Binding of Pentachlorophenol by Actomyosin*

William J. Bowen, Harold L. Martin, and William E. Jacobus

ABSTRACT: A study has been made of the binding of pentachlorophenol (PCP) to the muscle protein actomyosin because it causes extensive shortening of glycerol-treated muscle fibers without degradation as occurs when shortening is induced by adenosine triphosphate (ATP). The binding is increased markedly by decreases of pH from 9 to 7 and by addition of KCl, tetramethylammonium chloride, and $MgCl_2$. Actomyosin in homogenized glycerol-treated muscle binds about 120 moles of PCP/10^5 g of protein, but as myosin B it binds about 175 moles/10^5 g. Affinities are low (30–70) and the addition of salts increases them. Acetylation of actomyosin greatly reduces the amount of PCP bound, which suggests that binding occurs on amino groups, probably of lysine and arginine residues. Methylation supposedly decreases the net negative charge of the protein and more PCP is bound.

It is well known that ATP[1] causes glycerol-treated fibers of rabbit muscle (*psoas*) to shorten, but it is not as well known that pentachlorophenol (PCP) also causes extensive shortening of this biological model of muscular contraction (Weinbach and Bowen, 1958). Shortening by PCP involves binding of ions to the protein without degradation of the phenolic compound, and in this respect is unlike the binding of ATP which results in phosphorolysis of the nucleotide. During exposure of the contractile protein to ATP dephosphorylation occurs so rapidly that it is difficult to measure the binding of ATP to the fibers (Nanninga and Mommaerts, 1960), but PCP is not altered. Therefore the extent of PCP binding which causes glycerol-treated muscle fibers to contract 40–50% of their initial length (Weinbach and Bowen, 1958) can be measured by centrifugal removal of PCP bound to the protein or by equilibrium dialysis. The concentration of PCP in the supernatant fluid or the dialysate can be determined by measurement of its optical density.

This paper is concerned with the binding of PCP to actomyosin in myosin B and in glycerol-treated muscle fibers and fibrils. The amounts bound were several-fold to infinitely more than those of ATPase modifiers such as *p*-mercuribenzoate, DNP, and EDTA and were strongly influenced by pH and the concentration of salt in the reaction mixtures. The binding sites are the amino groups of the protein. The affinity is low compared with those for ATP and PP.

Experimental Procedure

Myosin B was prepared essentially according to the procedure of Szent-Györgyi (1951) by extraction of ground rabbit muscle with alkaline 0.6 M KCl for 20 hr with occasional stirring and at 4°. The myosin was precipitated two times to reduce extraneous proteins.

Fiber bundles of rabbit *psoas* muscle were prepared in isometric condition by the method of Szent-Györgyi (1951). The glycerol was washed out with water and the large bundles split by means of watchmakers forceps into bundles varying from 175 to 800 μ in width. Measurements of width were made by means of a microscope equipped with an ocular micrometer. Homogenized material was prepared from either isometric *psoas* muscle or isotonic thigh muscle which was cut into small pieces and then put into equal parts of glycerol and water. After storage the glycerol was washed out and the size of the pieces reduced to that which could be homogenized first in a blender and then in a Teflon-glass tissue grinder.

Pentachlorophenol, an Eastman product, was dissolved as the sodium salt by adding the minimum quantity of NaOH. Then the pH was carefully adjusted with HCl to 7.5–7.8 according to the concentration of PCP. Stock concentrations (0.2–0.4 M) required high pH values (7.8) for complete solution.

All experiments on binding, except those to study the effects of hydrogen ion concentration, were done between pH 7.5 and 8.0 to avoid precipitation of PCP which occurs at neutrality. The methods of pH control are given in the legends of the figures and table. Exploratory experiments showed that PCP begins to precipitate from 0.05 M solution in each of phosphate, Veronal acetate, and Tris maleate buffers at about pH 7; *e.g.*, 0.05 M PCP at pH 7.1 was clear, while at pH 6.9 it developed a voluminous precipitate of PCP.

Both myosin B and glycerol-treated muscle were acetylated with acetic anhydride by the method of

*From the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, U.S. Department of Health, Education, and Welfare, Bethesda, Md. *Received May 12, 1965.*

[1] Abbreviations used in this work: PCP, pentachlorophenol; ATP, adenosine triphosphate.

Fraenkel-Conrat (1955). The protein was suspended in 0.2 ml of water, an equal volume of saturated sodium acetate (16 M) was added, and the suspension was immersed in crushed ice. Then 30 μl of acetic anhydride was added in increments of 5 μl during 1 hr. Since the protein was suspended in 8 M sodium acetate and probably went into solution during acetylation, the following precaution was taken to prevent the loss of myosin dissolved in this high concentration of salt. When acetylation was completed any protein in solution was precipitated by dilution with 80 volumes of water and the precipitated protein allowed to stand for 1 hr. Then the preparation was centrifuged at 1400 $\times$ *g*. The supernatant-diluted acetate was poured off. The sedimented muscle was washed by resuspending it in 50 volumes of water and concentrating it again by centrifugation. This washing was repeated once.

In addition to the above procedure homogenized glycerol-treated muscle was acetylated by 5-μl additions of an ice-cold saturated aqueous solution of acetic anhydride over a 90-min period while maintaining the pH of the mixture between 7.5 and 8.5 by additions of 0.5 M NaOH. Homogenized glycerol-treated muscle was esterified by the addition of dimethyl sulfate according to the method and proportions described by Saroff *et al.* (1953) for methylating the carboxyl groups of bovine serum albumin.

The amount of PCP bound to these forms of contractile muscle protein was estimated by determining the amount removed from 1 or 2 ml of a known concentration of PCP solution by known dry weights of protein, or by determining the amount bound to protein contained in dialysis tubing when equilibrium was reached. Tests by the first method were done in quadruplicate so each datum is an average of four values. The protein and solution were separated by centrifugation.

When equilibrium dialysis was done, 4 ml of the mixture with protein and PCP were closed in dialysis tubing and suspended in 36 ml of phosphate buffer and KCl solution. Dialysis was done with continuous rocking. Since homogenized glycerol-treated muscle fibers and myosin B are thermostable, all experiments were done at 27 $\pm$ 1° except dialyses which were done at 5° with terminal periods at 27°.

The concentration of PCP in the supernatant solution or the outside solution was determined spectrophotometrically by measuring the optical density at 320 mμ and calculating of the concentration on the basis that the molar extinction coefficient at 320 mμ is 4800 (Weinbach *et al.*, 1963). When necessary, the optical density was corrected for the contribution of protein. The amount of PCP bound was calculated as moles/10^5 g of protein.

Isotonic shortening of fiber bundles of glycerol-treated muscle was followed in about 0.4 ml of 0.02 M PCP solution spread out on a glass slide lying on a metric scale. In order to reduce ionic effects, the solutions were adjusted to pH 7.1–7.2 by adding HCl to the Na salt of PCP.

Results

Effects of pH and Salts. The three solid line plots of Figure 1 show the effect of pH, controlled by phosphate, Veronal acetate, and Tris maleate buffers on the binding of PCP to the unmodified homogenized muscle. As pH was decreased from 8.75 to 7.1 the amount of PCP bound increased greatly. Also, at any given value of pH the amount bound was influenced by the buffer used in the sequence of phosphate > Veronal acetate > Tris maleate. Similar enhancement by phosphate occurred when other preparations of glycerol-treated muscle were used in experiments done subsequent to those of Figure 1. The enhancement of the binding of PCP in the presence of phosphate buffer also occurred with methylated and acetylated protein (*vide infra*) and is possibly due to the ionic strength of phosphate buffer being greater than that of Veronal acetate and Tris maleate.

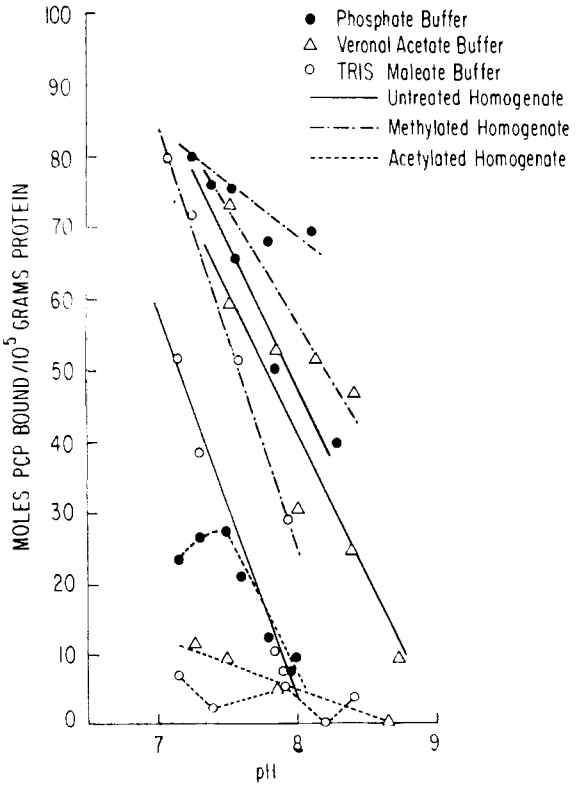

FIGURE 1: The effect of pH on the binding of pentachlorophenol by untreated, methylated, and acetylated homogenized glycerol-treated rabbit muscle. Solutions contained 5 mg of protein/ml, 0.01 M PCP, and 0.05 M buffer.

The latter is indicated in further experiments in which ions of several species with valences of one and two were studied. The addition of cations such as K^+, Mg^{2+}, and tetramethylammonium (TMA^+) enhanced binding of PCP to myosin B (Figure 2), the effect of Mg^{2+} being the greatest. Plots (not shown) of binding to homogenized glycerol-treated muscle showed the same enhancement by the addition of Mg^{2+}, K^+, and Na^+. In Figure 2 the molarity of salt refers to that

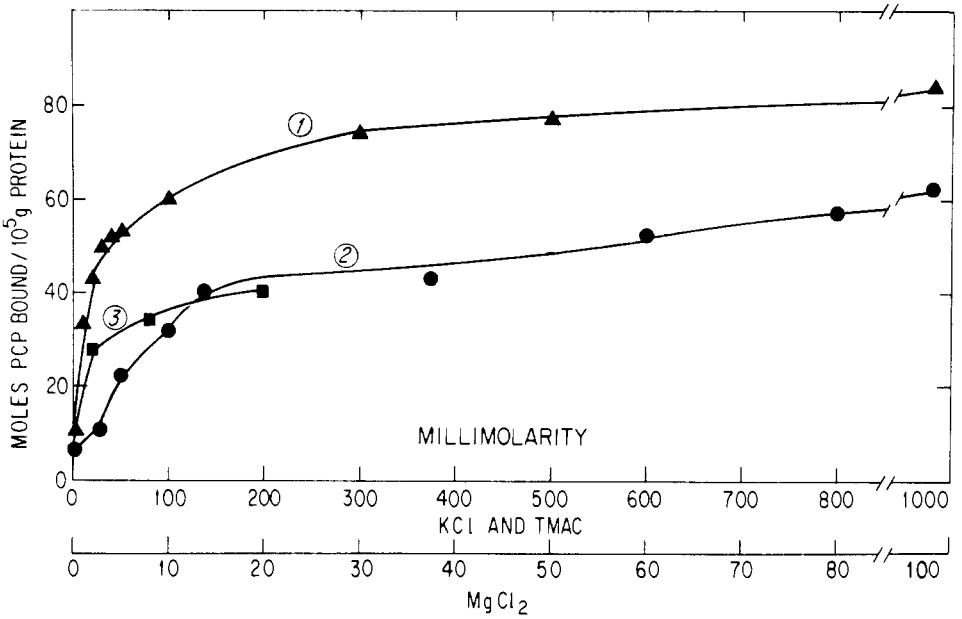

FIGURE 2: The effect of three salts on the binding of pentachlorophenol by myosin B. (1) $MgCl_2$; (2) KCl; (3) tetramethylammonium chloride. Solution contained 6 mg of myosin B/ml, 0.005 M PCP, and 0.05 M Tris maleate at pH 8.

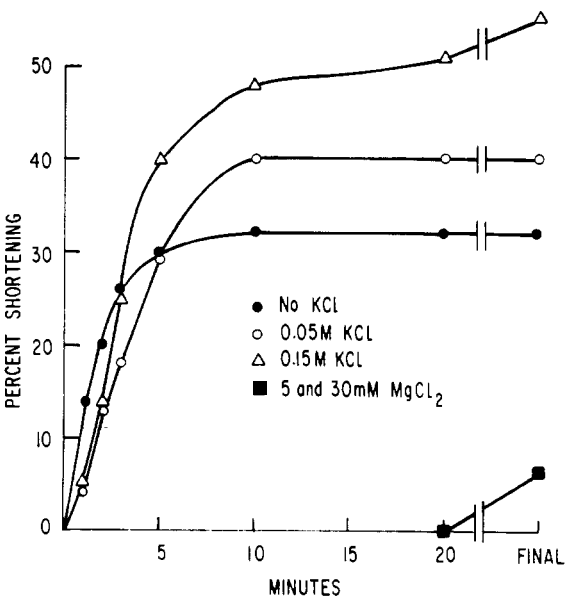

FIGURE 3: PCP-induced shortening of fiber bundles of glycerol-treated muscle in solutions of 0.02 M PCP with and without two concentrations of each of KCl and $MgCl_2$. pH values were adjusted to 7.1–7.2 with HCl to obtain maximum binding (see Figure 1). Shortening = $l_0/(l_0 - l_f)$.

added only and does not include the 0.015 M KCl which remained after precipitation of the myosin from 0.6 M KCl. When the concentration of KCl was reduced by a further washing of the myosin B (final concentration 7.5×10^{-4} M) 3 moles of PCP was still bound for every 10^5 g of protein.

Effect of KCl, TMAC, $MgCl_2$, and pH on PCP-Induced Shortening. Since addition of these salts and decrease of pH enhance binding of PCP, it is pertinent to ascertain whether they also enhance PCP-induced shortening. Isotonic contractions of bundles of glycerol-treated muscle fiber were followed. The results show (Figure 3) that increase of the concentration of KCl caused greater extent of shortening but at a slower rate. Additions of $MgCl_2$ inhibited shortening greatly. A similar decrease of the rates of PCP-induced shortening occurred in the first study of this phenomenon in PCP solution containing KCl and $MgCl_2$ (Weinbach and Bowen, 1958). TMAC also increased the extent of shortening (not shown), but there was little difference between the effects of 0.01 M and 0.1 M concentration. The extent of shortening was greater at pH 7.3 than at pH 8.2 (not shown), which is similar to the effect of increase of pH on the amount of PCP bound to glycerol-treated rabbit muscle.

Extent of Binding of PCP. The number of binding sites for PCP on the myosin molecule was estimated by direct measurement of the PCP removed from solution by known amounts of myosin B and homogenized glycerol-treated muscle or by equilibrium dialysis. Experimental solutions contained 4.4–5.0 mg of protein/ml of 0.05 M phosphate buffer at pH 7.8–8.0 and the indicated [KCl]. From the amounts bound in these solutions plots of curves were calculated using the mass law binding expression

$$\bar{\nu} = \frac{k'cn'}{1 + k'c}$$

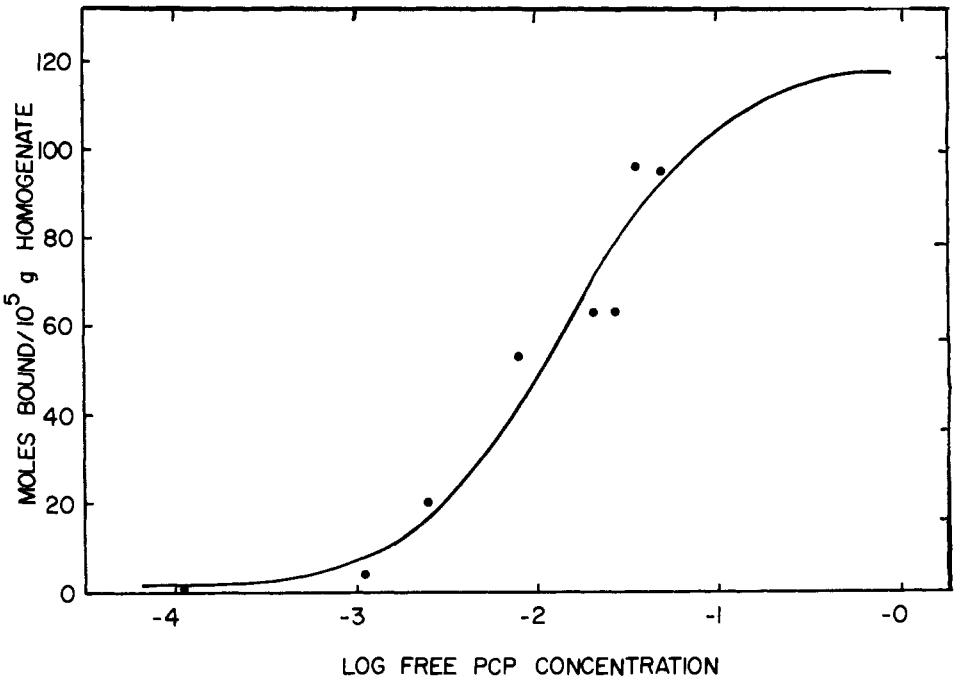

FIGURE 4: Extent of binding of pentachlorophenol by homogenized glycerol-treated rabbit muscle. Solutions contained 4.4–5.0 mg of protein/ml. The concentration of KCl was zero. pH was adjusted to 8 by 0.05 M phosphate buffer.

in which $\bar{\nu}$ is the number of moles PCP bound/10^5 g of protein, k' is the apparent binding constant, c is the concentration of PCP, and n' is the apparent number of binding sites. The results are given in Figures 4 and 5.

The value of the apparent binding constant was 50–70 for homogenate (Figure 4), 15–25 for myosin B in 0.02 M KCl, and 40–60 in 0.2 M KCl (Figure 5). Concentrations where higher values of $\bar{\nu}$ would be expected gave anomalous experimental results, probably because of the increase in solubility of myosin at [PCP] $> 10^{-1}$ M. This is undoubtedly related to the interactions of an insoluble protein with organic ions being unlike those of a soluble protein, thus yielding varying proportions of solid and aqueous phases as described by Klotz (1953).

Anionic vs. Cationic Binding Sites. Since actomyosin binds PCP freely (Figures 1, 2, 4, 5) it is likely there is a large number of sites on the muscle protein to which it is bound. Since PCP exists as an anion at pH 7 and above (pK = 4.8) the binding sites must be cationic. The role of cationic amino sites was investigated on homogenized glycerol-treated muscle by acetylating the protein. Likewise, the role of anionic carboxyl groups was investigated by methylating the protein.

The results are given in Figure 1 which also presents the effect of pH on binding of PCP. Figure 1 shows that acetylation drastically reduced the amount of PCP bound, indicating that the cationic amino groups of actomyosin are indeed responsible for most of the PCP binding which occurs. The moderate increase in PCP binding that follows methylation is probably due to removal of electrostatic repulsion of anionic carboxyl groups. It is to be noted that acetylation sharply reduced and that methylation slightly increased the binding in the presence of each of the three buffers used.

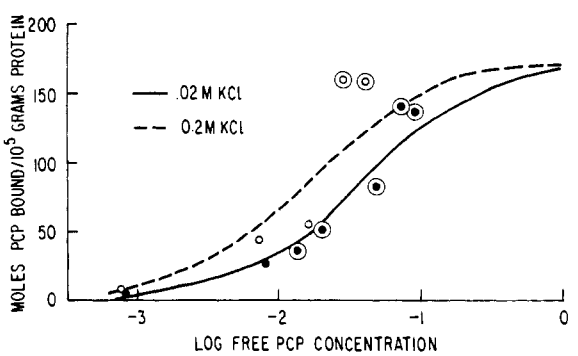

FIGURE 5: Extent of binding of pentachlorophenol by myosin B in 0.0225 and 0.2075 M KCl. Amounts bound were obtained by measuring the concentration of free PCP in the supernatant solution (uncircled points) or in outside solution of equilibrium dialyses (circled points). Solutions contained 4.55–5.0 mg of protein/ml; pH was adjusted to 8 by 0.05 M phosphate buffer.

Discussion

As indicated, this study was undertaken to quantitate the binding to actomyosin of a compound which causes

contraction of glycerol-treated muscle fibers.[2] It is not concerned with the effect of PCP on the enzymatic properties of actomyosin as these already have been considered (Weinbach and Bowen, 1958). Other compounds (EDTA, Ebashi, 1961; PCMB, Sekine *et al.*, 1962; DNP, Rainford *et al.*, 1964) which affect actomysin ATPase either do not bind or do so only in small quantities. These small quantities are not sufficient to cause contraction of glycerol-treated muscle fibers.

It is therefore of possible value to determine experimentally whether a compound like PCP which causes shortening binds to a greater extent than those compounds which affect only enzymatic properties. The amounts of PCP bound in the above study by three forms of actomyosin amply demonstrate that this is true. The affinity of PCP for homogenized glycerol-treated muscle estimated from Figure 4 is 50–70, while that for myosin B varies between 30 and 60 in the range from 0.02 to 0.2 M KCl. Both of these values are low for affinities but the number of sites on the molecule (120 for the homogenized muscle and about 160 for myosin B)[3] of actomyosin is sufficiently great that the muscle fibers shorten markedly in the presence of PCP.

The results of the studies with acetylated and methylated actomyosin indicated strongly that PCP is bound by cationic amino groups. This is contrary to the anion–anion reaction proposed by Giglio and Goncalves (1963) for binding of PCP to bovine serum albumin. From the results of Kominz *et al.* (1954) in their amino acid analyses of actin and myosin it can be calculated that actomyosin possesses about 120 cationic amino acid residues/10^5 g at pH 8 (lysine and arginine) if none are utilized in the myosin–F-actin interaction. This is slightly less than the number of binding sites found on myosin B and about equal to that found on homogenized glycerol-treated muscle. It appears that the procedures employed to prepare myosin B make more cationic sites available than exist in glycerol-treated muscle.

The contrasting effects of KCl and $MgCl_2$ on shortening of glycerol-treated fibers are of possible significance. The addition of KCl increased the extent of shortening in a manner similar to the increase of the binding of PCP to myosin, which is shown in Figure 2. Myosin B binds potassium (Lewis and Saroff, 1957) and appears to possess about 25 potassium binding sites/10^5 g. This is comparable to the increment in PCP bound produced by the addition of 1 M KCl (Figure 2) and makes it appear that the neutralization of anionic sites by K^+ enhances the binding of PCP. The extent of binding of Mg^{2+} to myosin B is not known, but in these experiments it is possibly significant that its effect on binding of PCP occurred at much lower concentration than did the effect of KCl. The possibility exists that Mg^{2+} links two fragments of H-meromyosin *via* an electrostatic bridge, thus immobilizing PCP-induced contraction of actomyosin.

The original investigation (Weinbach and Bowen, 1958) of the effect of PCP on the reaction between actomyosin and ATP indicated that ATP is considerably more readily bound to the protein than is PCP. This conclusion is based on the finding that the inhibition of ATPase by PCP occurred over a period of 5–8 min when ATP and PCP were applied to actomyosin simultaneously, but when the PCP was applied in advance no ATP was split (Table II, Weinbach and Bowen, 1958).

It is interesting that the number of sites involved in the ATPase activity of myosin A is much lower than the number of sites involved in PCP-induced shortening of glycerol-treated muscle fibers. Nanninga and Mommaerts (1960) found one ATP-binding site and Kubo *et al.* (1960) found two for every 4.2 × 10^5 g. The fact that ATP causes strong shortening of glycerol-treated muscle fibers indicates that the combination of myosin A and actin produces more sites, that binding to numerous nonenzymatic sites occurs, or that the turnover on each enzymatic site is sufficient to compensate for the small number of those sites.

Another comparison to be made is with the binding of pyrophosphate (PP). In the presence of 0.6 M KCl and 0.3 mM $MgCl_2$, Tonomura and Morita (1959) found that 1 mole equiv of PP is bound by 5.8 × 10^5 g of myosin A or B, while Brahms and Brezner (1961) found that 40 moles/4.2 × 10^5 g of myosin are bound in pure PP solution (0.025 M). Both investigators found that PP affects such parameters as flow birefringence, light-scattering, and viscosity of myosin. We have observed that no shortening of glycerol-treated muscle fibers occurs in solutions of this polyphosphate (Bowen and Martin, 1964). This raises the possibility that different sites bind PP and PCP or that the arrangement of the sites involved is such that when they are covered

[2] Microscopically the shortening of glycerinated muscle fibers induced by PCP is similar to that induced by ATP. In each, when shortening is between 25 and 40%, the I band narrows while the A band remains of constant width but the H zone is obliterated or reduced. All observations were made by phase-constant microscopy thanks to the kind proffer of the facilities of Dr. M. F. Morales, University of California, and Dr. R. J. Podolsky, National Institutes of Health. Publication of further details of the comparative microscopic changes occurring during these shortenings is contemplated.

[3] One preparation of myosin B consistently bound less PCP than any of the others used and appeared to be saturated when 30 moles of PCP was bound. One difference between the myosin B of this preparation and the others was that it settled rapidly (finished in 20 minute) when it was being purified by the usual 1:20 dilution and subsequent reconcentration by standing so as to settle into a small volume. If this was due to the electrostatic nature of the protein, it follows that numerous opposing charges had neutralized each other, discharged the protein, and caused rapid settling. The protein bound little PCP due to the discharged condition.

The other preparations of myosin B settled slowly (8 or more hours), thus giving the impression of being similarly charged. The possibility that this repelling charge was preponderantly positive is likely because the proteins bound five times as much PCP anion as did the slow settling preparation.

Two varieties of myosin B have been reported previously (Bowen and Gershfeld, 1957). The principal difference in character noted then was that ATPase activity of slowly settling preparations of myosin B responded only slightly to variations of the concentration of $MgCl_2$, while that of the rapid-settling variety responded markedly.

by PP the distribution of charges is not changed so as to produce electrostatic attractions.

If there are binding sites which bind both nucleotide and phenolic groups, the presence of ATP in mixtures of homogenized glycerol-treated muscle and PCP would possibly decrease the PCP bound. Such decrease was found experimentally (Table I), thus indicating

TABLE I: Effect of Sodium ATP on Binding of PCP by Homogenized Glycerol-Treated Muscle.[a]

mM ATP	mM NaCl	Moles PCP Bound/ 10^5 g Protein
0	200	44.2
5	150	41.2
10	100	36.2
20	0	27.2

[a] Ionic strength maintained at 0.2 by additions of NaCl. Homogenate/ml of reaction mixture, 5 mg; concentration of PCP, 5 mM. pH adjusted to 7.5 with HCl.

that some sites in the actomyosin molecule bind both ATP and PCP or that the molecular distortion induced by ATP makes PCP binding sites unaccessible. Whether there are nonenzymatic ATP-binding sites which contribute to shortening of these models of muscular contraction remains to be ascertained.

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