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The Aggregation of Myosin

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Light scattering and sedimentation data are employed to show that the spontaneous aggregation of myosin in 0.6 *M* KCl is a step-wise process in which the molecules join almost exclusively side-to-side up to weight average molecular weights of at least eight times the monomer weight. The aggregates are unaffected by Versene, base, ATP and high ionic strength. The rate of association, however, is somewhat decreased by Versene, ATP, and, particularly, inorganic phosphate, and increased in 1.5 *M* KCl. This suggests that the inhibitory action is caused by anion binding and subsequent enhanced repulsion of protein molecules. The experimental results argue against the recent hypothesis that the characteristic properties of Myosin B solutions are caused by aggregates of myosin monomers, since the aggregates formed in the present study bear no resemblance to Myosin B. The rate of aggregation is observed to be independent of protein concentration. This is explained in terms of a two step process involving a local configuration change, somewhere in the molecule, followed by a rapid association.

Introduction

Many of the difficulties encountered in a precise macromolecular characterization of myosin have been explained in terms of the occurrence of a spontaneous, temperature dependent aggregation of the molecules.[2] Since exact measurements on monomeric myosin require that the work be performed at an inconveniently rapid rate, using a great many precautions, a study of the factors affecting this aggregation process was undertaken in the hope of finding a method of controlling it.

Furthermore, there is at present considerable controversy over the nature of the molecular interactions involved in muscular contraction. In particular, the work of Morales and co-workers emphasizes the importance of myosin–myosin interactions, and, indeed, these workers have proposed recently that the dissociation produced by the action of ATP on solutions conventionally termed "actomyosin" or "Myosin B" (obtained by 5–24 hour extraction of the muscle) is a dissociation of a myosin–myosin complex rather than an actin–myosin complex.[3–5] By investigating the effects of various additives on the formation of aggregates of monomeric myosin we hoped to compare these phenomena with the dissociation caused by similar additives in Myosin B solutions. If Myosin B is merely a highly aggregated solution of myosin, we would expect that the aggregation of pure monomeric myosin would be reversed, or at least inhibited, by media that normally dissociate Myosin B.

In an earlier study,[2b] it was shown that the aggregation has the following characteristics: (1) it occurs step-wise, *i.e.*, yields dimers, trimers, etc.; (2) the molecules join side-to-side, the radius of gyration remaining substantially constant as the molecular weight increases; (3) the rate of aggregation increases markedly with temperature, being about ten times as fast at 25° as at 4°; (4) the reaction can be only slightly reversed by lowering the temperature; (5) the aggregation is not reversed by the action of Versene, base (*p*H 10) ATP or high ionic strengths (2 *M* KCl).

In the present work we report further studies on this aggregation reaction. In particular, we investigated whether the reaction can be *inhibited*, even if not reversed, by various additives. We also studied the rate of aggregation as a function of protein concentration.

Experimental

Samples, chemicals and experimental procedures are essentially as described in the previous paper. In this investigation we were concerned mainly with possible changes in behavior under various conditions. For this reason, no attempt was made to employ all the correction factors necessary when absolute molecular weights are required, or to use only monomeric starting material. However, a suitable control (0.6 *M* KCl, *p*H 6–7) was always run concurrently.

Results

The Aggregation Process in 0.6 *M* KCl, *p*H 6–7, 25°.—The aggregation has been discussed as proceeding step-wise to dimers, trimers, etc.[2b] Evidence for this step-wise picture is based on a semi-quantitative correlation of light scattering and ultracentrifuge experiments. In the previous work, even when almost all of the (presumed) monomer (molecular weight, 493,000) had disappeared and the appearance of the plates suggested about equal amounts of "dimer" and "trimer" and a fair amount of higher aggregates, a weight average molecular weight of only 1.6 million was found. This is about what one would expect from the foregoing interpretation. If the two main faster peaks were in reality higher aggregates, rather than dimer and trimer, such a small value for M_w could not have been obtained.

To check this point we note that, if the monomer is rod-shaped, and aggregates side-to-side, then the mass and dimensions of the dimer are known from the monomer values, and we can calculate a theoretical intrinsic sedimentation constant from the Kirkwood–Riseman equation (see equation 1 of the previous paper). This should then agree with the measured value for the dimer peak. We have $M_{dimer} = 2 \times 493{,}000 = 986{,}000$, but we must be careful about choosing the diameter d. The theory applies to a rigid string of spherical beads. If we pack two such molecules side-to-side the geometry of the aggregate does not conform to the model used in the theory. In fact, the dimer has

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(2) (a) K. Laki and W. R. Carroll, *Nature*, **175**, 389 (1955); (b) A. Holtzer, *Arch. Biochem. Biophys.*, **64**, 507 (1956).

(3) J. J. Blum and M. Morales, *ibid.*, **43**, 208 (1953).

(4) M. Morales, in "Enzymes; Units of Biological Structure and Function," Academic Press, New York, N. Y., 1956, p. 325.

(5) P. H. Von Hippel, M. Gellert and M. Morales, "Proceedings of the Conference on Muscle Contraction," International Enzyme Symposium, Tokyo, Japan, 1957.

no unique diameter, being $2d$ thick and only d wide. Therefore, to use equation 1 we must assume some average diameter.

The hydrodynamically reasonable choice, we feel, is to replace the two packed chains by a single average chain of the same length and volume, *i.e.*

$$\frac{2\pi d^2 L}{6} = \frac{\pi d_{av}^2 L}{6} \text{ and } d_{av}^2 = 2d^2$$

Therefore the average diameter we require is simply the monomer diameter $\times \sqrt{2} = 36.8$ Å. Using these values in the Kirkwood–Riseman equation we calculate: $[S_{20,w}] = 10.7\,S$.

The experimental value was obtained by allowing solutions to stand at room temperature for a few hours (at the end of which they could be expected to have appreciable quantities of dimer but not much higher polymer), and then running them in the ultracentrifuge. The dimer sedimentation constant then was determined readily and plotted as $1/S_{20,w}$ *vs.* the total protein concentration. An example of the photographs is shown in Fig. 1, and the plot of the data is given in Fig. 2. As

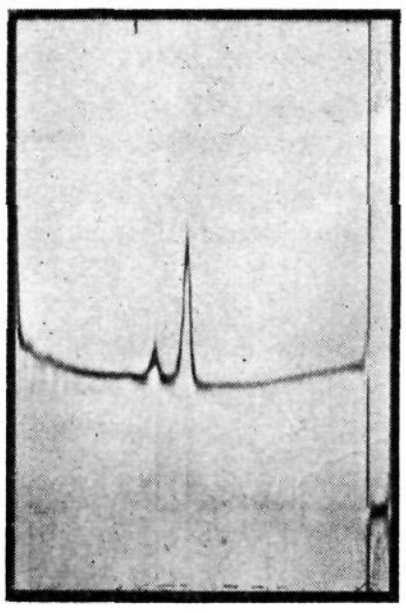


Fig. 1.—Sample MXVI, showing dimer and monomer peaks. Total protein concentration, 0.292% in 0.6 *M* KCl, *p*H 6.3, 25°. Speed, 59,780; bar angle, 50°; 77 min. after reaching top speed.

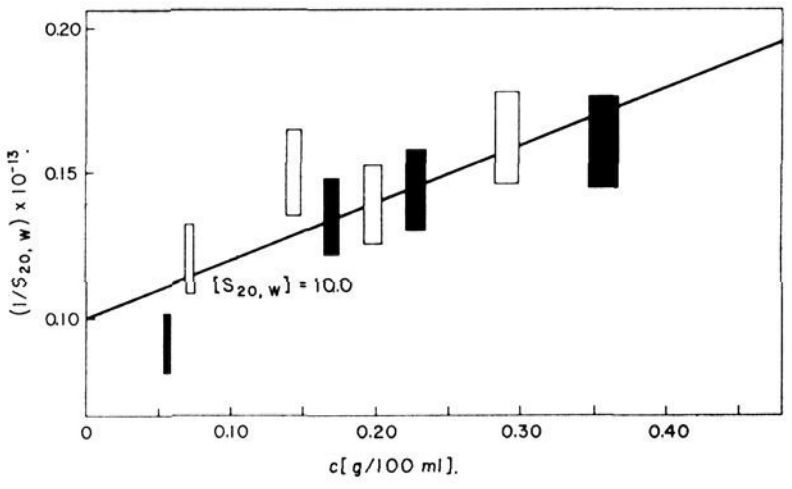


Fig. 2.—Reciprocal sedimentation constant of dimer in 0.6 *M* KCl at 25°, as a function of concentration of total protein; unfilled rectangles, MVIII; filled rectangles, MXVI.

might be expected, the reproducibility here was considerably less than is obtained for a pure sample, and correspondingly larger error estimates have been made. The data can be represented by the (least squares) line

$$(1/S_{20,w}) \times 10^{-13} = 0.100 + 0.197C$$

The experimental sedimentation constant, 10.0 *S*, is seen to be in satisfactory accord with the expected value, and the agreement is strong evidence for the step-wise picture of the process.

In an attempt to verify these conclusions further, some viscosity measurements were made. Theory insists that the intrinsic viscosity be an increasing function of axial ratio. Consequently the intrinsic viscosity of solutions containing some side-to-side aggregates should be smaller than the monomer viscosity.

We therefore left some myosin stock at room temperature until the analytical ultracentrifuge showed that a considerable amount of dimer had formed. This stock solution then was clarified in the preparative centrifuge (0.5 hr. at 15,000 r.p.m., 4°), and the upper 3/4 of the solution was pipetted out, diluted and measured in the viscometer. The results are shown as the upper line on Fig. 3 and yield an intrinsic viscosity of 3.1 dl./g., as compared to 2.2 dl./g. for the monomer!

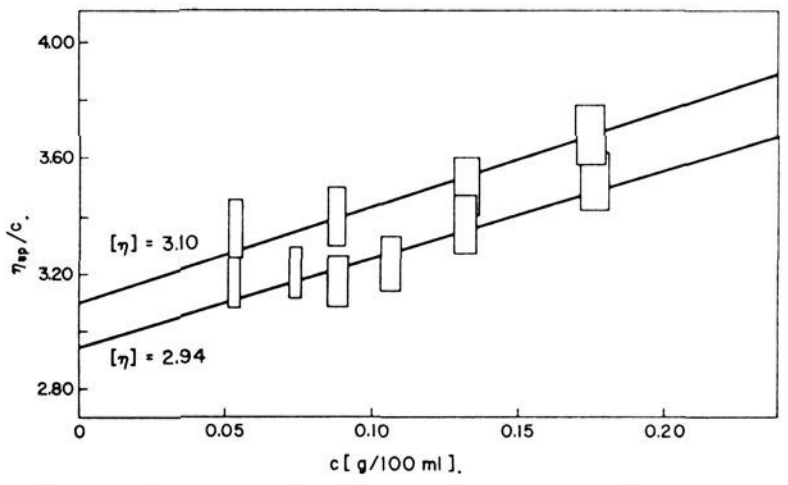


Fig. 3.—Concentration dependence of reduced specific viscosity for aggregated myosin in 0.6 *M* KCl at 25°. Lower curve shows data when solutions are re-centrifuged (see text).

Instead of a decrease in viscosity we find a rise of about 40%. In view of the high molecular weight of this aggregated sample, the viscosity must be admitted to be somewhat insensitive to the aggregation, but the increase is definitely significant. Nevertheless, considering the unequivocal evidence from sedimentation and light scattering cited above, one must examine the alternative explanation, that perhaps really large aggregates were being formed by the shear in the viscometer in these already highly aggregated samples. This would lead to an anomalous rise in viscosity. Some evidence for this latter view was noted in that measurements on aggregated samples were substantially less reproducible.

Accordingly, we re-centrifuged the previous solution ($[\eta] = 3.1$) for 1 hr. and remeasured the viscosity and concentration. The value obtained this time (Fig. 3) was 2.9 dl./g., representing a substantial decrease but still 30% over monomer. In all probability, then, the viscosity experiments merely form and measure higher aggregates and are therefore not relevant. The convection-free

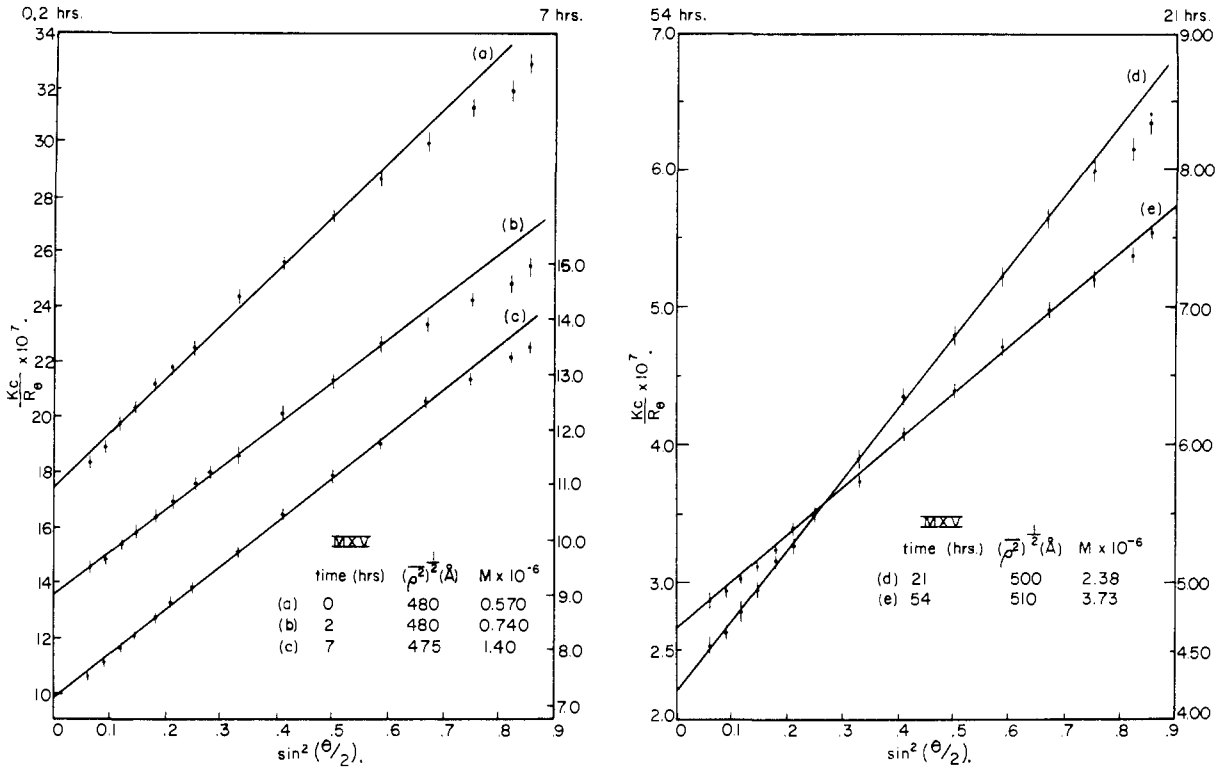


Fig. 4.—Reciprocal scattering envelopes for MXV in 0.6 M KCl as a function of time of incubation at 25°. Note differing ordinate scales at left and right of each graph.

analytical ultracentrifuge (in which one can "pick out" the dimer peak), and light scattering, in which long preparative centrifugations are carried out at low concentrations, would be expected to be more reliable, and these point unambiguously to the step-wise mechanism. Indeed, even in monomeric solutions, exposure to shear frequently results in aggregation and often to the appearance of visible fibers.

In order to check further the side-to-side nature of aggregation, the scattering of a sample at room temperature was followed up to a molecular weight of almost four million (Fig. 4). In spite of the eightfold increase in weight average molecular weight the radius of gyration remains constant. Even the small increase indicated is well within experimental error, as a brief examination, ruler in hand, will convince the reader. Since the measured light scattering radius in a polydisperse system of rod-like particles is a higher average than the weight average, we must conclude that the rods join almost exactly side-to-side.

The increase in molecular weight with time at 25° follows the course shown in the upper curve of Fig. 5. The value rises rapidly, then more slowly. However, it should be emphasized that no upper limit is attained. In fact, at molecular weights substantially above 4×10^6 a sufficient quantity of larger, visible aggregates appears so that the collection of meaningful scattering data would require re-centrifugation. This, however, would merely provide data on those aggregates not centrifuged down and would tell little about the nature of the process. In other words, the protein begins to precipitate, as careful visual observation in the light beam readily reveals. The variation of M_w with time, while quite reproducible for the same protein preparation, varies measurably from preparation to preparation, for unknown reasons. The general course followed is always the same, however.

The effect of protein concentration on the rate of aggregation also was measured. Because of the nature of the reaction, we anticipated a strong dependence on the concentration at which the protein was incubated. The results (Fig. 6), on the contrary, indicate no dependence on concentration. The weight average molecular weight attained in a given time is independent of the protein concentration. This point will be discussed further on.

The Effect of Additives.—In our study of additives no substance was found to be capable of reversing the aggregation to any measurable extent. In all cases, therefore, we focus now on the effectiveness of the added substance in inhibiting the aggregation.

The reagents that must be chosen for testing depend on the hypothesis made concerning the chemical group responsible for the aggregation. The three most likely kinds of bonding were thought to be: (1) metal chelation, (2) disulfide links, (3) hydrogen bonds. Of these, the latter would be extremely difficult to test, since all the common hydrogen bond breaking agents are known to have irreversible effects on myosin, quite apart from any effect they may have on the aggregates. Our studies were therefore confined to the first two hypotheses and, anticipating the results, neither was proven to be involved. In fact, no additives tried showed results that could not be explained in terms of simple, rather non-specific,

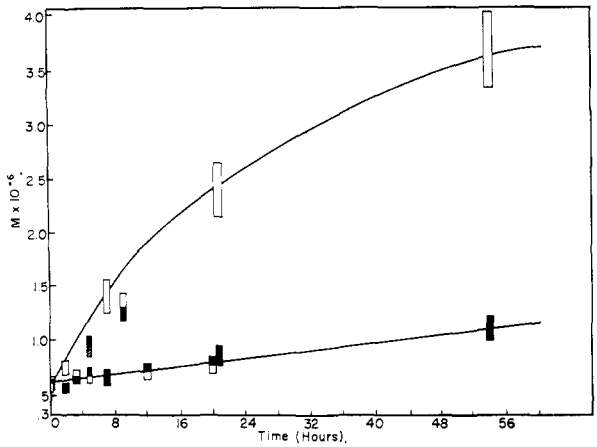


Fig. 5.—Weight average molecular weight of myosin as a function of time of incubation at 25°: unfilled rectangles, MXV in 0.6 *M* KCl, *p*H 6.3; filled rectangles, MXV in 0.1 *M* phosphate-KCl, *p*H 6.7; top filled, bottom lined, MXVI, 0.6 *M* KCl, *p*H 6.3; top filled, bottom unfilled, MXVI, 0.1 *M* phosphate-KCl, *p*H 7.8; top unfilled, bottom filled, MXVII, 0.6 *M* KCl, *p*H 6.3.

charge effects. Nevertheless, the data may be of interest in other connections.

The Disulfide Hypothesis.—This hypothesis at first appeared to be the most likely one. The formation of intermolecular S–S links has been amply demonstrated in other proteins. Air oxidation, for example, readily could cause this effect. To test this idea we tried two reducing agents, 0.001 *M* thioglycolate and a sulfite mixture (0.031 *M* Na_2SO_3 and 0.014 *M* $NaHSO_3$). The latter has been found to be almost 100% effective in eliminating dimerization in solutions of mercaptalbumin or urease.[6] However, neither reagent inhibited the myosin aggregation to an appreciable extent.

In the hope of blocking the –SH groups we tried an excess of $HgCl_2$ (0.001 and 0.01 *M*). This was entirely unsuccessful, it increased the aggregation, and a broad, polydisperse peak was observed in the ultracentrifuge. The addition of an organic mercurial, CH_3–Hg–Br, to myosin only resulted in precipitation of the protein.

We may tentatively infer that disulfide bonds are not involved, although the possibility exists that the aggregation induced by the presence of the mercury is a separate reaction obscuring any blocking effect and that the reducing agents used were simply not powerful enough.

The Metal Chelate Hypothesis.—The relevant additives chosen here were ATP (10^{-4} *M*), Versene (0.1 *M*) and $MgCl_2$ (10^{-3} *M*). These were added (to the final concentrations noted) to 0.2% myosin solutions in 0.6 *M* KCl, and allowed to stand, with a control, at 25° for 4 hr. At the end of that time ultracentrifuge runs were made. Solutions waiting to be run were kept in the refrigerator. The control showed appreciable dimer formation, while the Versene and ATP stabilized the protein slightly (Fig. 7A,B). The small preserving action of ATP is lost after longer incubation. This undoubtedly is caused by the enzymatic destruction of ATP by the

(6) S. Epstein, personal communication.

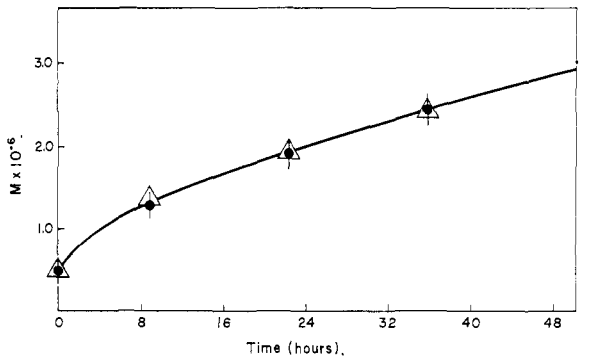


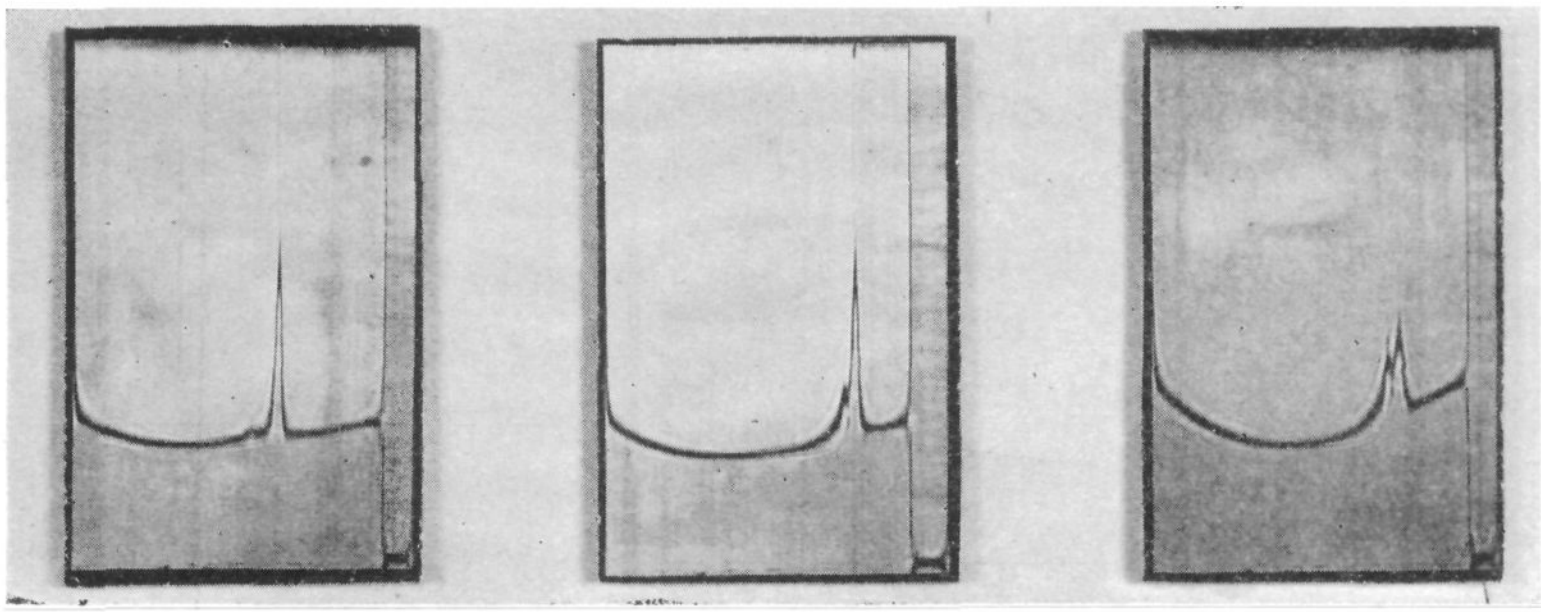
Fig. 6.—Weight average molecular weight of myosin MXX in 0.6 *M* KCl as a function of time of incubation at 25° and protein concentration; circles, 0.0217%; triangles, 0.0495%.

myosin. Addition of $MgCl_2$, on the other hand, increased the aggregation to where trimers were visible, although none appeared in the control.

Superficially, perhaps, these results might suggest that this hypothesis is correct, but closer inspection reveals that such is not the case. It is possible that the small effect of Versene and ATP is caused by the general binding of these anions by myosin; the resulting increase in negative charge would then simply increase, in a non-specific way, the repulsion between two protein molecules and thus inhibit the aggregation. Three tests were made of this latter hypothesis. First, the salt concentration was increased to 1.5 *M* to swamp out electrostatic effects. This resulted (Fig. 7C) in an enhancement of the aggregation rate, arguing strongly that the inhibition previously observed was non-specific. Further, the preservative action of simple phosphate (0.3 and 0.1 *M*), was investigated (Fig. 8A,B), and it also was somewhat effective. In fact, phosphate ions turned out to be the most effective inhibitor we encountered. A comparison of the curves of Fig. 5 shows clearly the magnitude of the effect. It should be emphasized that the phosphate concentrations used were far larger than the ATP concentration (10^{-4} *M*) in the previous experiments. However, a comparison of these observations with the well known effects of the two reagents on Myosin B is striking.

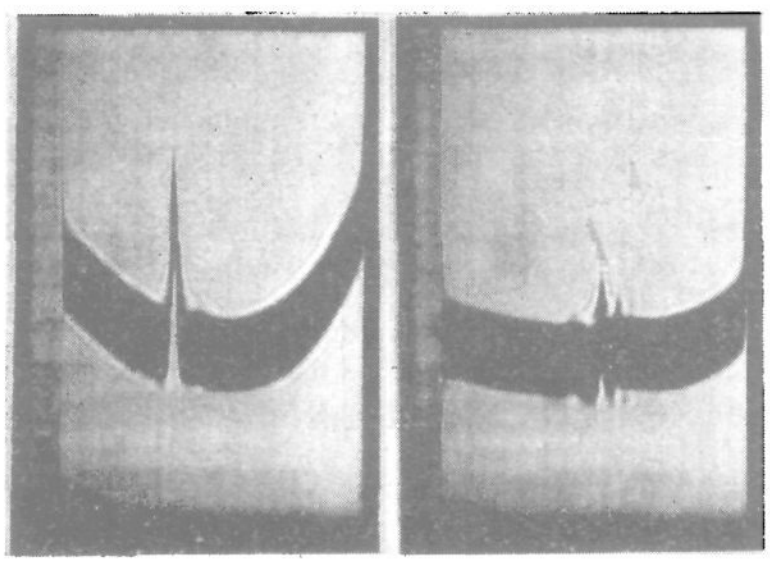
A final test was made to decide whether the observed extra aggregation in the presence of 0.001 *M* Mg^{++} was of the same type as the spontaneous aggregation or merely a separate chelating reaction commonly observed between divalent ions and proteins or polymers. The experiment here involved adding both ATP *and* Mg^{++}. In this case the solution, after 4 hr., showed an amount of dimer equal to that of the control. It may be inferred that the ATP complexes the Mg^{++} and this cuts out the *extra* Mg^{++}-chelated aggregation but leaves the same old spontaneous aggregation going on undisturbed. We may conclude therefore that the Mg^{++} induced aggregation is probably a separate and irrelevant reaction and that all the effects observed here are a result of non-specific alterations in the electrostatic charge carried by the protein.

The nature of the myosin–myosin bond is, therefore, still unknown. It may involve strictly van der Waals attraction.



a b c

Fig. 7.—Aggregation of MXIII in various media after 4 hr. at 25°: (a) 0.6 *M* KCl, 10^{-4} *M* ATP, 48 min.; (b) 0.6 *M* KCl, 24 min.; (c) 1.5 *M* KCl, 36 min. In all cases protein concentration was 0.200%; *p*H 6.3; speed, 59,780 r.p.m.; and bar angle, 50°.



a b

Fig. 8.—Aggregation of MXIII in various media: (a) 0.3 *M* phosphate + KCl, ionic strength 0.6; (b) 0.6 *M* KCl, speed 59,780 r.p.m., and bar angle 50° in both cases.

Discussion

The Nature of Myosin B.—Since it recently has been proposed[5] that Myosin B is, in reality, a polymer of myosin (Myosin A), the properties of our aggregates should bear some relationship to the properties of Myosin B. In point of fact, it will be seen, there is no clear relationship, and it is our feeling that the lack of correspondence between the properties of Myosin B and of aggregates formed from pure myosin, is strong evidence against the hypothesis that the protein obtained by long extraction (Myosin B) is simply an aggregate of the protein obtained by short extraction (myosin). In other words, some other substance (actin) must be present in long-extracted solutions.

To make the comparison we first summarize some of the relevant conclusions of Morales and coworkers on this question. They conclude that solutions of Myosin B contain these several classes of particles, all of which are fundamentally myosin, or aggregates of myosin: (1) very large aggregates, which remain associated, but extend in the presence of ATP, (2) smaller aggregates which dissociate to monomeric myosin in the presence of ATP, (3) myosin monomer.

They found that high *p*H or dilution also dissociated the class (2) aggregates, although dilution resulted in a slower approach to the final value. We may presume, from the earlier literature,[7] that high ionic strength (2 *M*) would lead to results analogous to those obtained on addition of ATP.

In complete disagreement with these findings, we see from the above that aggregates that form from pure myosin monomers are almost entirely unaffected by ATP, high *p*H or high ionic strength. Addition of ATP, base or 2 *M* KCl fails completely to dissociate the aggregates and results in no alteration of the radius of gyration. In fact, ATP is not even very effective in *inhibiting* the aggregation, except in a non-specific manner. The irrelevance of the small inhibitory action of ATP is conclusively demonstrated by the fact that the most effective inhibitor known, inorganic phosphate, does not dissociate Myosin B at all.

In addition, we find that the *rate* of aggregation is independent of protein concentration, and the process shows no evidence of reaching a stable equilibrium but continues until precipitation.

Finally, we observe a constant radius of gyration of about 500 Å. up to molecular weights of near 4 million. In contrast, solutions of Myosin B of comparable molecular weights have radii of about 1200 Å.

Reconciliation of the data reported in this investigation with the conclusions of Morales and co-workers would require that the intermolecular bond that forms between myosin molecules in solution be chemically distinct from that which forms on long extraction of the muscle; and that up to molecular weights of at least four million, the former process results in entirely side-to-side, but the latter in some end-to-end association. Furthermore, since the changes induced in Myosin B by these additives are reversible, one would have to assume that something is present in Myosin B

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

solutions that causes myosin molecules to reaggregate in a manner totally distinct from the way in which they combine in Myosin A solutions.

We would infer that it is far more likely that actin is a separate and relevant protein present in Myosin B solutions and that it is the interaction of actin and myosin that gives Myosin B its distinctive properties. It remains a fact, however, that actin never has been identified clearly as a dissociation product in Myosin B solutions. Nevertheless, it is clear from the above that the assumption that only myosin–myosin polymers are present is an inadequate explanation of the properties of Myosin B solutions.

The Concentration Independence of the Rate of Aggregation.—We have noted that the rate of association does not depend on the concentration. We wish to present here a rather speculative explanation for this peculiar feature.

We propose the following as a mechanism for the reaction. Suppose that a given myosin molecule cannot react until it has undergone some small, local, internal rearrangement which makes the relevent chemical group available for reaction. Let us suppose also that once this rearrangement has occurred the ensuing dimerization is rapid; *i.e.*, we propose the mechanism

$$\mathrm{P} \longrightarrow \mathrm{P}^*$$

$$2\mathrm{P}^* \longrightarrow \mathrm{P}_2 \text{ (rapid)}$$

To analyze this further, we confine ourselves to the early stages of the reaction, when the dimer concentration is small and there is effectively no trimer.

Note that, since each molecule undergoes the postulated rearrangement independently and dimerizes immediately thereafter, the number of monomers disappearing in time dt is simply proportional to the number of monomers present. We therefore have the first-order equation

$$\frac{dN_1}{dt} = -kN_1 \qquad (1)$$

where N_1 is the number of monomer molecules present at time t. Expressing (1) in terms of f_1, the weight fraction of monomers, we obtain

$$\frac{AW}{M_1}\frac{df_1}{dt} = -k\frac{AW}{M_1}f_1 \qquad (2)$$

where M_1 is the molecular weight of monomer, A is Avogadro's number, and W the total weight of protein present.

Since the property measured is the weight average molecular weight, we may write

$$M_w = M_1(2 - f_1) \qquad (3)$$

recognizing that $M_2 = 2M_1$ = dimer molecular weight, and $f_2 = 1 - f_1$ = weight fraction of dimers. From (3) we find

$$\frac{df_1}{dt} = \frac{-1}{M_1}\frac{dM_w}{dt} \qquad (4)$$

Inserting (3) and (4) into (2) we obtain

$$\frac{dM_w}{dt} = kM_1\left(2 - \frac{M_w}{M_1}\right) \qquad (5)$$

which gives explicitly the time rate of change of the measured property.

Rearranging (5) and integrating from time zero to time t we have

$$\int_{M_1}^{M_w}\frac{dM_w}{2M_1 - M_w} = \int_0^t k\,dt \qquad (6)$$

which is

$$\ln\left[\frac{M_1}{2M_1 - M_w}\right] = kt \qquad (7)$$

Equation 7 shows M_w to be independent of concentration and should give the dependence of M_w on time in the early stages of the reaction, before the appearance of appreciable trimer.

If we apply equation 7 to the early part of the experimental data of Fig. 6, we obtain for the rate constant of the rearrangement

$$k = 1.8 \times 10^{-5}\ \text{sec.}^{-1}$$

which corresponds to the half-life: $t_{1/2} = 0.693/k = 3.9 \times 10^4$ seconds, or about 11 hours. This implies that in 11 hours half of all monomers present are "ready" to aggregate and, since the aggregation step is presumed rapid, have already done so. The production of higher aggregates, which is appreciable in times as long as this, complicates the issue. However, it should still be true that, after 11 hr., half of the monomers have disappeared. Insofar as one can judge the areas under sedimentation patterns in this system, this is in accord with the facts.

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