again to the conclusion that some molecular inflation must occur.

Acknowledgment.—The authors are indebted to Dr. Bruno H. Zimm for reading the manuscript and especially for acquainting them with his treatment of the phase shift problem.

BETHESDA, MARYLAND
BERKELEY, CALIF.

[CONTRIBUTION FROM THE DIVISION OF PHYSICAL BIOCHEMISTRY, NAVAL MEDICAL RESEARCH INSTITUTE]

Studies on the Contractile Proteins of Muscle. II. Polymerization Reactions in the Myosin B System[1,2]

BY PETER H. VON HIPPEL, MARTIN F. GELLERT[3] AND MANUEL F. MORALES

RECEIVED JULY 22, 1958

Dilution of a concentrated solution of myosin B (to $c \simeq 0.01$ g./100 ml.) initiates a spontaneous turbidity decay. The turbidity falls gradually over a period of 24 hr. or more, eventually approaching the level reached immediately on adding "saturation" concentrations of ATP. A Zimm-type analysis of the light-scattering data shows a concomitant drop of the average molecular weight with time, indicating that the turbidity decay mirrors a slow depolymerization. The equilibrium level to which this reaction tends is shown to be a function of protein concentration; the rate of approach to equilibrium is a function of temperature, the decay proceeding more rapidly at low temperatures. Increasing the pH increases the rate of depolymerization and may displace the equilibrium. The interaction of this effect with that of ATP has been studied. Previously[4] ATP binding to particles of myosin B has been shown to "inflate" some particles and to depolymerize others. A reversible ATP-induced effect, presumably "inflation," is here demonstrated in dilute solutions and shown by light scattering to be qualitatively distinct from the effect of dilution. Sedimentation analysis is used to show that the depolymerization brought about by ATP is also reversed, in concentrated solutions, when ATP is removed. Drastic centrifugation in the presence of ATP leaves the system unable to re-aggregate, thus showing that some "cement" (possibly actin) has been removed.

Introduction

In the preceding paper[4] we concluded on the basis of light-scattering and sedimentation data that myosin B solutions are polydisperse and contain three general size classes of particles. The addition of adenosinetriphosphate (ATP) inflates the largest class (I) of particles at essentially constant weight average molecular weight ($\bar{M}_w$ = 10 to 50 $\times$ 10^6), depolymerizes a large portion of the intermediate class (II) of particles ($\bar{M}_w$ = 1 to 5 $\times$ 10^6) and does not physically alter the class (III) of smallest particles. Elsewhere[5] we have reported that the particles of class III are identical with those of myosin and have a molecular weight of 4.2 $\times$ 10^5. Solutions made from a 5-hr. extraction of muscle mince contain about 35% of classes I and II and 65% of class III. Longer extraction, *e.g.*, 24 hours, increases the proportion of classes I and II over class III. Given such a system, it is natural to inquire whether even in the absence of added ATP there may not exist reactions between particles of different size classes and whether the effect of ATP may not be considered a displacement of the equilibria of these pre-existing reactions. Suggestions along these lines are already in the literature, notably in the work of Laki, *et al.*[6,7] Recent work from this Laboratory[8] has furthered this idea, for it has shown that merely on standing cold at high dilution the turbidity of myosin B solutions falls slowly to roughly the same value as is reached on ATP addition. In the present work this "dilution effect" and its interplay with the ATP effect has been investigated and is shown to provide evidence supporting our previous conclusions[4] about myosin B. By means of the ultracentrifuge we have also examined the displacements which ATP brings about in more concentrated solutions, and their reversal following the removal of ATP. These results too are harmonious with the structural conclusions cited above, but additionally they point to the presence of a non-myosin, high molecular weight "cementing substance," possibly actin.

Methods

Most of the methods employed in this investigation, including the preparation of 5- and 24-hr. extracted myosin B, measurement of protein concentration and ATPase activity, light-scattering and sedimentation techniques and calculations, etc., have been described in detail in the preceding paper.[4] Here we need only add that the light-scattering experiments, in which the intensity of 90° (or 45°) scattering was followed as a function of time at various temperatures, were mostly carried out in a light-scattering photometer situated in a 5° room. Measurements at higher temperatures were achieved by warming the cell to the appropriate temperature in a water-bath before inserting it into the photometer.

Results

1. The "Dilution Effect." (a) Influence of Temperature.—If stock solutions (*ca.* 0.5 g./100 ml.) of 5-hr. or 24-hr. extracted myosin B are diluted 25-fold and are allowed to stand in the cold, the ratio of the intensity of 90° scattering at time t following dilution to the intensity immediately after dilution, $R_{90}(t)/R_{90}(0)$, falls as shown by the solid lines of Figs. 1a and 1b, respectively. If, just prior to taking a reading, the cell is warmed

(1) The opinions expressed in this article are those of the authors and do not necessarily reflect the opinions of the Navy Department or the Naval Service at large. (Statement added in accordance with U. S. Navy regulations.)

(2) Presented in part at the 132nd Meeting of the American Chemical Society, September 9, 1957, New York, and in part at the Conference on the Chemistry of Muscle Contraction, October 12, 1957, Tokyo, Japan.

(3) Postdoctoral Fellow of the National Heart Institute (National Institutes of Health), 1956–1958.

(4) M. F. Gellert, P. H. von Hippel and M. F. Morales, THIS JOURNAL, **81**, 1384 (1959).

(5) P. H. von Hippel, H. K. Schachman, P. Appel and M. F. Morales, *Biochim. et Biophys. Acta*, **28**, 504 (1958).

(6) K. Laki, S. S. Spicer and W. R. Carroll, *Nature*, **169**, 328 (1952).

(7) K. Laki and W. R. Carroll, *ibid.*, **175**, 389 (1955).

(8) M. F. Morales, A. J. Osbahr, H. L. Martin and R. W. Chambers, *Arch. Biochem. and Biophys.*, **72**, 54 (1957).

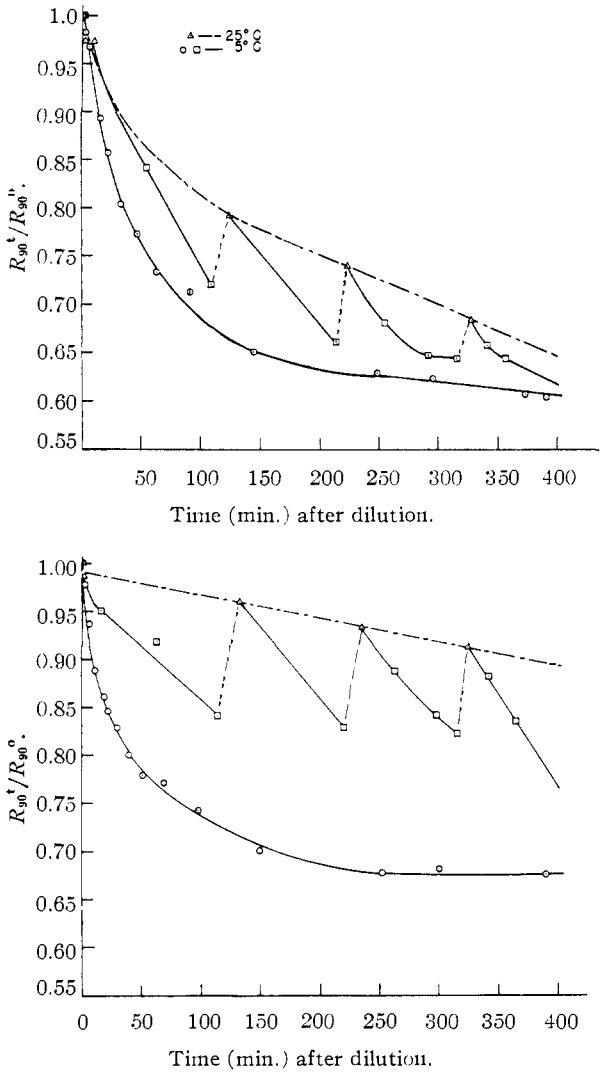

Fig. 1.—(a) "Dilution effect" in 5-hr. extracted myosin B. Protein concentration (c) is 0.012 g./100 ml. 0.6 M KCl, pH 7.0; diluted at zero-time; (⊙) measured at 5°; (⊡) measured at 25°. Maintained at 5° between measurements. (b) "Dilution effect" in 24-hr. extracted myosin B. c = 0.008 g./100 ml. 0.6 M KCl, pH 7.0; diluted at zero-time, (⊙) measured at 5°, (⊡) measured at 25°. Maintained at 5° between measurements.

(*ca.*, 5 minutes) to 25° $R_{90}(t)/R_{90}(0)$ rises sharply ("instantaneously," relative to our time scale). If the cell is then returned to 5°, $R_{90}(t)/R_{90}(0)$ decays back toward the uninterrupted 5° curve. The rate of this decay closely resembles the corresponding rate of decay of the uninterrupted 5° curve. An indefinite number of such cycles is possible. It might be thought that a line (dashed lines of Fig. 1) passed through all the points measured at 25° would coincide with the $R_{90}(t)/R_{90}(0)$ curve of a solution standing continuously at 25°. This is not the case; under the latter conditions an "irreversible aggregation" and a deterioration of ATPase occur. Qualitatively, solutions of 5-hr. and 24-hr. extracts behave alike; however, the extent of reversal achieved by warming 5-hr. extracts is much less and is progressively reduced on standing.[9] At times in excess of 800 to 1000 minutes after dilution, the 5 and 25° relative scattering curves for 5-hour myosin B (Fig. 1a) merge, and then further temperature changes have no effect. On the contrary, the 25° curve for the 24-hr. extracts does not (even in 10 days after dilution) fall much below $R_{90}(t)/R_{90}(0)$ = 0.85 to 0.90, and thus the "dilution effect" in 24-hr. extracts may be largely reversed at any time by warming to 25°. Particularly for 5-hr. extracts, the level to which $R_{90}(t)/R_{90}(0)$ decays on standing at dilutions of *ca.* 0.01 g./100 ml. is very close to that to which $R_{90}(t)/R_{90}(0)$ falls instantaneously upon the addition of excess ATP (*vide supra*).

(b) **The Influence of Protein Concentration.**—The following experiment showed that the "dilution effect" proceeds, to some extent, at all concentrations. Various dilutions of a 5-hr. myosin B extract, ranging in protein concentration from 0.8 g./100 ml. to 0.01 g./100 ml., were made at zero time and were allowed to stand for 24 hr. at 5°. Each solution was then made 0.01 g./100 ml. in protein by adding an appropriate amount of solvent and the 90° scattering was immediately measured. Figure 2 shows that the higher the

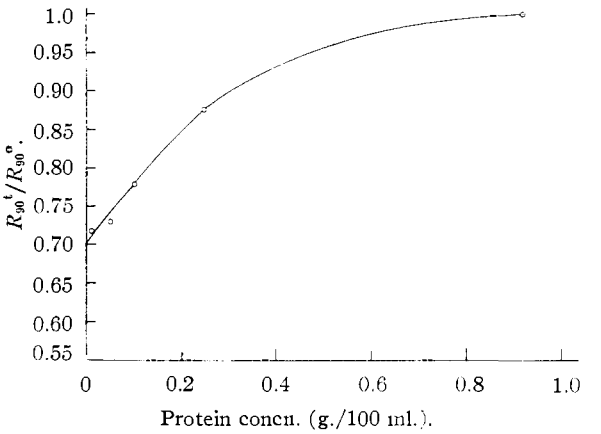

Fig. 2.—"Dilution effect" in 5-hr. extracted myosin B as a function of protein concentration. Stood at concentrations indicated (at 5°) for 24 hr., then all were diluted to c = 0.01 g./100 ml., and (90°) relative scattered intensity was immediately measured; solvent 0.6 M KCl, pH 7.0.

dilution at which the protein stood for the 24 hr. the lower was the relative scattering of the solution at the time of measurement, but there is no "threshold" concentration above which the "dilution effect" fails to occur. Earlier work,[8] as well as current experiments in which these diluted solutions were held at 5° for more than 24 hr., indicate that at each concentration, $R_{90}(t)/R_{90}(0)$ reaches a corresponding asymptotic value,[10] suggesting that what is observed as a result of dilution is a slow

(9) The first observations on the "dilution effect,"[8] made before the effect of temperature had been realized, were made on diluted 5-hour extracts which stood in the cold but were warmed for measurement. The curves obtained therefore corresponded to the dashed line of Fig. 1a. Fortunately, in 5-hour preparations, the dashed and the solid lines are rather similar, and both approach asymptotically the same $R_{90}(\infty)/R_{90}(0)$ level, so the effect was detected.

(10) The precise asymptotic value to which $R_{90}(t)/R_{90}(0)$ falls varies somewhat from preparation to preparation and with the age of the preparation.

transition between two concentration-dependent equilibria.

(c) **Influence of Ions Other than ATP.**—As might be expected from the work of Blum,[11] increasing the *p*H accelerates the "dilution effect" markedly. At levels above about *p*H 10, it is essentially instantaneous, mimicking the effect of excess ATP. However, we have found that the influence of increased *p*H is often suppressed or altered by the presence of anions such as phosphate[12] and lysinate, which presumably bind to the myosin B particles and therefore also influence the net charge. For this reason the most straightforward results were obtained by rendering the myosin B solutions alkaline with KOH.

(d) **Structural Nature of the "Dilution Effect."**—From the fact that it is a process triggered by dilution *per se*,[8] and from a preliminary "Zimm analysis" by one of us, (MFG, cited in ref. 8) the "dilution effect" could be surmised to result from a dissociation of particles into smaller particles. In the present work this inference has been confirmed. The results of a typical experiment in which a stock solution of 24-hr. extracted myosin B was diluted to 0.013 g./100 ml. and held at 5°, are presented in Fig. 3 and Table I. Table I

TABLE I

"DILUTION EFFECT"—24 HR. MYOSIN B (PREP. 81)

Time (after dilution),[a] min.	$\bar{M}_w$ (× 10⁻⁶)	$\bar{r}_g$ (× 10⁻² Å.)
0	28.6	19.1
10	25.0	18.7
50	18.6	18.2
24 hr.	10.6	17.6

[a] Diluted to 0.013 g./100 ml. Diluted sample stood, and its scattering was measured, at 5°.

shows that at the chosen dilution, the weight average molecular weight ($\bar{M}_w$) falls to about $^1/_3$ of its initial value during 24 hr. in the cold and that a large portion of this fall occurs in the first hour. The average radius of gyration ($\bar{r}_g$) also falls with time, though only slightly.

The existence of the "dilution effect" imposes noteworthy technical limitations on the study of myosin B. As Gergely[13] has emphasized, it would be better practice, when making light scattering measurements, to make up the final dilution to be studied and then to centrifuge it, rather than to centrifuge a stock solution and then make up the final dilution. Applied unknowingly, however, the "preferable" schedule leads to a different state altogether, since during the hour or more of centrifugation the structure of the particles is changing rapidly with time. In the case of a 5-hr. preparation, Fig. 1a suggests that it matters little whether, for the purpose of measurement, the solution is transiently warmed or not. Thus, in a 5-hr. extract the only way to study the properties of undissociated particles is to centrifuge the stock solution and then make the desired dilution. With a 24-hr. preparation, Fig. 1b shows that even after

(11) J. J. Blum, *Arch. Biochem. and Biophys.*, **43**, 176 (1953).

(12) The inhibition of the "dilution effect" by higher temperatures (*e.g.*, 25°) and by phosphate ion were first observed and communicated to us by our colleague, Dr. Shizuo Watanabe.

(13) J. Gergely, *J. Biol. Chem.*, **220**, 917 (1956).

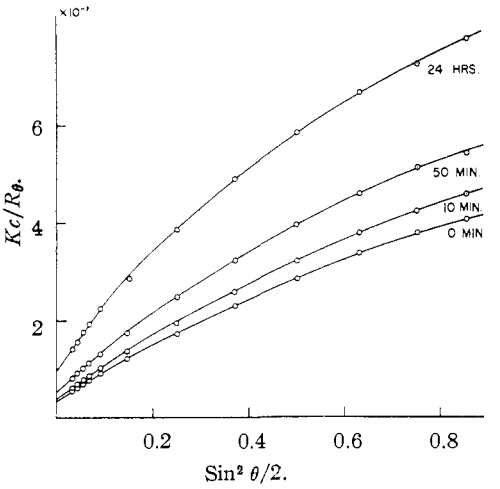

Fig. 3.—Angular scattering envelopes (Zimm Plots) of 24-hr. extracted myosin B, at various times after dilution from $c \simeq 0.5$ to 0.013 g./100 ml.; solvent 0.6 *M* KCl, *p*H 7.0.

a few hours of standing diluted the state immediately following dilution can be reconstructed by the simple expedient of transiently warming the solution to 25° for measurement. Thus in a 24-hr. extract, one may dilute and then centrifuge, provided the final solution is momentarily warmed to 25° prior to measurement. In the work reported in the present paper, measurements of the "dilution effect" were made on 24-hr. myosin B using both the centrifugation–dilution and the dilution–centrifugation–warming sequences and on 5-hr. extracts using the centrifugation–dilution scheme. In every case standing diluted led to marked dissociation, measured as a large fall in $\bar{M}_w$.

2. The ATP Effect in Highly Dilute Solutions.—In the preceding paper,[4] we reported and interpreted results of adding ATP to myosin B solutions under conditions in which the concentration of ATP remained at "saturation" levels throughout the experiment. Such simplifying conditions were achieved in relatively short experiments (*i.e.*, light scattering) by adding ATP in considerable excess, and in longer experiments (*i.e.*, sedimentation) by inhibiting the ATPase activity of the protein. Much can also be learned from experiments in which small ("non-saturating," in the enzyme kinetics sense) amounts of ATP are added to ATPase-active solutions of myosin B, thus leading to transitory displacements of the system. Experiments of this sort have long been performed with myosin B. In the earlier work,[14,15] viscosity or flow birefringence was used to follow these displacements, and more recently light scattering has been employed.[16,17] The displacement observed by light scattering in *dilute* solutions will be considered in the present section. Elucidation of the displacement observed (for instance, by

(14) A. Szent-Gyorgyi, "Chemistry of Muscular Contraction," 2nd Ed., Academic Press, New York, N. Y., 1951.

(15) M. Dainty, A. Kleinzeller, A. S. C. Lawrence, M. Miall, J. Needham, D. M. Needham and S.-C. Shen, *J. Gen. Physiol.*, **27**, 355 (1944).

(16) Y. Tonomura and S. Watanabe, *Nature*, **169**, 113 (1952).

(17) J. J. Blum and M. F. Morales, *Arch. Biochem. and Biophys.*, **43**, 208 (1953).

viscosity) in more concentrated solutions has required experiments of a different sort (see Section 3).

The results of the previous section indicate that, even in the absence of ATP, there occur in myosin B solutions reversible polymerizations whose kinetics and equilibria depend on temperature, protein concentration, etc. It is natural to try to understand one of the effects of ATP, *viz.*, that of depolymerizing Class II particles, (see preceding paper[4]) as a displacement of the previously existing polymer equilibrium. The experiments to be reported encourage this interpretation, and they also provide additional indications of the other structural ATP effect, *viz.*, inflation of Class I particles.

Figure 4 shows the time course of relative (45°) scattering, $R_{45}(t)/R_{45}(0)$, in a 5-hour extracted preparation, following dilution (at zero time) and the imposition (at 400 seconds) of decreasing (curves 1–4) small concentrations of ATP. Since the solutions employed in this experiment had been prepared from a concentrated stock solution just prior to adding ATP, the effects of ATP were superimposed on the usual "dilution effect" (curve 5). The measurements were made at 25°.

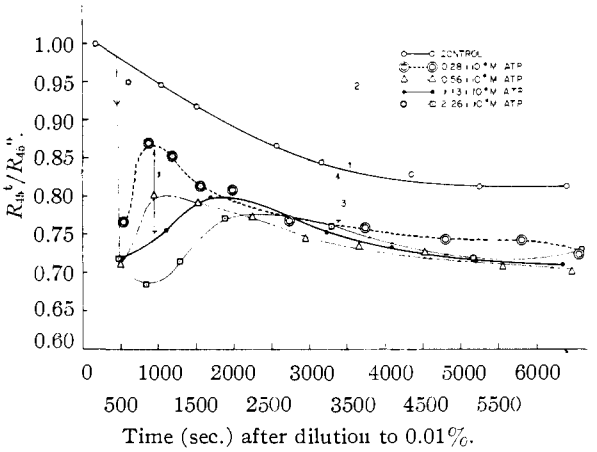

Fig. 4.—Relative (45°) intensity of light scattered from 5-hr. extracted myosin B in the presence of various ATP concentrations. Diluted to c = 0.01 g./100 ml. at zero-time, ATP added at 400 seconds; solvent 0.6 M KCl pH 7.0. In the text, curves 1–4 are in order of decreasing ATP concentration, curve 5 is control.

The dependence of the general shape of curve 1–4 on the instantaneous ATP concentration has, of course, been recognized in the past. Certain features, however, are of special concern here. Considering, to be concrete, curve 4, it will be noted that (a) long after the transitory ATP displacement, curve 4 decays with time, reassuring us that the "dilution effect" proceeded in the solution of curve 4, just as in that of curve 5. Moreover (b) after this long time, curve 4 decays below and roughly *parallel* to curve 5, that is, ATP addition irreversibly reduced the value of $R_{45}(t)/R_{45}(0)$ in solution 4 relative to solution 5. Finally (c) from the minimum which it reached immediately following ATP addition, curve 4 actually *rose* for a considerable time.

As seen above, the myosin B system, suddenly diluted, is unstable, and a spontaneous depolymerization carries it toward an equilibrium state in which the degree of polymerization is less than that originally present. Facts (a) and (b) suggest that *one* of the effects of ATP—presumably its depolymerizing effect—merely superimposes on the dilution effect and displaces the system toward the equilibrium state of the latter. Such a displacement, even if insufficient to reach the equilibrium state, is nevertheless irreversible on removal of the ATP (by enzymatic action), since a system is not apt to more *away* from its equilibrium spontaneously.[18] It is this irreversibility that expresses itself in fact (b). By the same token, fact (c)—a clear indication that there also occurs a reversible displacement—implies that ATP does also something different from mere displacement of the polymerization reaction and does this against a restoring force. This qualitatively different process could be, for example, the displacement of another type of polymerization reaction; however, in view of the evidence given previously[4,17] the most natural interpretation is that fact (c) reflects the inflation–deflation process among Class I particles.

Essentially similar results can be obtained with a 24-hr. extract measured at 5° (Fig. 5), except

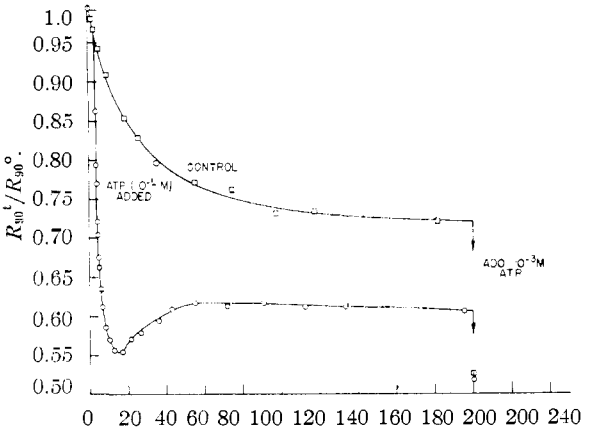

Fig. 5.—Relative (90°) intensity of light scattered from 24-hr. extracted myosin B. Diluted to c = 0.01 g./100 ml. at zero-time, upper curve is control, lower curve made 10^{-5} M in ATP at 3 min. Both made 10^{-3} M in ATP at 200 minutes; solvent 0.6 M KCl, pH 7.0.

that a depressed ATPase activity and the endothermic polymerization of the "dilution effect" distort the curve in the expected way. It should be noted, however, that at 5° the immediate response of the system to ATP is sufficiently slowed up to make its kinetics measurable with simple apparatus.

3. The ATP Effect in More Concentrated Solutions.—It was concluded in the preceding paper[4] that the imposition of a "saturation" ATP concentration on a myosin B solution causes a depolymerization of Class II particles. The only optically detectable product of this depolymerization is myosin, the production of which is manifested by an increase in the amount of Class III particles. It has been suggested above that this effect of adding ATP can be considered to be a displacement of

(18) The products of ATP hydrolysis, *viz.*, ADP + P, are not known to cause any polymerization of the system.

a pre-existing polymerization equilibrium. In the preceding section it was argued that in a solution initially made very dilute, the equilibrium greatly favors "monomers" or low-degree "polymers." However, by using higher protein concentrations (0.1 to 1.0 g./100 ml., traditionally employed in viscometric and flow birefringence investigations) one can get an equilibrium which calls for a higher degree of polymerization than is possible in the presence of ATP. Then, following the sudden imposition of a small concentration of ATP, there should result a transitory displacement of the Class II-Class III reaction toward depolymerization, (*i.e.*, toward Class III) followed, upon ATP withdrawal, by a repolymerization that restores the pre-existing equilibrium. Simultaneously, of course, the reversible inflation of class I particles occurs, and here a method (*e.g.*, light scattering) sensitive to *both* processes would yield an ambiguous result. Sedimentation analysis, however, makes it possible to follow shifts in the depolymerization–repolymerization equilibria alone. The procedure is illustrated in Fig. 6.

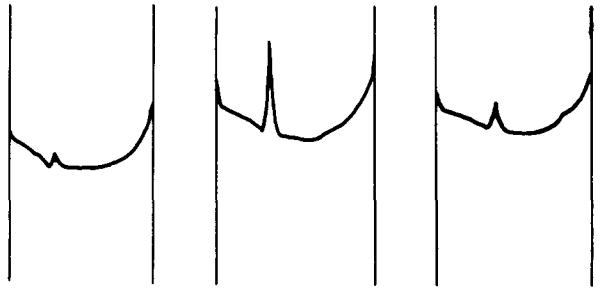

Fig. 6.—Sedimentation (schlieren) diagrams of 24-hr. extracted myosin B. c = 0.17 g./100 ml. Solvent 0.6 M KCl, pH 7.0. (a) control 20 min. at 52,640 r.p.m.; bar angle = 35°; temp. = 24.5°. (b) Plus 10^{-3} M ATP, 10^{-2} M $MgCl_2$: 64 min. at 52,640 r.p.m.; bar angle = 35°, temp. = 23.9°. (c) Same solution—ATP removed by dialysis: 62 min. at 52,640 r.p.m., bar angle = 35°, temp. = 25.5°.

Figure 6a is the schlieren diagram of a sedimenting 24-hr. extract (*ca.* 0.2 g./100 ml.); clearly, this pattern contains no peak due to free myosin ($S_{20} \simeq 5.5$ S). Figure 6b is the schlieren pattern of the same solution, except that 10^{-3} M ATP (and 10^{-2} M $MgCl_2$, an inhibitor of ATPase)[19] have been added. The *de novo* appearance of a myosin component is obvious. As much of the ATP and Mg^{++} as possible was then removed from this solution by exhaustive dialysis; the remaining material sedimented as shown in Fig. 6c. Although the ATP-induced free myosin peak has not totally disappeared, enough of it (about $^2/_3$) is gone to assure that most of the myosin seen in Fig. 6b repolymerized back into polydisperse aggregates of much larger size following ATP removal. In the foregoing experiments, all manipulations were carried out at about 5°. However, the ultracentrifuge runs, for speed and convenience, were carried out at room temperature. In view of the work of Laki, Spicer and Carroll[6] on the effects of temperature on the myosin B system (amply supported by our own observations reported above), it was necessary to ascertain that the qualitative conclusions drawn from Fig. 6 were not jeopardized by temperature-induced aggregations occurring during centrifugation. Figure 7 shows the results of comparable experiments, with all operations,

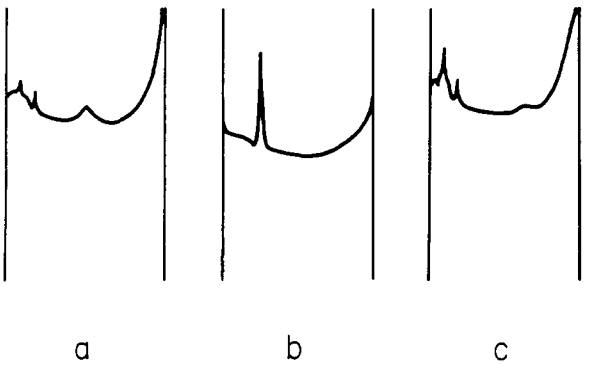

Fig. 7.—Sedimentation (schlieren) diagrams of 24-hr. extracted myosin B at low temperature. c = 0.17 g./100 ml. Solvent: 0.6 M KCl, pH 7.0. (a) Control 24 min. at 52,640 r.p.m.; bar angle = 30°; temp. = 4.9°. (b) Plus 10^{-3} M ATP, 10^{-2} $MgCl_2$: 76 min. at 52,640 r.p.m., bar angle = 40°; temp. = 4.0°. (c) Same solution—ATP removed by dialysis: 22 min. at 52,640 r.p.m.; bar angle = 30°; temp. = 5.3°.

including the actual sedimentation runs, performed at 5°. While appropriate comparisons (*i.e.*, Fig. 6a with Fig. 7a) provide additional confirmation of the work of Laki, *et al.*,[6] the conclusions to be drawn relative to the appearance of the myosin peak with ATP and its substantial disappearance with dialysis are obviously the same as those we have drawn from the clearer situation responsible for Fig. 6.

The foregoing phenomenon (the ATP-induced depolymerization and its reversal on ATP removal) can also be demonstrated by light scattering, provided we keep in mind that this method, being especially sensitive to large-sized particles, may indicate an apparently slower rate of repolymerization than is inferred from the rate of disappearance of the myosin peak from the schlieren diagram obtained in sedimentation experiments. Solutions such as those employed for Plates A, B and C in Figs. 6 and 7, when diluted to *ca.* 0.009 g./100 ml., can be immediately measured in the light scattering photometer.[20] In the experiment illustrated in Fig. 8, the 90° scattering (R_{90}) of a diluted "solution A" was taken as unity. A diluted "solution B" (ATP just added) shows the expected reduction in R_{90} relative to that of solution A. Beginning at this same zero time, an aliquot of "solution B" was dialyzed for 4 hr., thus becoming a "solution C." At various times after the beginning of dialysis, portions of "solu-

(19) Added Mg^{++} appears to play no role in these phenomena, except as an ATPase inhibitor, since many of these experiments were repeated without added $MgCl_2$, and no differences were noted.

(20) It should be kept in mind that because the "dilution effect" is relatively slow, light-scattering measurements made immediately after dilution still reflect properties of the particles which existed in the concentrated system.

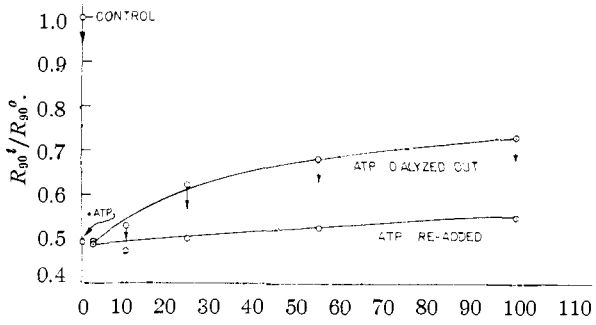

Fig. 8.—Relative (90°) intensity of light scattered from 24-hr. extracted myosin B at various times after ATP removal by dialysis. Measured at c = 0.009 g./100 ml., immediately after dilution from 0.17 g./100 ml. (See text.)

tion C" were diluted and measured.[21] These relative turbidities plot on a slowly rising curve, indicating the progressive reconstruction of the depolymerized aggregates. At any point on this curve, ATP addition drops the relative R_{90} to nearly what it was for the diluted "solution B."

It seems, *a priori*, that it should also be possible to demonstrate the concentration dependent equilibria between aggregates and free myosin particles by allowing a system initially containing ATP responsive particles to stand at high dilution until its ATP response is essentially gone, and then, by reconcentrating the system, to restore the response. Our attempts to achieve this result have thus far been unsuccessful. The difficulties are in part technical, *i.e.*, having to do with devising a sufficiently rapid and effective method of concentrating the protein while maintaining the ionic strength of the solvent constant. Concentration by precipitation (at low ionic strength), centrifugation and redissolution (at high ionic strength) has also failed to restore the ATP response; but it is very possible that the marked changes in ionic strength involved in this technique have either modified or excluded a component necessary for the repolymerization (see next section).

4. The "Cementing Component."—A major objective of our investigations has been to reach an unambiguous conclusion regarding the role (if any) played by actin in the ATP–myosin B interaction. The discovery that there existed in myosin B solutions, particles which inflated but did not dissociate on ATP addition, led Blum and Morales[17] to question an independent[22] role for actin. Even though the present investigations have established that only a portion of the heavy components fail to depolymerize on ATP addition, it continues to be true and disturbing that to date no one has unambiguously "seen" actin released from aggregates as a consequence of ATP addition (for instance, as a new component in an ultracentrifuge plate) in either the G- or the F-form. It is true that in her important work demonstrating *myosin* release on ATP addition, A. Weber[23] also obtained pellets which she considered to be actin, but her qualitative identification of this protein was uncertain, since the material failed to exhibit the characteristic G-F transformation; moreover, in our hands, comparable experiments (see below) failed to yield such pellets. Barany, *et al.*,[24] and Gergely and Martonosi[25] found it possible to extract actin *from* their preparations of myosin B, using (in the latter instance) a specific anti-actin for identification, but the amount thus found was unascertained, and it was not shown whether the actin was released by ATP addition. Finally, the attempts to demonstrate, by salting-out analysis, the appearance of free actin following ATP or PP addition, are in controversy. Sasaki[26] found that addition of pyrophosphate, even to "synthetic actomyosin" (*i.e.*, made by mixing actin and myosin in the ratio 1:4) did not result in a separation of components. On repeating these experiments Gergely[27] has recently reported such separation. The experiments and results which we report in this section also fall somewhat short in terms of component identification and thus should be considered in the nature of a progress report. They do, however, show that ATP liberates a component from myosin B which has some of the properties expected of F-actin.

Experiments resembling those of the previous section were performed with 24-hr. extracted solutions. They differed, however, in that portions of a "solution B" were subjected to centrifugation at $6.5 \times 10^4 \times g$ for varying lengths of time before dialyzing (to remove ATP and Mg^{++}). On completing the centrifugation, samples carefully drawn from the upper halves of the tubes were dialyzed, and it was these samples which then constituted "solution C." Figure 9 and Table II show that from "solution B" which had been centrifuged for 2 hr. (in the presence of ATP) roughly $^1/_5$ of the protein of the supernatant had been lost (accompanied, however, by essentially no decrease in free myosin concentration)[28] and following dialysis practically no repolymerization had occurred. Table II also shows that with one hour of centrifugation, roughly $^1/_{10}$ of the protein was lost and after dialysis about $^1/_3$ of the myosin released by ATP had polymerized back into large aggregates.

The foregoing observations indicate that in the presence of excess ATP (and in 0.6 M KCl) about $^1/_5$ of the protein forms particles of a size that can very easily be separated from myosin by centrifugation and that at least some of this protein is essential to the repolymerization of the myosin liberated by ATP.

Interpretation of the results reported in this section encounters certain difficulties. The "classical" view of myosin B envisions monomers of

(21) After about 4 hr., this curve is unaffected by continued dialysis and is a function of time of standing only.

(22) That is, such a particle was considered to behave as a unit in the course of the interaction, so for the purpose of the interaction it did not matter whether it was "pure myosin" or "acto-myosin."

(23) A. Weber, *Biochim. et Biophys. Acta*, **19**, 345 (1956).

(24) M. Barany, K. Barany and F. Guba, *Nature*, **179**, 818 (1957).

(25) J. Gergely and A. Martonosi, *Federation Proc.*, **17**, 894 (1958).

(26) A. Sasaki (private communication).

(27) J. Gergely, "Proceedings of the N. Y. Acad. of Sci. Conf. on Metabolic Factors in Cardiac Contractility," New York, March 18, 1958 (in press).

(28) This could be demonstrated by comparing the area under the myosin peak of "solution B" (containing ATP) which had undergone centrifugation with a control which had not.

TABLE II

EFFECT OF PRE-CENTRIFUGATION, IN THE PRESENCE OF ATP, ON THE REVERSAL OF THE ATP EFFECT IN 24-HOUR EXTRACTED MYOSIN B

Centrifugation (hr. at 65,000 × g)	Decrease in total protein concn. of supernatant[a]	Concn. of free myosin remaining after dialysis of supernatant[b]
0	0%	33 ± 4%
1	8	65 ± 5
2	19	90 ± 11

[a] Supernatant is material removed from the upper halves of centrifuge tubes. Thus these concentrations represent the percent decrease in average protein concentration of this supernatant with respect to the concentration before centrifugation. [b] Free myosin concentration in presence of ATP taken as 100%. This is not appreciably altered by centrifugation.

myosin attached to long threads of F-actin. Were ATP to dissociate particles of such a complex, it would be expected that the resulting particles of separated myosin and actin would be smaller than particles of complex. If so, then the application of $6.5 \times 10^4 \times g$ in the *absence* of ATP might rid the solution of its ATP response even more rapidly than in the presence of ATP, but this is not the case. To circumvent this difficulty, one might speculate that F-actin, once released from the complex by ATP, polymerizes to a higher degree, thus becoming more susceptible to sedimentation. But the generation of particles larger than those of the original complex should lead to an *increase* in the weight-average molecular weight given by light scattering (through Zimm plots) and this is not observed.[4] A remaining possibility is, of course, a highly improbable cancellation of this hypothetical aggregation and a depolymerization, resulting each time in a constant $\bar{M}_w$.

Despite these difficulties of interpretation, the experiments presented in this section are quite reproducible and worthy of reporting here for their qualitative implications.

Discussion

The results reported in the preceding paper[4] and in the present one bear upon several questions of current interest. One question, of importance to chemo-mechanical energy transduction, is whether there exist in myosin B solutions particles capable of suffering a shape change only when reacting with ATP (as claimed originally by Blum and Morales[17]) or whether the effect of ATP on all particles is solely dissociative (as believed by most workers and urged especially by Gergely).[18] In reinvestigating this question by the method of light scattering, following the best preparative techniques and pressing measurements to angles lower than those used by previous workers, we have failed to observe the large changes in $\bar{M}_w$ which Gergely has reported to result from ATP addition; rather, we have confirmed that on ATP addition $\bar{M}_w$ remains sensibly constant. We suggest that the discrepancy between Gergely and ourselves with respect to experimental results may arise from the complications of the "dilution effect" (not known at the time of Gergely's work) or from the fact that his preparations are precipitated at much higher ionic strength than ours, (0.3 as against 0.06)

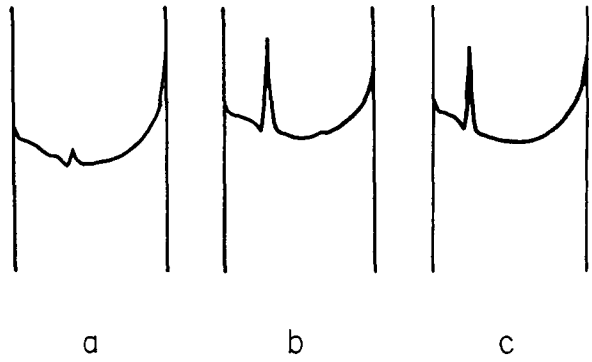

Fig. 9.—Sedimentation (schlieren) diagrams of 24-hr. extracted myosin B. $c \simeq 0.17$ g./100 ml. (see Table II). Solvent 0.6 M KCl, pH 7.0. (a) Control 28 min. at 52,640 r.p.m.; bar angle = 35°; temp. = 24.5°. (b) Plus 10^{-3} M ATP, 10^{-2} M $MgCl_2$. Centrifuged for 2 hr. at 65,000 × g in preparative ultracentrifuge. Supernatant removed, and sedimented for 56 min. at 52,640 r.p.m.; bar angle = 35°; temp. = 21.0°. (c) Same solution—ATP removed by dialysis: 54 min. at 52,640 r.pm.; bar angle = 35°; temp. = 17.1°.

and therefore may differ in composition or the nature and extent of intra-particle, cohesive bonding.

While the present work has substantiated the existence of a non-dissociating fraction of myosin B particles, it has, at the same time, provided ample evidence that another fraction (about 1/3 of the heavy components of 5-hour extracted myosin B, by weight), does dissociate. Moreover, it does so very much along "classical" lines, in that one of the products of dissociation consists of myosin "monomers" (particle weight *ca.* 4.2×10^5); a second product has not been separated in gross fractionation or located in sedimentation patterns, but has been shown to be very heavy (heavier, perhaps, than any component present prior to ATP addition), to be necessary for polymerization at ATP-dissociated particles and to be present in comparatively small amounts—all attributes commonly ascribed to F-actin.

Distinct from the question of what happens when ATP is added to a solution of myosin B is the question of *why* this happens. Consideration of the highly anionic character of the polyphosphates capable of causing structural changes in myosin B solution early[17] led to the suggestion that the adsorption of such substances on anionic protein particles generated an "outward" electrostatic stress on the particle structure. If the cohesive forces in the particle are sufficiently strong, such a stress might result in an "inflation," while if the cohesive forces are somewhat weaker, the stress might bring about dissociation. Thus these two qualitatively different results may both be reactions to the *same* stimulus; which reaction occurs may depend upon the strength and distribution of the cohesive bonds and forces relative to the positions of ATP binding sites.

The nature of the polyphosphate *binding* is not clear; obviously it is not simply electrostatic, since under the conditions usually employed both adsorbent and adsorbate bear net charges of the

same sign (though, of course, the protein bears both plus and minus charges and doubtless has regions of net *positive* charge) and also because ring substitutions[29,30] manifestly influence the strength of binding. It is also well known that divalent cations, particularly Mg^{++}, influence the extent of binding.

In the previous work of Blum,[11] which has been confirmed and extended in the course of the present investigation, the electrostatic explanation has received at least some indirect support, in that effects rather similar to those brought about by ATP can be evoked by imposing fairly high (>10) *p*H's[31]: Although the number of proton-binding sites certainly exceeds the number of ATP binding sites, the effects brought about by high *p*H must unequivocally result from the imposition of an electrostatic stress onto the protein particle. At the same time it must be conceded that the electrostatic explanation of the ATP effect cannot at this time be put quantitatively. Measurements of the number of ATP binding sites, albeit dissonant and not on the firmest theoretical basis,[32] suggest that saturation with ATP would not sufficiently alter the *net* charge to bring about the considerable structural changes observed, so that the electrostatic explanation must also be combined with some assumptions about strategic location of the ATP binding sites.

A third question on which this work bears has to do with the general composition of the myosin B system. The polydispersity of the system, and more specifically its distribution into a minimum of three size classes, has been demonstrated, and it also has been shown that polymerization (or perhaps in some instances copolymerization) reactions connect these various classes. These reactions have been shown to be easily displaceable by concentration, *p*H and temperature changes; this property probably rules out polymerization through covalent bonding. Thus the myosin B system appears to resemble comparable aggregating protein systems, except that the particles of the myosin B system seem specialized to adsorb polyphosphates such as ATP, thus providing a special means of displacing the polymerization reactions.

(29) J. J. Blum, *Arch. Biochem. and Biophys.*, **55**, 486 (1955).

(30) E. T. Friess and M. F. Morales, *ibid.*, **56**, 326 (1955).

(31) The reproducibility achieved to date by adding KOH to myosin B solution (to *p*H 11) is inferior to that attained with ATP addition. However, in several Zimm-type light-scattering experiments, the similarity with the ATP result is striking. For example, in one such case, (with 5-hr. extracted myosin B) the weight average molecular weight of the particles remained almost constant ($\overline{M}_w$ went from 22.2×10^6 to 20.0×10^6), while the average radius of gyration, $\bar{r}_g$, rose sharply (from 3310 to 4270 Å.). It is interesting that the stress imposed in such experiments cannot be borne indefinitely. Thus, in this case, after 45 minutes at high *p*H, $\overline{M}_w$ had fallen to 17.5×10^6 and $\bar{r}_g$ to 4030 Å.

(32) M. F. Morales, J. Botts, J. J. Blum and T. L. Hill, *Physiol. Revs.*, **35**, 475 (1955).

BETHESDA, MD.

[FROM THE DEPARTMENT OF BIOLOGICAL CHEMISTRY, HARVARD MEDICAL SCHOOL]

Molecular Weight Studies on Human Serum Albumin after Reduction and Alkylation of Disulfide Bonds[1]

BY MARGARET J. HUNTER AND FREDERIC C. MCDUFFIE[2]

RECEIVED JULY 23, 1958

The disulfide groups of human serum albumin were reduced by sodium thioglycolate in the presence of urea or sodium dodecyl sulfate to render all the disulfide bonds available for reaction. After reduction, an excess of iodoacetamide was added to block the protein sulfhydryl groups and prevent their subsequent reoxidation. Viscosity, light scattering and ultracentrifuge studies were performed on the reduced protein and on an unreduced control. Upon correcting for bound detergent, the molecular weight of the protein appeared unchanged. No amino acids nor small peptides were found in the dialysates, and the protein solution appeared homogeneous in the ultracentrifuge. The appearance of carboxy-S-methylcysteine and the disappearance of cystine in the hydrolysates of the reduced protein was confirmed by paper chromatography. The results suggest that the human serum albumin molecule is a continuous peptide chain internally crosslinked by disulfide bonds.

I. Introduction

Amino acid end-group analyses of human serum albumin have shown the presence of only one terminal amino group per molecule[3,4] and studies with carboxypeptidase have indicated that only one terminal carboxyl group is present.[5] While this evidence might suggest that the albumin molecule is composed of a single polypeptide chain, it is not conclusive as cyclic peptides, interlinked by disulfide bonds, could be present in the native protein.

The existence of cyclic peptides, at least in bovine serum albumin, would appear to have been demonstrated by the studies of Reichmann and Colvin.[6,7] These workers have shown that oxidation of the disulfide bonds of bovine serum albumin with performic acid resulted in the appearance of several lower molecular weight species as determined by ultracentrifugal analyses and light scattering measurements.

It seemed of interest to determine whether similar results would be obtained on rupture of the disulfide bonds by reduction rather than by oxidation and the present study was therefore undertaken to determine the molecular weight of thioglycolate-reduced human serum albumin. Disulfide bonds can be reduced by thiol compounds under some-

(1) This work has been supported by funds of Harvard University, by grants from the National Institutes of Health, and by contributions from industry.

(2) Fellow of the National Heart Institute, 1953–1954.

(3) E. O. P. Thompson, *J. Biol. Chem.*, **208**, 565 (1954).

(4) H. Brown, *Science*, **121**, 106 (1955).

(5) W. F. White, J. Shields and K. C. Robbins, THIS JOURNAL, **77**, 1267 (1955).

(6) M. E. Reichmann and R. Colvin, *Can. J. Chem.*, **33**, 163 (1955).

(7) M. E. Reichmann and R. Colvin, *ibid.*, **34**, 160 (1956).