosin first, subsequent addition of actin causes no increase in turbidity, indicating that in the absence of actin both types of sites are immediately available for the mercurials. This view should not be taken to imply that actin is, of necessity, bound to an SH group; we simply suggest that the SH group is in the domain in which the contact between myosin and actin is established.

IV. Summary

(1) PP combines with skeletal and cardiac myosin and with the tryptic and chymotryptic "heavy-meromyosins" in a ratio of about one mole of PP per mole of protein. Binding constants have been evaluated. Actin and the "light-meromyosins" do not combine with PP.

(2) Mercurials and F-actin interfere with the combination of PP with the above proteins. The effect of some other compounds, including ADP, on the binding has been investigated.

(3) Light scattering studies have been carried out to study the interrelation of PP, ADP, and mercurials on the actin-myosin binding.

(4) The results are discussed from the point of view of the structure of the active center of myosin.

References

1. K. Bailey, *in* "The Proteins" (H. Neurath and K. Bailey, eds.), p. 951. Academic Press, New York, 1954.
2. K. Bailey and S. V. Perry, *Biochim. et Biophys. Acta* **1**, 506 (1947).
3. W. W. Kielley and L. B. Bradley, *J. Biol. Chem.* **218**, 653 (1956).
4. J. Gergely and H. Kohler, "Conference on the Chemistry of Muscular Contraction," p. 14. Igaku Shoin, Tokyo, 1957.
5. Y. Tonomura, F. Morita, and K. Yagi, *J. Phys. Chem.* **61**, 605 (1957).
6. P. Gallop, C. Franzblau, and E. Meilman, *Biochim. et Biophys. Acta* **24**, 644 (1957).
7. A. Szent-Györgyi, "Chemistry of Muscular Contraction." Academic Press, New York, 1951.
8. J. Gergely, M. Gouvea, and D. Karibian, *J. Biol. Chem.* **212**, 165 (1955).
9. G. Feuer, F. Molnár, E. Pettko, and F. B. Straub, *Acta Physiol. Acad. Sci. Hung.* **1**, 150 (1948).
10. W. F. H. M. Mommaerts, *J. Biol. Chem.* **188**, 559 (1951).
11. A. G. Szent-Györgyi, *Arch. Biochem. Biophys.* **42**, 305 (1953).
12. J. Gergely, H. Kohler, W. Ritschard, and L. Varga, *Abstr. Biophys. Soc.* p. 46 (1958).
13. R. B. Hurlbert, H. Schmitz, A. Brumm, and V. R. Potter, *J. Biol. Chem.* **209**, 23 (1954).
14. I. M. Klotz, *in* "The Proteins" (H. Neurath and K. Bailey, eds.), p. 727. Academic Press, New York, 1953.
15. K. Laki, and W. R. Carroll, *Nature* **175**, 389 (1955).
16. A. Holtzer and S. Lowey, *J. Am. Chem. Soc.* **78**, 5955 (1956).
17. W. F. H. M. Mommaerts and B. B. Aldrich, *Science* **126**, 1294 (1957).
18. P. D. Boyer, *J. Am. Chem. Soc.* **76**, 4331 (1954).
19. J. P. Greenstein and J. T. Edsall, *J. Biol. Chem.* **133**, 401 (1940).
20. J. Gergely, *J. Biol. Chem.* **220**, 917 (1956).
21. H. M. Kalckar, *J. Biol. Chem.* **153**, 355 (1944).
22. G. D. Greville and D. M. Needham, *Biochim. et Biophys. Acta.* **16**, 284 (1955).
23. J. B. Chappell and S. V. Perry, *Biochim. et Biophys. Acta.* **16**, 285 (1955).

24. W. J. Bowen and T. D. Kerwin, *J. Biol. Chem.* **211**, 237 (1954).
25. S. V. Perry, *Physiol. Revs.* **36**, 1 (1956).

Discussion

MORALES: I think that Dr. Gergely has given us several demonstrations that pyrophosphate is similar to ATP which is very gratifying. This had been thought for a long time because pyrophosphate has been well known to be a competitive inhibitor of ATPase. We differ on some things he said but I think those differences are beyond the scope of this meeting. However, there is one little item that I think fits into this puzzle, and perhaps somebody has a good idea about this. A couple of years ago Dr. Kielley wrote a very interesting paper in which he showed that as you add PCMB to myosin in 0.6 *M* KCl, that the first half of the PCMB that goes on actually stimulates the ATPase activity. Recently we have been experimenting along lines which now seem very related. We have been studying substances like cysteine ethyl ester and AET* on ATPase activity, and we now think that these compounds form mixed disulfides with the enzyme, so that probably they are doing the same thing as Dr. Kielley's PCMB. But the curious thing is that they do not *of themselves* increase the ATPase activity. What they do is to make the ATPase enormously sensitive to calcium. If you always test in calcium, then you observe simply an acceleration of the splitting rate. But it can be shown that the effect is that when you tie up the SH group with either PCMB or by forming a mixed disulfide, you then make the system very much more sensitive to calcium. So something is going on long before you get an interference with pyrophosphate bond splitting.

Very recently Dr. Blum has communicated to me some work which appears to point to the fact that what is going on is some interference with the binding of the ring end of the ATP. I think this is very interesting. I think it is maybe also harmonious with the fact that somehow the first PCMB's that go on or the first AET's that go on, interfere with the ring end and the later ones interfere with the splitting of the pyrophosphate structure.

RACKER: I would like to draw your attention to a procedure for measuring ATPase activity which we find more accurate and reproducible than the conventional method. We use catalytic amounts of ATP together with an ATP regenerating system, e.g., excess phosphoenolpyruvate and pyruvatekinase, and measure liberation of orthophosphate. While in the conventional system an excess of ATP is used and ADP is allowed to accumulate, by the above procedure ADP is continuously removed. Several ATPases have been reported to be inhibited by ADP. With these enzymes, much higher activity values can be obtained in the ATP regenerating system than by the conventional assay. For example, in the case of mitochondrial ATPase, which Kielley found to be inhibited by ADP, and which is known to be difficult to analyze, good proportionality between concentration and activity can be obtained. Moreover, the activity values are 4–5 times higher than without the regenerating system. I would like to ask Dr. Gergely whether some of his findings may not be explained by ADP inhibition which may be more pronounced under certain experimental conditions.

GERGELY: In reply to Dr. Racker's suggestion, I think this is a very good suggestion and very often useful. In the case of myosin—and I think Dr. Morales agrees with me—the amount of ADP needed for inhibition is much larger than the amount that

* Aminoethylthiouronium salts.

is liberated in the course of the enzymatic reaction. Furthermore, the phosphate liberated is proportional to time, showing no falling off as ADP is formed.

MORALES: I was going to say that it can be shown that the velocity is strictly linear with enzyme concentration.