[CONTRIBUTION FROM THE DIVISION OF PHYSICAL BIOCHEMISTRY, NAVAL MEDICAL RESEARCH INSTITUTE AND THE VIRUS LABORATORY, UNIVERSITY OF CALIFORNIA]

Studies on the Contractile Proteins of Muscle. I. The ATP–Myosin B Interaction[1,2]

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

RECEIVED JULY 22, 1958

Myosin B, a purified high ionic strength extract containing the contractile proteins of rabbit skeletal muscle, has been subjected to physico-chemical examination before and after the addition of adenosine triphosphate (ATP) or pyrophosphate (PP). Light-scattering studies demonstrate that the weight average molecular weight ($\bar{M}_w$) of both 5- and 24-hr. extracts of myosin B is essentially unchanged by ATP addition but that ATP induces a marked increase in the average radius of gyration, r_g. By sedimentation analysis (using both absorption and schlieren optics) 5-hour extracted myosin B is shown to consist of about 35% heavy components, which show a structural response to ATP, and 65% free myosin, which does not. Myosin B extracted for 24 hr. reveals the ultracentrifuge peak characteristic of myosin only after the addition of ATP. In 5-hour extracts, the heavy components are shown to fall into two classes. ATP and PP largely depolymerize the lighter class, primarily to myosin, without detectably altering the sedimentation behavior of the heavier class. After this treatment, the total amount of heavy components is decreased by about 25%. Considering both sedimentation and light-scattering, myosin B is shown to contain, in addition to free myosin, larger particles covering a broad spectrum of sizes. Of these, the smaller tend to dissociate on the addition of ATP, while the larger inflate at constant $\bar{M}_w$.

Introduction

It has been the cherished hope of many investigators that from knowledge of the structural response of "contractile protein" or "myosin B" solutions to adenosine triphosphate there could be inferred knowledge about the molecular nature of muscle contraction. Accordingly, the armamentarium of physical biochemistry has been brought to bear on the problem. From the results, many workers have concluded that the effect of such substances as adenosine triphosphate (ATP) or pyrophosphate (PP) is simply to dissociate particles of a protein complex thought to exist in myosin B solutions, *viz.*, particles of actin–myosin, or "actomyosin." Some years ago an effort by Blum and Morales[4] to show this dissociation by the (then new to this field) method of light scattering led to the dissonant conclusion that the constituent particles, rather than dissociate, appeared to expand or inflate (*i.e.*, the weight average molecular weight remained constant, but the average radius of gyration increased markedly). Blum and Morales considered two implications of this result. Most important was the suggestion that the adsorption of polyvalent anions to the particles caused them to expand. Since under different conditions the same anions caused the particles to contract, the suggestion that electrostatic forces governed the particle dimensions was strong, and consistent with the "polyelectrolyte view" of muscle contraction.[4–7] Apart from this implication, which is independent of whether the particles consisted of myosin, or of actin and myosin, Blum and Morales also felt that their results imperiled the conventional idea of an actin–myosin complex. The Blum–Morales proposal that the myosin B particles were not "acto-myosin," but perhaps "poly-myosin," was challenged by A. Weber,[8] in an important paper in which she showed that as a result of ATP addition there was released from myosin B, certainly free myosin, and perhaps (but with considerable ambiguity) actin. Moreover, Gergely[9] concurrently reported a light-scattering investigation from which he concluded that on addition of ATP the weight-average molecular weight of myosin B particles was in fact reduced 3- to 7-fold. Especially Gergely's conclusion was therefore a flat *experimental as well as deductive* contradiction of the Blum–Morales result.

Thus despite a great deal of effort, there has persisted a lack of agreement with regard to the behavior of this important system. We therefore undertook a complete reinvestigation of the problem, as reported in this and the succeeding paper.[10] This reinvestigation has shown that the myosin B system is more complicated than previously realized, due to polydispersity (with different size classes of particles responding differently to ATP) and to reversible polymerizations which proceed even in the absence of added reagents. In reaching an understanding of the ATP effect it has been necessary, as reported in this paper, to use not only light scattering, but also sedimentation followed with absorption optics, since the more important protein components of myosin B are polydisperse and are often completely overlooked in the conventional schlieren ultracentrifuge patterns.

The general conclusions are that myosin B contains, in addition to myosin, larger particles covering a broad spectrum of sizes. Of these, the smaller tend to dissociate on the addition of ATP, while the larger inflate without dissociation.

Nomenclature.—Early investigators of muscle proteins have introduced several overlapping sets of names for the various protein fractions they ob-

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

(5) J. Riseman and J. G. Kirkwood, THIS JOURNAL, **70**, 2820 (1948).

(6) M. F. Morales and J. Botts, *Arch. Biochem. Biophys.*, **37**, 283 (1952).

(7) M. F. Morales and J. Botts, in "Currents in Biochemical Research," edited by D. E. Green, Interscience Publishers, New York, N. Y., 1956, p. 609.

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

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

(10) P. H. von Hippel, M. F. Gellert and M. F. Morales, THIS JOURNAL, **81**, 1393 (1959).

tained. Later workers have sometimes used the same names in a different sense, making it difficult to write unambiguously about muscle proteins. For simplicity, we will here define our notation and usage.

The terms "myosin A" and "myosin B" will be used, as originally defined, to refer to purified muscle extracts obtained by certain preparative procedures. Myosin A refers to a preparation resulting from a 10- to 30-minute extraction with Weber–Edsall solution, or an equivalent procedure.[11] Myosin B refers to a 5- to 24-hr. extraction with Weber–Edsall solution. Note that these names do *not* refer to molecular species but only to methods of preparation.

The term "myosin," without postscript, refers to the principal molecular constituent of myosin A solutions. Myosin has a molecular weight of *ca.* 4.2×10^5 g.,[12] a large ATPase activity, and shows no detectable structural response to the addition of ATP. We will often refer to protein aggregates and complexes containing myosin.

We will not, as is often done, use the term "actomyosin" to designate the protein aggregates found in solutions of myosin B, for this would amount to prejudging the result of these investigations.

For a detailed discussion of muscle protein nomenclature, see ref. 11.

Experimental

1. **Preparation of Myosin B.**—Myosin B solutions were prepared by extracting (Latapie-)minced rabbit back muscles with a 3-fold weight of high ionic strength, mildly alkaline carbonate buffer for 5 or 24 hr. The resulting extracts were then purified by three alternate precipitations at an ionic strength of 0.06 and resolubilizations at 0.6. The procedure used, which is a modification of the basic Weber–Edsall technique, differs only in minor details from that previously described.[13] The product, dissolved in 0.6 *M* KCl at *p*H 6.8, was stored in the refrigerator at 5° and generally remained stable for periods of several weeks. The final protein concentration was usually *ca.* 0.5 g./100 ml.

The ATPase activity of each new batch of myosin B was measured immediately, the release of inorganic phosphate being followed by the usual Fiske–Subbarow method,[14] and was taken as an index of the success of that particular preparation. Activities of 5 hr. extracted myosin B generally ranged between 3.5 and 5.0 μmoles P/sec./g. protein/liter and between 2.0 and 4.0 μmoles P/sec./g. protein/liter for 24 hr. extracts. Activities were measured at *p*H 8.0, in 0.6 *M* KCl, 0.1 *M* Tris and 10^{-3} *M* $CaCl_2$, using an initial ATP concentration of 2×10^{-3} *M*. The fall of ATPase activity during storage was monitored and used as a measure of the deterioration of the sample. In cases where a 5-hr. and a 24-hr. preparation were made concurrently from muscle tissue from the same animal, the ATPase activity of the purified 24-hr. extract was usually 30–40% below that of the purified 5-hr. extract. (In an earlier study, Blum and Morales[4] found equal activities for 5- and 24-hr. extracts.)

2. **Protein Concentration Measurements.**—Protein concentrations were routinely measured using a modified form of the Folin–Biuret procedure described by Lowry, *et al.*[15] Using this method, protein concentrations of the order of 0.01 g./100 ml. could be measured with an error of *ca.* ±3%. Thus, direct measurements could be made on solutions diluted to light scattering levels. The procedure was calibrated against micro-Kjeldahl determinations of protein nitrogen in myosin B solutions. Several modifications of the Lowry method were found necessary or useful.[16]

a.—The copper sulfate–sodium tartrate solution (solution B), is unstable as described.[15] Therefore separate solutions of copper sulfate (solution B_1) and sodium tartrate (solution B_2) were prepared and diluted into the alkaline sodium carbonate solution (solution A) prior to use each day.

b.—An aliquot of a standard bovine serum albumin solution of known concentration (obtained as 10 mg. protein nitrogen per ml. solution from Armour Laboratories, Kankakee, Ill., and diluted 1:1000 for use) was included in each set of determinations as an "internal standard." This served as an indicator of unsuspected variations in reagents or procedure.

c.—Potassium ion at concentrations above 0.1 *M* was found to form a precipitate with the Folin reagent (solution E[15]). As our experiments on myosin B solutions were almost all carried out in 0.6 *M* KCl and since the proteins of myosin B become insoluble at ionic strengths much below 0.3, 0.6 *M* NaCl was used as a diluent to lower the K^+ concentration to acceptable levels. (High Na^+ concentrations do not interfere.) Dialysis against 0.6 *M* NaCl was employed with dilute solutions, to avoid further dilution.

d.—Certain buffers (amino acids, tris, etc.) interfere at concentrations of about 0.1 *M* and were dialyzed out when sufficient dilution was not possible.

3. **Reagents and Chemicals.**—Chemicals and reagents of C.P. grade or the equivalent were used throughout. Sodium ATP was obtained from Pabst Laboratories, Milwaukee, Wis., and from Sigma Chemical Co., St. Louis, Mo., and was used without further purification.

4. **Light Scattering.**—Light-scattering measurements were carried out in a Brice–Phoenix Light-Scattering Photometer, Series 1150, using a beam collimated to a width of 4 mm. The scattering cells used were cylindrical, with flat entrance and exit windows for the undeviated beam. The cells were calibrated with dilute fluorescein solutions to determine their angular dependence of scattering, and the absolute magnitude of scattering was established using "Ludox" suspensions. (The transmittance of the "Ludox" was measured at the appropriate wave length in a Cary spectrophotometer.) Since cylindrical cells were used, the appropriate Sheffer–Hyde corrections[17] were applied to the intensities measured at all angles.

Scattered intensities, using incident light of 436 mμ, were measured at angles ranging from 21 to 135° relative to the undeviated beam (27 to 135° in earlier experiments). The required value of specific refractive index increment was determined using a Brice–Phoenix Differential Refractometer. A value of 0.209 ml./g. at 436 mμ was used throughout, although some variability in this value is recognized.

Buffers, ATP solutions, etc., intended for use in light-scattering measurements were optically cleaned by several passages through Millipore filters. Protein solutions were clarified by 1 to 2 hours of centrifugation (in a Spinco Model L Ultracentrifuge) at 35,000 × *g* before each light-scattering experiment. The centrifuged solutions were then pipetted from the centrifuge tubes, taking care to avoid stirring up any sedimented material, and transferred to the light scattering cell. Solutions were routinely placed in the beam and examined visually for scattering from floating dust particles. If such scattering was noted, clarification was continued.

It had been suggested by Gergely[9] and confirmed in preliminary work (on 5-hour extracted preparations) from this Laboratory,[18] that the order in which centrifugation and dilution are carried out can materially affect the results. In the present work this point was examined using both 5- and 24-hour extracts. Each technique has its disadvantages. On the one hand, it is more difficult to clarify the concentrated protein by centrifugation; dilution before centrifugation obviates this problem. On the other hand, the slow dilution-initiated decay of turbidity (and ATP response) proceeds during the *ca.* 2-hour time lapse between dilution and scattering (see ref. 10). The ATP response may then be less than half its original value. In 5-hour

(11) K. Bailey in "The Proteins," edited by H. Neurath and K. Bailey, Academic Press, New York, N. Y., 1954, p. 951.

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

(13) J. Botts and M. F. Morales, *J. Cellular Comp. Physiol.*, **37**, 27 (1951).

(14) C. H. Fiske and Y. SubbaRow, *J. Biol. Chem.*, **66**, 375 (1925).

(15) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *ibid.*, **193**, 265 (1951).

(16) We wish to thank Dr. Paul M. Gallop of the Long Island Jewish Hospital, N. Y., for very helpful discussions of this method and specifically for recommending the first two modifications to us.

(17) H. Sheffer and J. C. Hyde, *Canad. J. Chem.*, **30**, 817 (1952).

(18) J. J. Blum and M. F. Morales, *Federation Proc.*, **15**, 21 (1956).

extracted preparations this "dilution effect" reduces the ATP response to such an extent that interpretation is difficult; therefore such preparations have to be studied in the sequence: centrifugation, then dilution.

We have found that 24-hr. extracted preparations retain an ATP response sufficient for accurate work, even when dilution precedes centrifugation. Moreover, it was found in the course of the present work that in 24-hr. extracts the turbidity decay can be largely reversed by briefly warming the protein to room temperature (see ref. 10). Thus for 24-hr. extracts, one can dilute, centrifuge, warm briefly, cool and measure scattering. With such extracts we find that, with care, identical results can be obtained with either sequence.

Typical experiments were carried out as follows. A stock solution of myosin B was centrifuged and an aliquot diluted (in the light-scattering cell) into 25 ml. of clean buffer (usually 0.6 *M* KCl, 0.1 *M* histidine, *p*H 7.0), to a final protein concentration of about 0.01 g./100 ml. Scattering was measured over the entire angular range. Then 1 ml. of ATP solution was added, resulting in a final ATP concentration of 10^{-3} *M*, and mixed by slow swirling of the cell. (Care must be taken to avoid excessive stirring, which often results in some aggregation.) The scattering was then measured again and corrected for the dilution resulting from ATP addition. Finally the solution was dialyzed against 0.6 *M* NaCl and its protein concentration determined.

Experiments of this type were done both at room temperature (20–30°) and at 5°. To obtain the latter temperature in the absence of a thermostated cell, the entire photometer was transferred into a cold room. This necessitated wrapping the galvanometer in a heating pad, as otherwise the oil-damped suspension performed sluggishly and erratically. Also it proved necessary to recalibrate the neutral filters in the photometer for the lower temperature.

The results were calculated in the usual way and are presented in the form of "Zimm-type" plots.[19] At first, full Zimm plots based on measurements at several concentrations were made. These demonstrated that within experimental error, the values of Kc/R_θ obtained are independent of concentration at all angles. Therefore all the experiments reported here were carried out at only one concentration, and the data were plotted against $\sin^2(\theta/2)$ only, thus corresponding to the zero-concentration curve on a conventional Zimm plot.

5. Sedimentation.—The appearance of a single quasi-symmetrical peak in a schlieren diagram does not guarantee that the system under consideration is monodisperse. A polydisperse system in which the average sedimentation coefficient falls with increasing concentration exhibits "boundary sharpening" and thereby false homogeneity. A solution of 5-hr. extracted myosin B showing but one schlieren peak is not at all homogeneous, but rather a mixture of myosin (responsible for the peak) and heavier material which is revealed in the most unfavorable cases only as a slight elevation of the apparent base line on the solution side of the myosin boundary.[12] For the sedimentation analysis of such a system the observational method of choice is ultraviolet absorbance, which in effect gives protein concentration, c, as a function of radial position in the cell, x, and therefore does not depend on the formation of a sharp boundary.

The sedimentation work reported herein was performed at the University of California at Berkeley. All runs were made in a Spinco Model E Analytical Ultracentrifuge, modified for ultraviolet absorbance measurements. The apparatus, the modifications and the method of operation have been fully described.[20,21]

Successive absorption (and, simultaneously, also schlieren) photographs[22] were obtained and converted into plots of optical density *versus* distance from the axis of rotation in the cell, using a modified Spinco Model R Analytrol microdensitometer.[21] Each sedimentation run generated ten to fifteen such plots. Two typical examples are presented in Fig. 1a, with significant features labelled.

Vertical distances in Fig. 1a are proportional to absorbance at 254 mμ. Since the intensity of the light source and the quality of development may fluctuate slightly from one exposure to the next, vertical distances must be referred to a calibration standard on each plate, *viz.*, the height, h, corresponding to the transmission of the dummy cell. After a solution of myosin B containing protein and low molecular weight substances (*i.e.*, "solvent"—0.6 *M* KCl, ATP, etc.) has been centrifuged for 30 minutes or more at fields of *ca.* 200,000 × *g*, the boundary due to myosin ($M \sim 4.2 \times 10^5$ g.)[12] has moved a considerable distance from the meniscus, and the absorbance on the solvent side of this boundary then corresponds to that of "solvent" plus perhaps proteins of low molecular weight, such as G-actin. By the present method, at least, no such proteins were found; this was established by the fact that (in a separate run) solvent alone, centrifuged for an equal time at the same speed, gave the same absorbance as solvent plus the hypothetical low molecular weight proteins.[23,24] We therefore accepted the absorbance in this myosin-free region as the solvent base-line and considered that absorbances greater than this, seen in early plates, arose from the presence in the cell of myosin or proteins heavier than myosin. In a late photograph, the heavier proteins have been swept out of the visible portion of the cell, and the distance $C_L(t)$ measures the myosin ("L" for "light-component") concentration. In the radial direction we also measured d_m, the distance from a reference position to the meniscus, and $d_b(t)$, the distance from the same reference position to the center of the myosin boundary (located as the abscissa at $C_L(t)/2$). From such data, as well as from the schlieren diagrams, we computed the sedimentation coefficient of myosin. Knowing this (and therefore the position of the myosin boundary at any time, t), we applied the radial dilution square law[25] and calculated the absorbance contributed by myosin at $t = 0$[26] and at the time of each successive exposure. This contribution was represented on each densitometer tracing by a horizontal line an appropriate distance above the solvent base line. Absorbance in excess of this horizontal line was then taken as the contribution of "H" (for "heavy") protein components. On each plate, $C_H(t)$, the concentration of the heavy material, was plotted as a function of radial distance in the cell (Fig. 1b), and by planimeter integration of graphs such as those of Fig. 1b, we obtained $A_H(t)$, the amount of heavy material in the cell at time, t. Finally, the ratio $A_H(t)/A_T(0)$, ("T" for "total protein") was plotted as a function of effective centrifugation time for each run (Fig. 4); such curves will be interpreted below (Results section), on the basis of the analysis developed in Appendix I.

These concentration measurements are based on the 254 mμ absorbance of proteins. It might be asked whether such measurements are confused by nucleic acid impurities[27] or by ATP bound to the sedimenting proteins. We have sought to avoid nucleic acid complications by dealing only with *ratios*, such as $A_H(t)/A_T(0)$, hoping that, if indeed there be nucleic acid contaminants, the various fractions (see below) of myosin B are homogeneously contaminated and thus not disturbing to a ratio. Bound ATP does not seem to be a complication because (1) the amount of ATP which sediments with the protein appears to be insufficient to alter significantly the absorbance of the remaining solvent, compared to appropriate blanks, and (2) essentially identical results are obtained with PP (pyrophosphate), which has been shown to have the same physical effect on myosin B as ATP,[28] but has no intrinsic UV absorbance.

(19) B. Zimm, *J. Chem. Phys.*, **16**, 1099 (1948).

(20) K. V. Shooter and J. A. V. Butler, *Trans. Faraday Soc.*, **52**, 734 (1956).

(21) V. N. Schumaker and H. K. Schachman, *Biochim. et Biophys. Acta*, **23**, 628 (1957).

(22) In the Model E Ultracentrifuge, visualization of both the schlieren and the absorption images is possible, since the two optical systems are entirely independent.

(23) D. Kominz, W. Carroll, E. Smith and A. Mitchell, *Arch. Biochem. and Biophys.*, in press.

(24) D. Kominz, *et al.* (ref. 23), as well as we (in preliminary work) have noted the appearance of a new small component in the schlieren diagram when a myosin B solution is made alkaline, but according to ref. 23 this substance is a sub-unit of myosin and is certainly not actin.

(25) R. Trautman and V. Schumaker, *J. Chem. Phys.*, **22**, 551 (1954).

(26) For the present purposes, the sedimentation which occurs during acceleration was taken to be equivalent to that obtained during 3 minutes at speed. The first photograph ("zero time") was taken at 3000 r.p.m.

(27) E. Mihalyi, D. F. Bradley and M. I. Knoller, THIS JOURNAL, **79**, 6387 (1957).

(28) W. F. H. M. Mommaerts, *J. Gen. Physiol.*, **31**, 361 (1948).

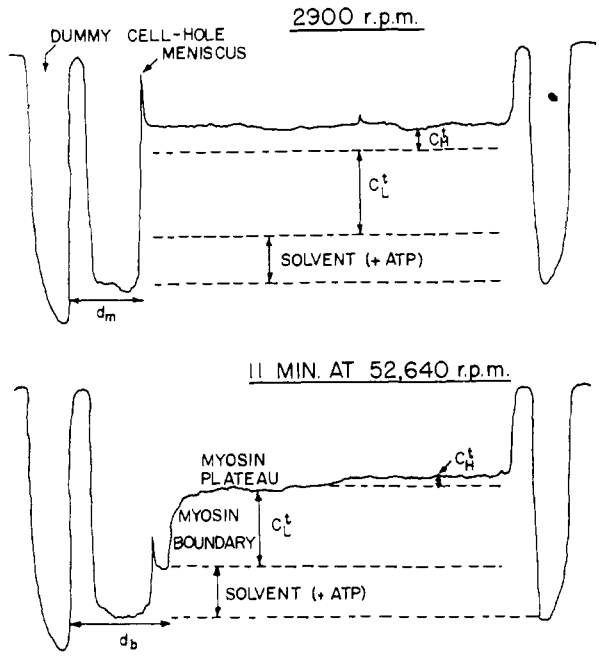
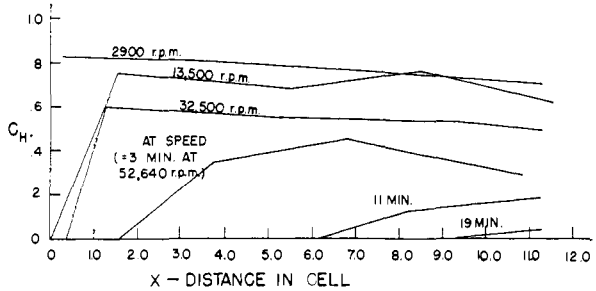


Fig. 1.—(a) microdensitometer tracings of ultraviolet absorption photographs for sedimentation of 5-hr. extracted myosin B in the presence of 2×10^{-5} *M* ATP, 0.01 *M* $MgCl_2$, 0.6 *M* KCl, *p*H 7.0. Protein concentration = 0.08 g./100 ml. (b) Concentration of heavy components *versus* distance from the axis of rotation in the cell (in arbitrary units) from microdensitometer tracings.

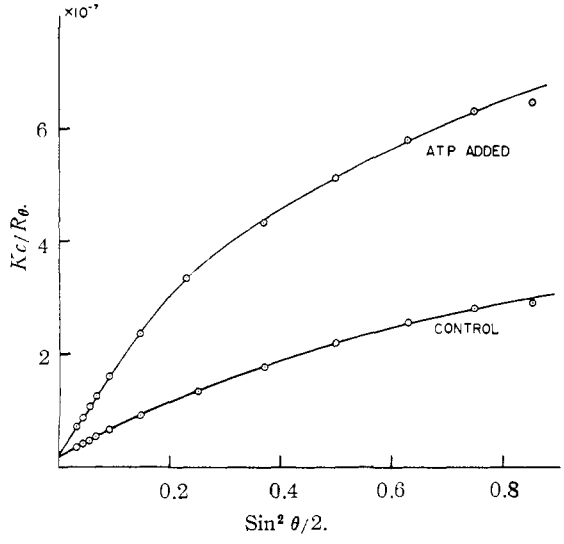
Results

Light Scattering.—The scattering from a sample of 24-hr. myosin B before (lower curve) and after (upper curve) the addition of excess ATP is plotted in Fig. 2a. The numerical value of the intercept of each curve at zero angle (obtained by linear extrapolation through the low angle points) is equal to the reciprocal of the weight average molecular weight ($1/\bar{M}_w$) of the scattering particles. Note that, within experimental error, this intercept is unchanged by ATP addition, and thus $\bar{M}_w$ is also unchanged.



The limiting slope at zero angle, on the other hand, has increased markedly. Thus the average radius of gyration of the scattering particles, defined by $\bar{r}_g \equiv [(\sqrt{3}\lambda)/(4\pi n)][\sqrt{\text{slope/intercept}}]$, with λ = wave length of the light and n = refractive index of the solution, must also have increased. The $\bar{r}_g$ thus measured is the "light-scattering" average, defined by

$$\overline{r_g^2} = \frac{\Sigma_i c_i M_i r_i^2}{\Sigma_i c_i M_i}$$



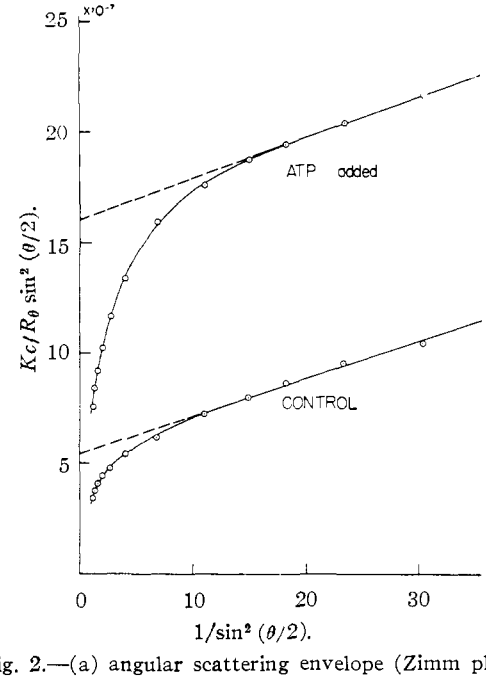


Fig. 2.—(a) angular scattering envelope (Zimm plot) of 24-hr. extracted myosin B (lower curve) plus 10^{-3} *M* ATP (upper curve). Protein concn. = 0.018 g./100 ml. Solvent: 0.6 *M* KCl, *p*H 7.0. (b) Yang plot (see text) of same data on 24-hr. extracted myosin B (lower curve) plus 10^{-3} *M* ATP (upper curve).

where c_i, M_i and r_1 are the weight concentration, molecular weight and radius of gyration of the *i*th component, respectively. $\bar{r}_g$ is approximately a "*z*-average" for common models and is thus very heavily dependent upon the behavior of the largest particles.

In the data representation recently suggested by Yang,[29] $1/\bar{M}_w$ is the limiting slope reached at large values of the abscissa (small values of θ). Such a plot is therefore a sensitive means of examining for changes in $\bar{M}_w$ in large praticles. The constancy of $\bar{M}_w$ on ATP addition can therefore be judged more critically by replotting the data

(29) J. T. Yang, *J. Polymer Sci.*, **26**, 305 (1957).

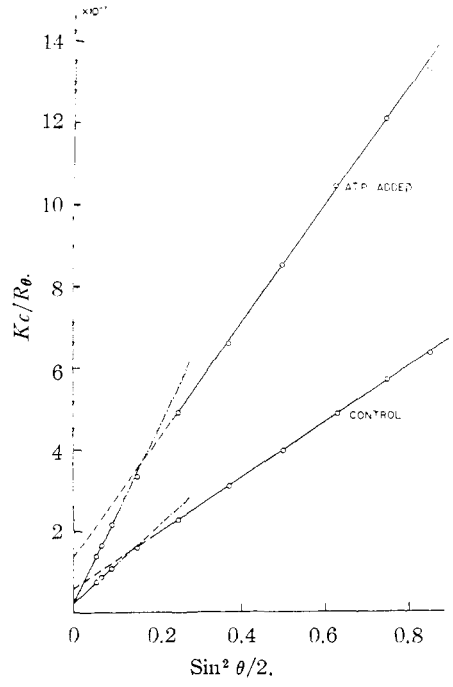


Fig. 3.—Zimm plot of 24-hr. extracted myosin B (lower curve) plus 10^{-3} M ATP (upper curve). Protein concn. = 0.01 g./100 ml., in 0.6 M KCl, 0.1 M histidine, pH 7.0. Shows both asymptotic (---) and limiting (·–·–·) extrapolations.

of Fig. 2a in the manner of Yang (Fig. 2b); it is obvious that also in the Yang plot in such experiments $\bar{M}_w$ varies very little. In this representation, the average radius of gyration is defined as

$$\bar{r}_g \equiv [(\sqrt{3}\,\lambda)/(4\pi n)][\sqrt{\text{intercept/slope}}]$$

Since the slope remains approximately constant, $\bar{r}_g$ clearly increases markedly upon ATP addition.

Table I cites values of $\bar{M}_w$ and $\bar{r}_g$ obtained by Zimm and Yang plots in a number of experiments

TABLE I

EFFECT OF 10^{-3} M ATP ON THE MOLECULAR WEIGHT AND RADIUS OF GYRATION OF VARIOUS MYOSIN B PREPARATIONS

Prep. no.	Extn. time (hr.)	Before ATP $\bar{M}_w \times 10^{-6}$	Before ATP $\bar{r}_g \times 10^{-2}$, Å.	After ATP $\bar{M}_w \times 10^{-6}$	After ATP $\bar{r}_g \times 10^{-2}$, Å.
76	5	21	32	20	39
77	5	17	29	15	36
76	24	52	27	45	43
77	24	40	28	45	47
77	24	42	28	50	47
80	24	56	23	50	39
80	24	48	25	36	35

on various preparations of 5-hr. and 24-hr. extracted myosin B. There is some variation in average particle size from one preparation to another, but the result of essentially constant $\bar{M}_w$ before and after ATP addition, coupled with large increases in $\bar{r}_g$, can be seen throughout for both 5- and 24-hr. extracts. The present results are therefore in agreement with those obtained by Blum and Morales[4] and in disagreement with those of Gergely.[9]

However, consideration of the scattering data obtained at high angles suggests that the situation might be more complicated. Benoit[30] has shown that for a solution of large polydisperse random coils, the Zimm plot at large angles is asymptotic to a straight line from whose slope and intercept the *number average* molecular weight (intercept = $1/2\bar{M}_n$) and radius of gyration can be calculated. Since our Zimm plots frequently do approach a straight line at large angles and since *ad hoc* aggregates of particles are apt to have mass distributions similar to those of random coils it is tempting to apply the Benoit analysis. The increase in the intercept of the high angle asymptote, which such Zimm plots show after ATP addition, then leads to the conclusion that the *number average* molecular weight ($\bar{M}_n$) has dropped significantly, while the *weight average*, as we have seen, is unchanged. A typical plot exhibiting such a linear asymptote is presented in Fig. 3. Here both the low angle and the asymptotic extrapolations are drawn in, and it is easily seen that while the intercept of the low angle extrapolation (and thus $\bar{M}_w$) remains constant, the intercept of the asymptotic extrapolation rises after ATP addition, and thus $\bar{M}_n$ falls.

This interpretation, however, is only suggestive because in a significant number of experiments the Zimm plots obtained do not show a linear asymptote at large angles. (Figure 2a is a typical example of such a plot.) Also, increased downward curvature at high angles can equally well be caused by expansion of the particles toward a more rod-like form. Since one of the effects of ATP is to produce an inflation of the larger aggregates, this effect is undoubtedly intertwined with the consequences of polydispersity in such a way that an unambiguous interpretation is difficult. Nevertheless, to the extent to which it may be applicable, the Benoit treatment suggests an interpretation of the ATP effect in terms of two general particle size classes. The largest particles, which we will call class I, seem to inflate at constant molecular weight, while the smaller, class II, appear to depolymerize. The sedimentation results presented below re-enforce this interpretation.

Sedimentation.—Figure 4 shows $A_H(t)/A_T(0)$ (ratio of amount of heavy components at time t to amount of total protein, including myosin, at time zero) as a function of centrifugation time[31] for solutions of 5-hr. extracted myosin B which are 0.08 g./100 ml. in total protein and contain various concentrations (see legend) of other substances. Close inspection of this figure shows that sedimentation in the presence of saturating concentrations of ATP and PP markedly affects the "elimination curve." To bring out this point, the average curve, with and without ATP (or PP), has been plotted in Fig. 5, together with the average deviations of the data. It can be seen that, even before centrifugation begins, the two solutions have different proportions of heavy material. The "control" contains *ca.* 35% "heavy material" and

(30) H. Benoit, *J. Polymer Sci.*, **11**, 507 (1953).

(31) The methods used in obtaining $A_H(t)/A_T(0)$ have been described in the Experimental section.

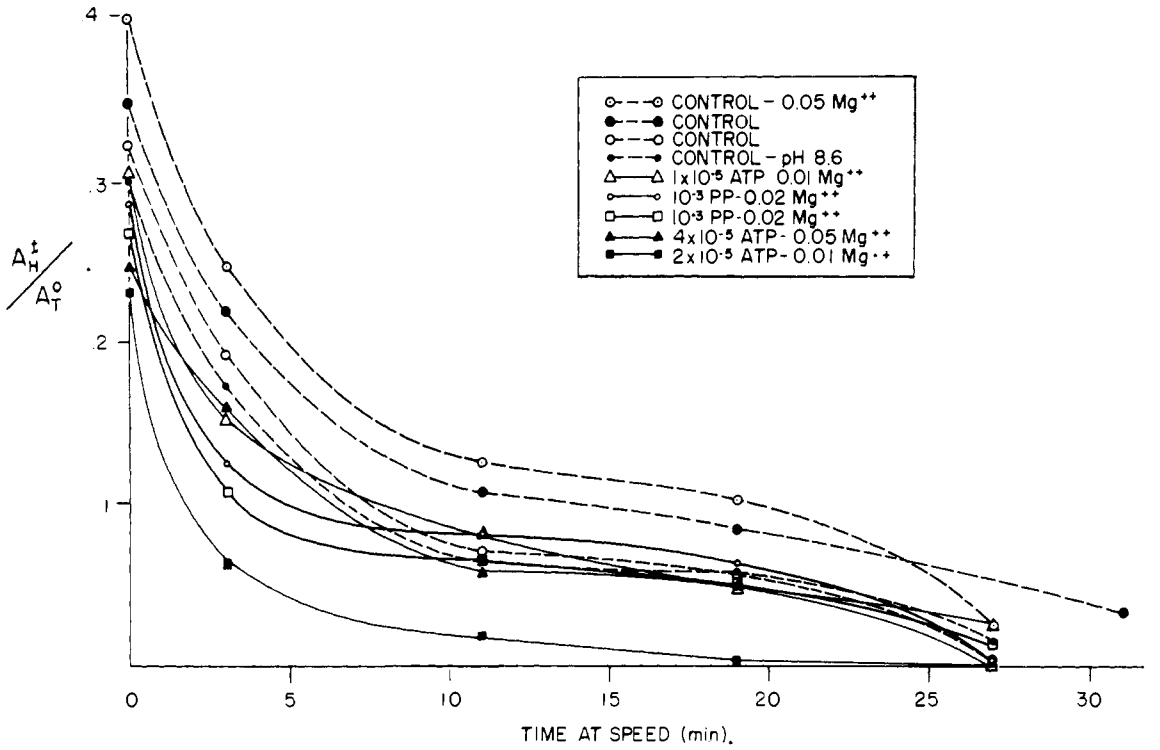


Fig. 4.—Sedimentation "elimination curves" showing the fraction of heavy components of 5-hr. extracted myosin B remaining in solution as a function of centrifugation time, in the presence of various reagents (see figure). Protein concentration = 0.08 g./100 ml. Measurements derived from ultraviolet absorbance–sedimentation data.

65% free myosin. On the addition of ATP or PP, some 29% of the heavy material (10% of the total protein) has depolymerized into "light material." This light material is probably myosin and nothing else, because of considerations already cited above (Experimental section) and because of the following argument. The ultraviolet data indicate that as a result of the depolymerization the amount of "light material" (myosin and anything of equal or lesser molecular weight) increases from 65 to 76%. On the other hand, in the schlieren diagram, the area under the peak which has a sedimentation coefficient of *ca.* 5.5 Svedbergs (*i.e.*, the myosin peak) increases in the proportion 65:78. One result of the sedimentation studies, therefore, is that addition of these polyphosphates brings about a depolymerization, the only *low* molecular weight ($M \leqslant 5 \times 10^5$) product of which is myosin.

Considerably more information can be derived from Fig. 5 by analysis of the time course of the elimination curves. In Appendix I we have derived the equation relating $A_H(t)/A_T(0)$ (the ratio of heavy components remaining in the cell at time t to total material at time zero), to t (the effective centrifugation time)

$$\frac{A_H(t)}{A_T(0)} = \frac{A_H(0)}{A_T(0)} - K\frac{A_H(0)}{A_T(0)}\bar{s}t \qquad (1)$$

where K is a lumped set of constants. For the 5-hr. myosin B system, average values of $A_H(t)/A_T(0)$ before and after the addition of ATP (or PP) are plotted against t in Fig. 5. Equation 1 shows that the intercept of these curves with the ordinate give $A_H(0)/A_T(0)$, while the instantaneous slope at any time t is proportional to $A_H(0)/A_T(0)$ times the

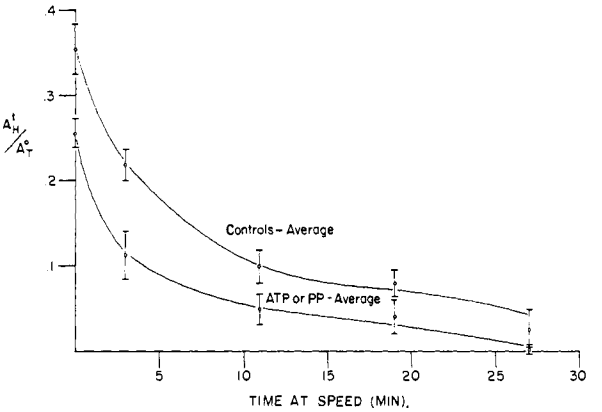


Fig. 5.—Average sedimentation "elimination curves" for 5-hr. extracted myosin B (upper curve) plus ATP or PP (lower curve). (Data from Fig. 4.)

weight average sedimentation coefficient of the system, $\bar{s}$ as it is composed at that time. Since for each curve, $A_H(0)/A_T(0)$ is a constant, $\bar{s}$ clearly decreases monotonically with time, as might be expected from the fact that we are dealing with a polydisperse system from which the heaviest particles are being preferentially sedimented.

Guided by the tentative deduction discussed in the light-scattering work, we shall assume that the heavy ATP-responsive material consists of two size classes (we shall, of course, admit to some polydispersity within these classes), a heavier (I) and a lighter (II). Toward the end of the period of observation ($t > 15$ min.) we shall assume that class I material has been largely swept out of view

so that eq. 1 now becomes essentially

$$\frac{A_{II}(t)}{A_T(0)} = \frac{A_{II}(0)}{A_T(0)} - K\frac{A_{II}(0)}{A_T(0)}\bar{s}_{II}t \qquad (2)$$

In other words, a linear extrapolation backward from the tail end of the elimination curve gives us an experimental value of

$$\frac{A_{II}(0)}{A_T(0)}, \text{ and } K\frac{A_{II}(0)}{A_T(0)}\bar{s}_{II}$$

We may now subtract eq. 2 from eq. 1 to obtain the sedimentation of class I particles

$$\frac{A_I(t)}{A_T(0)} = \frac{A_I(0)}{A_T(0)} - K\frac{A_I(0)}{A_T(0)}\bar{s}_I t \qquad (3)$$

and compare the results with the experimental residue curve. If the assumption of two gross classes of heavy components is correct, then eq. 3 should describe the experimental residue curve. Figure 6, in which the preceding steps are carried out graphically, shows that, at least roughly, this is the case. The ratio of the intercepts in eq. 3 and 2 is $A_I(0)/A_{II}(0) \simeq 1.2$; the ratio of slopes is ~ 5, whence $s_I/s_{II} \sim 6$. If we assume that we are dealing with particles whose diffusion coefficients are large and not too different, then it is a consistent assumption that $M_I/M_{II} \sim s_I/s_{II} \sim 6$. Such a ratio of molecular weights is compatible with the light-scattering results cited above.

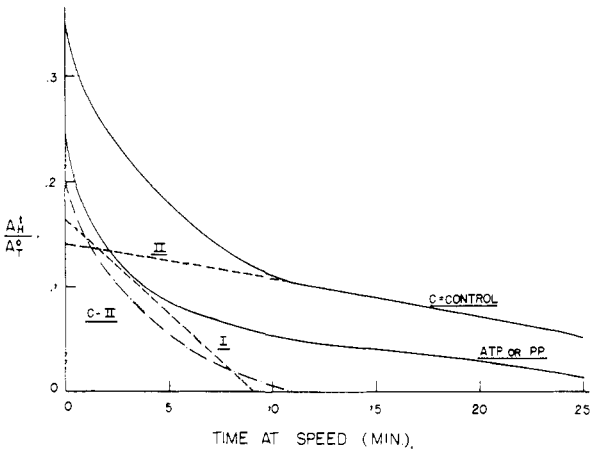


Fig. 6.—Average sedimentation "elimination curves" for 5-hr. extracted myosin B (upper curve) plus ATP or PP (lower curve). (See Fig. 5.) Line II represents a linear approximation to the sedimentation of class II particles; curve C-II is then the derived curve for the sedimentation of class I particles, and line I is the linear approximation to curve C-II.

The effect of adding ATP or PP can be understood in similar terms and displayed on the same graph, since $A_T(0)$ is the same with or without added polyphosphates. A comparison of the lower (ATP or PP) experimental curve of Fig. 6 with the calculated curves for class I particles shows that the addition of ATP or PP does essentially nothing to class I particles (the frictional properties and therefore the sedimentation behavior of particles of this size should be essentially unchanged by the inflation deduced from light scattering) but substantially, though not completely, reduces the number of class II particles.

Considering the sedimentation data as a whole, we find that certain particles of intermediate size (class II) contained in myosin B are partially dissociated by ATP. These aggregates are much larger than myosin but smaller than the largest aggregates present. Within experimental error, all the *low* molecular weight material which disappears from the heavy fraction appears as myosin.[32] However, this does not exclude the possibility that a small quantity of material sedimenting at a rate comparable to the heavy particles of myosin B is simultaneously released. The existence of such a material is examined in the following paper.[10]

Discussion

Because light-scattering measurements of the myosin B system are controversial, it is both useful and necessary to consider in some detail whether the results we have obtained using this method are meaningful and definitive. The following questions must be examined.

(1) Can the Zimm treatment properly be applied to particles of the size of those studied here? With sufficiently large particles, deviations from the simple theory arise, due to phase shifts, secondary scattering, etc. However, these effects are most important for compact particles (*i.e.*, spheres) rather than extended particles[33] such as those of myosin B. Since the Zimm theory, applied to particles such as tobacco mosaic virus which are of comparable molecular weights and much more compact, has been validated by several independent methods,[34] our use of the conventional treatment seems justified. The contribution of phase shifts, in particular, can be shown to be negligible, by using the analysis of Zimm and Dandliker.[35]

(2) Granting, then, that the Zimm method is in principle applicable to myosin B, is a valid extrapolation to zero angle possible in practice? That is, do the experimental points reach small enough angles to make Kc/R_θ a linear function of $\sin^2(\theta/2)$? It was to assure this situation as far as possible that measurements were extended to 21°, that is, to angles smaller than previously examined by other workers. When this is done, the five lowest angle points (21–35°) do fall on a straight line, making possible an accurate linear extrapolation.

(3) To what extent can our results be influenced by the presence of "dust" (or large, inert contaminants)? First, of course, great pains were taken to detect and remove such contaminants. But since "dust" does scatter most prominently at low angles, our extension of the angular range to 21° does increase the danger of some unreliability on this account. The linearity of the line through the low angle points, plus the consistency with which the same results were obtained in repeated

(32) This release of myosin from aggregates by ATP is more easily demonstrated using 24-hr. extracted myosin B. Before ATP, it contains no free myosin, and therefore there is no sign of the *ca.* 5.5 *S* schlieren peak characteristic of myosin. After ATP addition, a large myosin peak appears.

(33) P. Doty and J. T. Edsall in "Advances in Protein Chemistry," Vol. VI, edited by M. L. Anson, K. Bailey and J. T. Edsall, Academic Press, Inc., New York, N. Y., 1951, p. 37.

(34) (a) H. Boedtker and N. S. Simmons, THIS JOURNAL, **80**, 2550 (1958); (b) C. E. Hall, *ibid.*, **80**, 2556 (1958).

(35) B. H. Zimm and W. B. Dandliker, *J. Phys. Chem.*, **58**, 644 (1954).

experiments with solutions containing presumably variable amounts of "dust," indicate that scattering by "dust" is not responsible for our results.

(4) A related question is whether the addition of ATP to myosin B with subsequent stirring might produce large aggregates, thus causing an apparent increase in $\bar{r}_g$.[36] If so, the observed constancy of $\bar{M}_w$ must be accounted for by supposing a balance between this hypothetical aggregation and the dissociation reported by Gergely.[9] Such an explanation would require that the degree of aggregation produced by ATP be remarkably similar from one experiment to another. It also would imply that only ATP (and other ATP analogs, such as PP) cause this aggregation, and that a *second* addition of these agents produces no further effect.

(5) Gergely has questioned whether the difference between his results and those of Blum and Morales[4] depends on differences in technique. Gergely's practice was to dilute myosin B, then centrifuge and measure scattering; Blum and Morales centrifuged the concentrated stock solution and then diluted it. Gergely surmises that the latter procedure fails to remove certain large aggregates unaffected by ATP. He suggests that the unaltered presence of these aggregates, combined with the dissociation of ATP-sensitive particles, could then give an increase in initial slope at constant $\bar{M}_w$. This argument seems unsatisfactory on two accounts. The first, examined in detail in Appendix II, is that while dissociation in the presence of large inert particles can indeed produce a rise in $\bar{r}_g$, it is not possible by any reasonable adjustment of parameters even to approach the observed increase quantitatively. The second is empirical; the experiments on 24-hr. preparations have now been repeatedly done by both techniques (*cf.* Experimental section) and lead to the same results. A more plausible reason for the differing results obtained by Gergely and ourselves lies in a point of preparative technique. Gergely precipitates myosin B at an ionic strength of 0.3, whereas we precipitate it at 0.06. The resulting myosin B samples may differ sufficiently in composition or intraparticle bonding to account for the divergent ATP responses.

We are thus unable to arrive at any interpretation of our light-scattering findings other than that a certain class of particles in our myosin B solutions actually does inflate, under the influence of ATP, at sensibly constant $\bar{M}_w$. Since it appears to be the largest particles which inflate, while smaller ones dissociate, it is undoubtedly possible to remove the larger ones preferentially (*i.e.*, by strong centrifugation). The resulting solution would then show only dissociation.

Combining the results from light scattering and from sedimentation, we arrive at the following picture of the myosin B system. There are three broad classes of particles, in terms of size. One is homogeneous myosin, which is structurally unaffected by ATP. The second is a class of much larger aggregates, dissociable by ATP and yielding myosin as the only discernible sub-unit of *low* molecular weight. Members of the third class—still larger particles—inflate in the presence of ATP without noticeable dissociation, as indicated by the sharp increase of $\bar{r}_g$ and the constancy of $\bar{M}_w$.[37]

Additional support for this interpretation may be drawn from the recent flow birefringence studies of Maruyama and Noda.[38] In addition to the commonly observed *increase* of the rotary diffusion constant of the system at moderate rates of shear (conventionally interpreted as a dissociation), they find that ATP, at *very low* shear rates, produces a *decrease* in rotational diffusion constant. The latter effect may well be due to the inflation of the larger particles. At high shear rates, these are always completely oriented, and any inflation will go unobserved. After intensive ultracentrifugation, which presumably removes the largest aggregates, Maruyama and Noda find an increase in the average rotary diffusion constant at all rates of shear.

We will postpone a consideration of the molecular mechanisms by which ATP brings about these structural alterations of myosin B particles to the following paper,[10] in which we present further experimental results pertinent to the subject.

Appendix I

The analysis of ultracentrifugal "elimination curves" (such as those of Fig. 5) can yield considerable information abut a polydisperse system. Assuming that the components of interest are of very high molecular weight and that the observational time is short (as is the case here), diffusion may be neglected. Under these circumstances the concentration $c(x,t)$ of a single component satisfies the equation of the centrifuge,[39]

$$\frac{1}{x}\frac{\partial}{\partial x}\left\{\left(D\frac{\partial c}{\partial x} - s\omega^2 xc\right)x\right\} = \frac{\partial c}{\partial t} \qquad \text{(i)}$$

with both $D = 0$ and $\partial c/\partial x = 0$. Direct integration then shows that

$$c(t) = c(0)\exp(-2s\omega^2 t) \qquad \text{(ii)}$$

As long as the component remains in the observed portion of the cell, *i.e.*, as long as $x(t)$, the x-coordinate of its boundary, is equal to or less than x_b, the coördinate of the "bottom" of the cell, the amount of component, $a(t)$ is given by

$$a(t) = \int_{x(t)}^{x_b} h\theta c(t)\,\xi d\xi$$

$$a(t) = (h\theta/2)c(t)\{x_b^2 - x^2(t)\} \qquad \text{(iii)}$$

where ξ is radial distance in the cell and h and θ are the depth and angle of the sector cell. Substituting from (ii) into the radial dilution square law[25]

$$x^2(t) = x^2(0)\exp(2s\omega^2 t) \qquad \text{(iv)}$$

Since $dx(t)/dt = s\omega^2 x(t)$, the maximum time, t^*, during which the component survives in the cell is given implicitly by

$$x_b/x(0) = \exp(s\omega^2 t^*) \qquad \text{(v)}$$

(36) A. Holtzer, private communication.

(37) The relative amounts of particles in each class, and their molecular weights, seem to be a function of the length of the extraction period. As Table I indicates, the mean particle size rises markedly in going from 5-hr. to 24-hr. extraction times. Simultaneously, the weight per cent. of material present as free myosin falls from *ca.* 65% to essentially zero.

(38) K. Maruyama and H. Noda (private communication).

(39) T. Svedberg and K. O. Pedersen, "The Ultracentrifuge," Oxford Press, London, 1940, p. 21.

In the Model E ultracentrifuge, the square of the ratio, $x_b/x(0)$, has approximately the measured value, 1.21; thus twice the argument of the exponent (eq. v) is 0.191. With good accuracy the square of such an exponential can be "linearized" (replaced by $1 + 2s\omega^2 t^*$). Substituting from (ii), (iv) and (v) into (iii) ,we obtain

$$a(t) = (h\theta/2)\, c(0)x^2(0)\, \{\exp\,[2s\omega^2(t^* - t)] - 1\} \quad \text{(vi)}$$
$$t \leqslant t^*$$

Since the restriction on t requires that the argument of the exponential in equation vi be not greater than $2s\omega^2\ t^*$ and since we have seen above that $\exp(2s\omega^2 t^*)$ can be safely linearized, eq. (vi) can be simplified to

$$\begin{aligned} a(t) &= [h\theta\omega^2x^2(0)]\ c(0)s(t^* - t) \\ &= kc(0)s(t^* - t) \\ &= a(0) - kc(0)st \end{aligned} \quad \text{(vii)}$$

An equation such as (vii) must hold for each individual (ith) component in a polydisperse system. Therefore, if we define

$$A(t) = \sum_i a_i\,(t)$$

and understand that the summation receives contributions only from the surviving components, then, summing over equations of the type(vii) we obtain

$$A(t) = A(0) - k(\sum_i c_i(0)s_i)t$$

or, if v is the observable volume of the cell

$$\frac{A(t)}{A(0)} = 1 - (k/v)\frac{\sum_i c_i(0)s_i}{\Sigma\, c_i(0)}t \quad \text{(viii)}$$

Equation viii shows that a plot of $A(t)/A(0)$ *vs.* t may be thought of as a line whose slope is proportional to $\bar{s}$, the weight-average sedimentation coefficient and which therefore keeps decreasing with time as components pass out of view in order of decreasing s_1. This is still true if eq. viii is multiplied through by $A(0)/\{M(0) + A(0)\}$, provided $M(0)$ (*e.g.*, the observable amount of myosin in the cell) remains constant while the heavier components are sedimenting out (this is very nearly so in the myosin B system). Equation viii then becomes

$$\frac{A_H(t)}{A_T(0)} = \frac{A_H(0)}{A_T(0)} - K\frac{A_H(0)}{A_T(0)}\bar{s}t \quad \text{(ix)}$$

which is the form applied above to the data of Fig. 5.

Appendix II

Light scattering measurements on myosin B solutions indicate that the light scattering average radius of gyration, $\bar{r}_g$, increases when ATP is added. It may be questioned, however, whether this increase is an artifact caused by the dissociation of some component in this polydisperse system; in other words, whether an increase in the length of any molecular species is required to account for the experimental result. We shall see that this increase in $\bar{r}_g$ cannot be artifactual, and must arise from a real lengthening.

Our ultracentrifuge data show that 5-hr. myosin B is about 65% by weight myosin and about 35% larger particles. When ATP is added, some of this heavier material, about 10% of the total protein, dissociates into myosin. This non-dissociating heavy fraction we have called component I, the dissociating heavy fraction component II, and the myosin fraction component III.

We will now estimate the greatest increase in $\bar{r}_g$ that can be found on such a model, if *no* molecular inflation occurs. By the usual definition

$$\bar{r}_g^2 = \frac{\sum_i x_i M_i r_i^2}{\sum_i x_i M_i} = \frac{1}{\bar{M}_w}\sum_i x_i M_i r_i^2 \quad \text{(i)}$$

where x_i = weight fraction, M_i = molecular weight, and r_i = radius of gyration. In order to solve the problem, we must assume some functional relation between M_i and r_i. For the myosin fraction we take the experimental value of 4.2×10^5 for M_{III},[10] and 477 Å. for r_{III}.[40] Since in 5-hr. myosin B, $\bar{r}_g$ is only about six times the myosin value while $\bar{M}_w$ is fifty times as great, we may plausibly assume for the larger fraction the relation between r_i and M_i that holds for random coils, *i.e.*, $r_i^2 = aM_i$. $\bar{M}_I$ and r_I must also be selected to bring the averages into line with the experimental values. A suitable choice is $\bar{M}_I = 75 \times 10^6$, $\bar{r}_I = 3000$ Å. With these values one obtains, for the "ATP added" case, $\bar{M}_w = 19.1 \times 10^6$, and $\bar{r}_g = 2980$ Å. In order to obtain the maximum increase in $\bar{r}_g$ in the absence of inflation, we must now minimize for $\bar{r}_g^2$ in the "no ATP" case with respect to M_{II}. One easily obtains $(M_{II})_{min} = 34.2 \times 10^6$, and therefore $\bar{M}_w = 22.5 \times 10^6$ and $\bar{r}_g = 2850$ Å.

Thus the *maximum* possible increase in $\bar{r}_g$ with this model is 4.6%, compared with the experimental value of ~25%.

So far, all three components in this system have been assumed individually monodisperse. This assumption must now be examined. Component III makes such a small contribution to both numerator and denominator of eq. i that any polydispersity in it may be neglected. Terms due to component I are the same before and after addition of ATP, and are uniquely determined once mean values of $\bar{M}_I$ and r_I have been chosen. Thus any polydispersity of component I may also be disregarded. Polydispersity of component II may *not* be ignored and, for the purposes of this calculation, must be so chosen as to give the minimum possible value of $\bar{r}_g$ before ATP. As a Z-average molecular weight is never less than the corresponding weight-average and is equal to it only for a monodisperse system, it is seen that choosing component II monodisperse will yield the smallest value of its term in the numerator of (i) for a given denominator term. Hence $\bar{r}_g$ will also be smallest with this choice. Thus the assumption that all three components are monodisperse is justified.

In summary, the largest possible increase in $\bar{r}_g$ produced by this mechanism is only a small fraction of the experimentally observed increase, leading

(40) A. Holtzer and S. Lowey, THIS JOURNAL, **78**, 5954 (1956).

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 *p*H 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).