THE EFFECT OF VARIOUS MONOVALENT ANIONS ON MYOSIN B SOLUTIONS THE IDENTIFICATION OF ACTIN AS A PRODUCT OF ATP ACTION

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SUMMARY

Physical studies on Myosin B solutions in KSCN, KI, and in a variety of other ionic media reveal that exposure to these media leads to a dissociation into myosin and actin moieties. The actin appears in the globular form and may be clearly identified in the ultracentrifuge. The peak formed under these conditions (G-actin) is indistinguishable from the peaks formed in the same media by conventionally prepared actin or by redissolution of the pellet obtained by addition of ATP to Myosin B and strong centrifugation. It is concluded that actin exists in Myosin B in combination with myosin but otherwise in substantially the same form as is obtained by conventional procedures, employing treatment with non-aqueous solvents; and that actin is indeed a product produced by the action of ATP on Myosin B.

INTRODUCTION

Since the early work on the proteins of muscle it has usually been assumed that solutions obtained from 5 to 24-h extracts of striated muscle, after successive precipitations at low ionic strength, contain a complex of myosin and actin, which may be dissociated by the action of adenosine triphosphate (ATP)*,[1].

Doubts concerning this interpretation have persisted, however, and these have partly been based on the consistent failure of many workers to observe a separate actin component in, say, the ultracentrifuge upon addition of ATP[2–5]. In the ultracentrifuge 24-h preparations usually show a single, sharp, fast-moving ($S_{20,w} > 10S$) peak (Fig. 1). When ATP is added, a single, sharp peak with the sedimentation rate of myosin invariably becomes visible, but no component appears that is identifiable as actin, which under the usual conditions of pH ~ 7, in 0.6 *M* KCl, would presumably be in the fibrous form (F-actin), rather than in the globular form (G-actin). Proponents of the classical view insist that the F-actin released is of such low concentration, so polydisperse, and/or so fast-moving that it never appears in the Schlieren photograph[6]; but since F-actin prepared from extracts of muscle acetone powder is visible in the

* We shall refer to these as "Myosin B" solutions and, in general, conform to the nomenclature suggested in ref. 4.

centrifuge, this argument is not entirely convincing and more definite proof is needed. Recently, several attempts have been made to obtain such proof. WEBER sedimented Myosin B solutions containing ATP in the preparative centrifuge and conclusively identified the supernatant as myosin by a variety of means[6]. However, it was only shown indirectly that the pellet contained actin by demonstrating that mixing a pellet extract with supernatant restored the super-precipitation property and the enzymic activity (measured in the presence of Mg^{++}), both characteristic of the original Myosin B solution. It has also been shown that the supernatant solutions do not reform the original fast component when the ATP has been enzymically destroyed[6], or when it is dialyzed out[5]. Thus it appears clear that a protein component essential to the formation of the characteristic particles in Myosin B can be separated from myosin by addition of ATP and strong centrifugation. A positive identification of this component as actin has not yet been made, however.

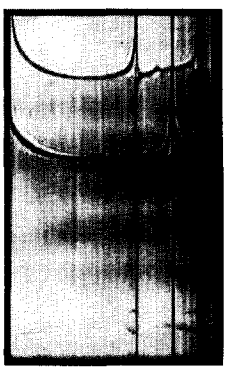

Fig. 1. Myosin B, 0.49%, centrifuged at 59,780 rev./min; bar angle, 60°; temperature, 11.9°; time, 32 min. Wedge cell, 0.6 *M* KCl, $S_{20,w} = 9.5$ S. Regular cell, 0.6 *M* KCl + $5 \cdot 10^{-3}$ *M* ATP, $S_{20,w} = 4.1$ S.

SZENT-GYÖRGYI was able to prepare actin from Myosin B by precipitating the myosin with alcohol[7], and GERGELY AND MARTONOSI prepared actin by making an acetone powder from a myosin B precipitate and subsequent extraction exactly as in the preparation of actin from muscle pulp[8]. Both of these preparations increased the viscosity of myosin solutions and displayed the G–F transformation and, in the latter case, reacted with actin antibody. It might be argued, however, that the use of organic solvents simply destroys the myosin, producing a fragment that one calls "actin", but which has no significance as far as the original myosin B is concerned. It is presumed that parallel experiments on Myosin A gave no such results, although no mention is made of this point. Furthermore, it has been pointed out that these experiments do not show that the actin is released from combination with myosin *by ATP treatment*[5].

One possible way out of the difficulties outlined above is to investigate the behavior of Myosin B solutions in media in which actin is expected to be entirely in the globular form, *e.g.*, 0.6 *M* KI or KSCN. Complete depolymerization to monomers should prevent rapid sedimentation of actin and should also wipe out the polydispersity presumably caused by the varying degrees of polymerization of the F-actin molecules. The G-actin peak should then be clearly visible in the ultracentrifuge. With this end

in view we have studied the ultracentrifugal, viscometric, and light-scattering properties of solutions of Myosin B in a variety of ionic media.

Incidental to this main objective was the desire to investigate the possible effects of these media themselves on Myosin B, exclusive of their use in identifying the products produced by action of ATP. The early observation that I^- and SCN^- decrease the birefringence of Myosin B solutions[9] and the more recent finding that these ions cause contraction of Myosin B fibers[10] suggest that their action is similar to that of ATP, but the point has never been conclusively demonstrated in solution studies.

Anticipating the results below, we will see that the action of I^- and SCN^- on Myosin B solutions makes it possible to identify G-actin clearly in the ultracentrifuge and to decide the question of ATP action in favor of the classical view of dissociation into myosin and actin moieties. Furthermore, we find, in agreement with expectations, that I^- and SCN^-, and a variety of other ions, themselves dissociate the complex when in proper concentration.

METHODS

Preparations and materials

Myosin B was prepared by 24-h extraction of rabbit muscle with WEBER-EDSALL solution[1], precipitation at ionic strength 0.06 *M*, redissolution in 0.6 *M* KCl, and two more precipitations at 0.28 *M* ionic strength. The final solution ($\sim$ 1 % protein) was cleaned up by a 3-h centrifugation at 50,000 $\times$ *g*.

Myosin was prepared and purified by conventional procedures as previously described[11].

The ATP used was the crystalline disodium salt (Sigma). It was neutralized with potassium hydroxide before use.

All other reagents used were of A.R. grade. Potassium iodide contains enough carbonate impurity so that the pH of fresh solutions is about 9. These were brought to pH 7.2 by adding potassium phosphates to 0.005 *M*. A small amount of sodium thiosulfate (10^{-3} *M*) was added to prevent formation of iodine. Checks of the action of pure iodide, using fresh solutions, showed that the thiosulfate had no detectable effects. Phosphates are similarly inert.

Whenever it was necessary to introduce iodide or thiocyanate ions into the Myosin B solutions by dialysis, this was accomplished in 2 h by stirring thoroughly and making frequent changes of dialyzate. Myosin aggregates rapidly in these media and the solutions must be studied immediately after the addition of these ions.

Sedimentation: All analytical ultracentrifuge runs were made in the Spinco Model E instrument, usually with both the regular and the "wedge window" cell. High speed preparative ultracentrifugations were carried out in the Spinco Model L.

Concentration: Protein concentrations were determined routinely by a Folin procedure, and by microkjeldahl analysis for the physical measurements (*e.g.*, viscosity).

Viscosity: Cannon-Fenske viscometers were used. These required a 5-ml charge and had water efflux times of 200–300 sec.

RESULTS

Action of iodide and thiocyanate ions

As anticipated by various workers[9,10], solutions of Myosin B in 0.6 *M* KI or 0.6 *M* KSCN behave in a manner similar to 0.6 *M* KCl solutions containing ATP.

Fig. 2-a shows the sedimentation pattern displayed by a solution obtained by dialysis *versus* 0.6 *M* KI. The main peak was found to have $S_{20,w} = 5.9$ S, a value consistent with the identification of this component as myosin. In this medium, actin, if present, would have to be in the globular form. Indeed, in spite of the considerable base line distortion caused by the solvent, a small peak is clearly visible behind the myosin peak, and having $S_{20,w} = 3.5$ S. This value is in satisfactory agreement with that expected from studies on pure actin[12], and is a clear argument in favor of the idea that the actin isolated using the action of organic solvents on muscle pulp (or Myosin B solutions), is not a result of this action, but actually exists as such in the original material. To emphasize this point further we present sedimentation results on a solution in 0.6 *M* KSCN (Fig. 2-b), which also depolymerizes actin completely, and causes less base line distortion. Again, the results, we feel, are clear cut.

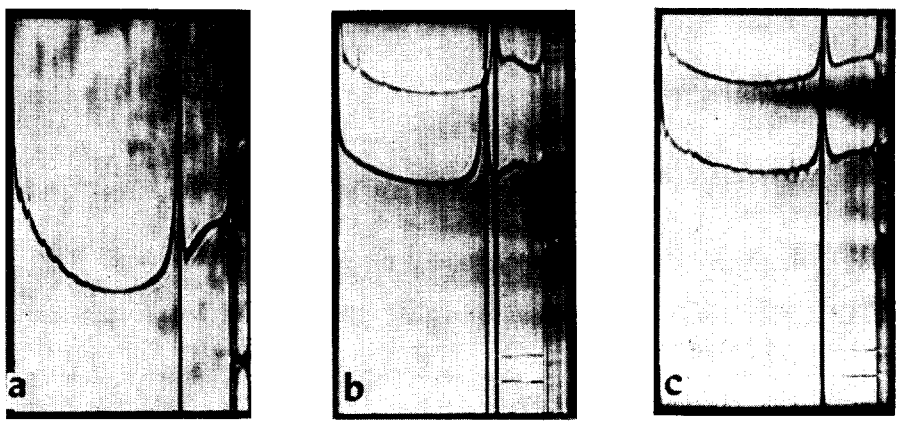

Fig. 2. Centrifugation at 59,780 rev./min; bar angle, 60°. (a) Myosin B in 0.6 *M* KI, 0.50% protein; temperature: 12.0°; time, 32 min. For the main peak: $S_{20,w} = 5.9$. For the slow peak: $S_{20,w} = 3.5$. (b) Myosin B in 0.6 *M* KSCN; temperature, 12.5°; time, 48 min. Wedge cell: 0.80% protein; $S_{20,w} = 4.7$, 3.1. Regular cell: 0.51% protein; $S_{20,w} = 6.0$, 2.9. (c) Myosin in 0.6 *M* KSCN; temperature, 10.1°; time, 96 min. Wedge and regular cells, 0.88% protein, $S_{20,w} = 3.8$ S.

On the other hand, the addition of iodide or thiocyanate to myosin solutions of higher protein concentration produces no slow peak that could be confused with G-actin (Fig. 2-c). In this case a faint convexity of the base line behind the myosin peak, probably representing traces of actin, is to be compared with the clear peaks seen in Myosin B, at a lower concentration of protein. This is especially noteworthy in view of the recent finding that 0.1 *M* Na_2CO_3 or 0.01 *M* KOH do produce a small fragment in both myosin[13] and Myosin B (see ref. 4).

The identification of the slow peak we observed with the actin obtained by acetone treatment of muscle and of Myosin B, or by alcohol treatment of Myosin B solutions, may be made more directly by adding such actin to solutions of Myosin B in 0.6 *M* KSCN. To this end, actin was prepared from Myosin B by a procedure involving precipitation of the myosin with alcohol[7]. The resulting actin was divided into two aliquots which were dialyzed *vs.* 0.6 *M* KSCN and 0.1 *M* KCl + 10^{-3} *M* $MgCl_2$, respectively. These centrifuge patterns are shown in Fig. 3-a. The thiocyanate solution shows nothing but the expected slow peak. The solution in potassium chloride shows a very small amount of this slow peak and an indeterminate amount of faster material.

Since both solutions contained the same amount of protein it would appear that it is possible to form F-actins that do not show up on the Schlieren plate*.

The actin solution in 0.6 *M* KSCN, when mixed with Myosin B solution in the same medium, gave the Schlieren pattern shown in Fig. 3-b. The characteristic slow peak of the Myosin B is seen to be augmented by the addition of actin, lending further credence to the view that they are one and the same. It would require an extraordinary coincidence for the action of such different reagents as KI or KSCN, alcohol, and acetone to produce such similar, yet artifactual, substances.

Further proof of the similarity in the effect on Myosin B of KI or KSCN and of ATP was obtained by viscosity measurements (Fig. 4). The intrinsic viscosity of a

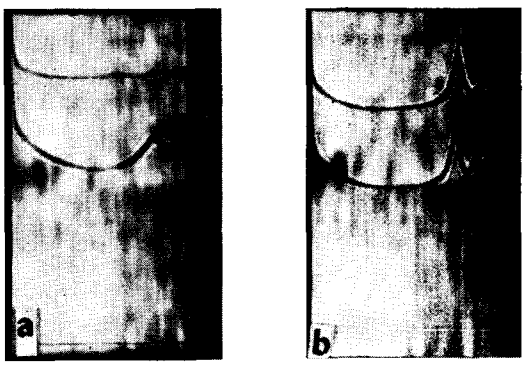

Fig. 3. (a) Actin (prepared from Myosin B) centrifuged at 59,780 rev./min; bar angle, 55°; temperature, 6.8°; time 64 min. Wedge cell: solution in 0.1 *M* KCl + 10^{-3} *M* $MgCl_2$. Reg. cell, 0.6 *M* KSCN; $S_{20,w}$ = 3.1 S. (b) Centrifugation at 59,780 rev./min; bar angle 60°; temperature, 14.4°; time, 16 min. Wedge cell: Myosin B, 0.33%, in 0.6 *M* KSCN; Regular cell: Myosin B, 0.66%, in 0.6 *M* KSCN + equal volume of actin solution (Fig. 3-A) in 0.6 *M* KSCN.

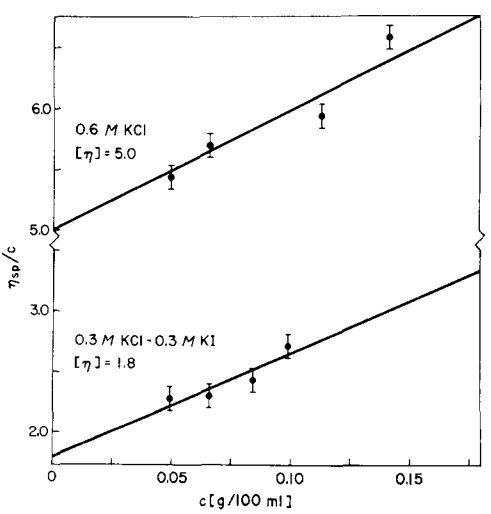

Fig. 4. Intrinsic viscosity of Myosin B in 0.6 *M* KCl and in 0.3 *M* KCl + 0.3 *M* KI.

* It is worth noting that this F-actin, after dialysis *vs.* 0.6 *M* KCl, readily formed a very fast moving component when mixed with a myosin solution; and that addition of ATP to this mixture resulted in regeneration of the myosin peak.

Myosin B sample in 0.6 *M* KCl at 25.3 ± 0.02° was found to be 5.0 dl/g. When measured immediately after adding an equal volume of 0.6 *M* KI* to solutions at various protein concentrations, the value fell to 1.8 dl/g. The latter is almost exactly what one would expect for a mixture of myosin and G-actin of the proper composition, and is essentially the value obtained in the presence of ATP[1]. The amount of actin present is sufficiently small so that its presence in the G or F forms makes little difference in the intrinsic viscosity of the solution.

Finally, we have consistently found that light-scattering measurements on these solutions show molecular weights and radii of gyration comparable to those produced by ATP. Qualitatively speaking, there is an immediate and visually obvious reduction in turbidity on addition of KI, KSCN, or ATP.

It is of interest to determine the minimum concentration of I^- or SCN^- required to produce complete dissociation. It is necessary in these experiments to maintain the ionic strength at 0.6 *M*, with a suitable amount of KCl, to retain the protein in solution. For a semiquantitative determination, Myosin B solutions (in 0.6 *M* KCl) were mixed with the appropriate amount of 0.6 *M* KI or KSCN and 0.6 *M* KCl to provide a series of solutions of varying ionic composition but with the same protein concentration. From the Schlieren diagrams it was estimated that at a total ionic strength of 0.6 *M* and 0.5 % protein ~ 0.3 *M* KI was required for complete dissociation and ~ 0.2 *M* KSCN. The sedimentation pattern in 0.4 *M* KCl + 0.2 *M* KSCN, indicating complete dissociation, is shown in Fig. 5-a. The partial dissociation caused by a lower concentration of KSCN may be seen in Fig. 5-b.

The effects caused by these reagents thus represent definite and positive evidence that a component exists in Myosin B solutions that is necessary for the retention of

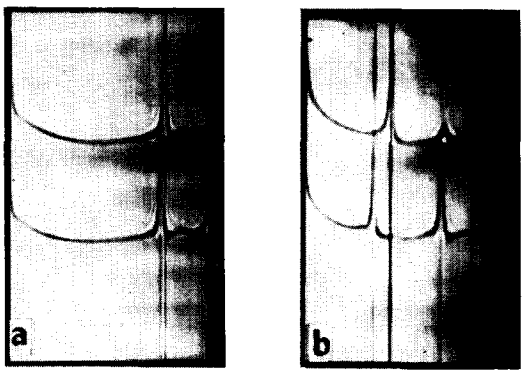

Fig. 5. Myosin B centrifuged at 59,780 rev./min; bar angle, 55°; time, 48 min. (a) 0.34 % protein in 0.4 *M* KCl + 0.2 *M* KSCN in both cells; temperature, 13.0°; $S_{20,w}$ = 5.2, 3.6. (b) 0.51 % protein in both cells; temperature, 11.7°. Wedge cell: solution in 0.6 *M* KCl; $S_{20,w}$ = 11.7, 5.9. Regular cell: solution in 0.5 *M* KCl + 0.1 *M* KSCN; $S_{20,w}$ = 13.6, 5.5 S.

the molecular integrity of the major component of 24-h Myosin B, and that this component is altered in the presence of suitable concentrations of KI or KSCN to form material with $S_{20,w} \cong 3$–4 S. The simplest interpretation consistent with these observations is that this component is the same as actin prepared from acetone

* Thus producing a solution containing 0.3 *M* KCl + 0.3 *M* KI. This is sufficient iodide to cause complete dissociation (see below).

powders of muscle pulp, whose properties it shares, and that, in Myosin B, it is in combination with the other product identifiable after addition of KI or KSCN: myosin. Of course, this does not prove directly that the action of ATP produces actin, but the evidence given here that actin is present in these solutions in combination with myosin and the fact that myosin is produced by ATP leaves little room for doubt. Nevertheless, more evidence on this point will be presented in the next section but one.

Action of other monovalent anions on Myosin B solutions

The results described above on effects of iodide and thiocyanate prompted us to investigate the effects of other anions on Myosin B.

It has been known for a long time, of course, that high concentrations of KCl cause effects similar to ATP addition[1], but the process has not been studied in detail and no evidence has come forward on whether this is a simple ionic strength effect or a result of chloride binding. These studies, a brief preliminary report of which follows, do not allow for quantitative interpretation, but do suggest that the effects are not simply caused by ionic strength.

Several monovalent anions were tested for their effects on Myosin B solutions at pH 7. In every case the total ionic strength was kept at least at 0.6 *M* by adding enough KCl, where necessary, to make up the difference. In Fig. 6 ultracentrifuge patterns are shown in 0.6 *M* KBr, 0.6 *M* KNO_3, 1 *M* and 2 *M* KCl and in a mixture of composition: 0.3 *M* NaCl + 0.3 *M* $NaClO_4$*. Table I indicates the minimum concentration needed to produce complete dissociation in this system for all the anions tested. The order is suggestive of the HOFMEISTER series, but not entirely in register with it. The steady change in minimum concentration of halogenide needed for complete

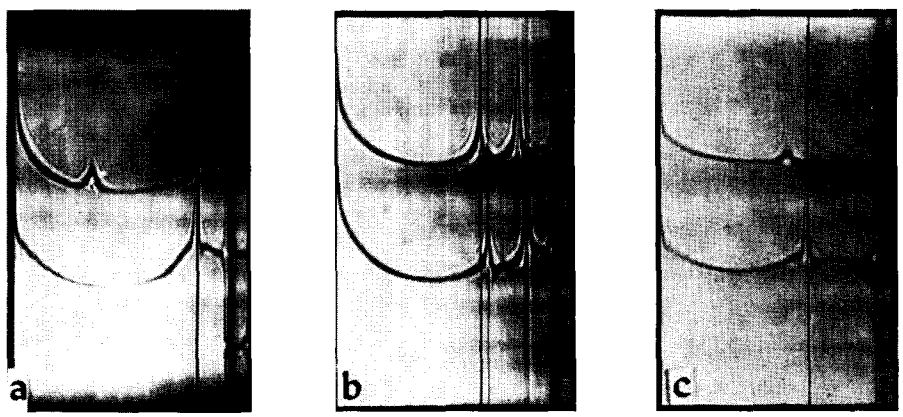

Fig. 6. Myosin B centrifuged in various media at 59,780 rev./min. (a) bar angle, 60°; temperature, 10.6°; time, 32 min. Wedge cell, 0.65% protein in 0.6 *M* KNO_3; $S_{20,w}$ = 21, 4.7, 4.0 (latter for peak, not yet visible in the frame shown, behind the myosin). Regular cell, 0.65% protein in 0.6 *M* KBr; $S_{20,w}$ = 4.9, 4.0 (also not yet visible). (b) bar angle, 55°; temperature, 11.0°; time, 32 min. Wedge cell, 0.51% protein in 1 *M* KCl; $S_{20,w}$ = 11.3, 5.3. Regular cell, 0.51% protein in 2 *M* KCl; $S_{20,w}$ = 12.9, 4.5. (c) bar angle, 55°; temperature, 12.0°; time, 80 min. Regular cell, 0.51% protein in 0.3 *M* NaCl + 0.3 *M* $NaClO_4$; $S_{20,w}$ = 4.4, 3.0 S. Wedge cell run is irrelevant in this case.

* Potassium perchlorate is insufficiently soluble so that sodium has to be used as the cation. This has no demonstrable effect, as was shown by comparing solutions in 0.6 *M* NaCl and 0.6 *M* KCl.

dissociation makes it doubtful that the dissociation produced by 2 *M* KCl is a simple ionic strength effect.

The question of the reversibility of this phenomenon is not easy to answer, partly because of the fairly rapid aggregation of myosin in these media, and partly because the conversion to G-actin becomes irreversible after a short time. However, preliminary work indicates that at least partial reversibility can be achieved, if the actin is "protected" with ADP[14] and if the exposure time is kept short.

TABLE I

In all cases the total ionic strength was at least 0.6 *M*, the difference being made up for with KCl, where necessary.

Anion	*Minimum concentration for complete dissociation (M)*
Paratoluene sulfonate	0.20
Thiocyanate	0.20
Iodide	0.30
Perchlorate	0.30
Bromide	0.60
Nitrate	0.65
Chloride	> 2.0

Products produced by ATP action

Recent work leaves little room for doubt that myosin is the major product produced by ATP addition to Myosin B solutions[6,15]. We have already noted that myosin itself shows no $S_{20,w} \cong 3$–4 S peak in 0.6 *M* KSCN. If, then, actin is the other product of dissociation by ATP, the pellet obtained after addition of ATP and centrifugation should show a slow peak in 0.6 *M* KSCN. The result of an experiment in which this pellet was dissolved in a small volume of 0.6 *M* KSCN is shown in Fig. 7. The only visible constituent has $S_{20,w} = 3.4$ S, which enables us to identify the "rapidly sedimenting" product of ATP addition (the "cementing component" of ref. 5), as rich in actin*. Since the "slowly sedimenting" product is myosin this would seem to be conclusive evidence that Myosin B is "actomyosin" and that the addition of ATP produces myosin and actin.

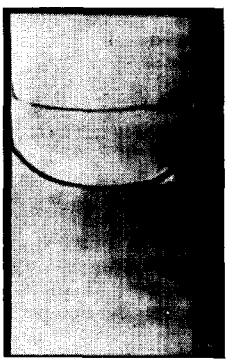

Fig. 7. Pellet produced from Myosin B by ATP addition and centrifugation and dissolved in 0.6 *M* KSCN. Ultracentrifuge run at 59,780 rev./min; bar angle, 60°; temperature, 7.8°; time, 8 min; $S_{20,w} = 3.4$ S in regular cell. Wedge cell run is irrelevant in this case.

* Of course the pellet should also contain myosin, but since a large portion of the precipitate is insoluble the absence of myosin in the solution obtained is not surprising.

DISCUSSION

There is one further objection that has been raised to the "classical" picture of ATP–Myosin B interaction that is worth mentioning. It has been remarked[5] that dissociation of an actomyosin complex by ATP should lead to "smaller" (*i.e.*, less massive) particles, and that, therefore, it should be easier to destroy the ATP response of Myosin B by centrifugation in the absence of ATP than in its presence—in disagreement with the facts. It would seem reasonable, however, that a thread-like F-actin molecule, relieved of its rod-like myosin partners, would coil up more tightly, thus decreasing its hydrodynamic resistance sufficiently to overcompensate for its diminished mass. Quantitative studies on purified actins are required in order to determine whether this effect is, in fact, occurring.

The work described here concerns only the major components of Myosin B, visible in ordinary Schlieren optics. We can say nothing, therefore, about the large "deformable" component proposed and discussed by GELLERT *et al.*[4]. We wish merely to point out here that Myosin B does contain actomyosin and that one result of ATP action is its dissociation into actin and myosin.

Thus far, only speculation is possible on the mechanism of the dissociation of actomyosin by these anions. The simplest assumption, of course, is that these ions are bound to the individual protein moieties and that charge repulsion leads to dissociation. Such measurements of ion binding by myosin as exist[16] are in rough accord with the data of Table I. Similar data are not available for actin, however, and the fact that the dissociation produced by monovalent ions occurs in the same range of ion concentration as actin depolymerization indicates that the state of charge of the actin is playing as important a role as that of the myosin. Furthermore, since ATP produces F-actin as a dissociation product, the mechanism of action of the nucleotide may differ from that of the other anions, where it is complicated by (or includes as an essential step) G-actin formation.

ACKNOWLEDGEMENT

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