

# THE ISOLATION OF MYXOMYOSIN, AN ATP- SENSITIVE PROTEIN FROM THE PLASMODIUM OF A MYXOMYCETE\*

BY PAUL O.P. TS'O, LUTHER EGGMAN, AND JEROME VINOGRAD

(From the Kerckhoff Laboratories of Biology and the Gates and Crellin Laboratories of Chemistry, † California Institute of Technology, Pasadena)

(Received for publication, October 3, 1955)

## INTRODUCTION

The plasmodium of the slime mold, *Physarum polycephalum*, has been extensively used as a model organism in studies of protoplasmic streaming (1). Recently both streaming in the living plasmodium and the viscosity of suitably prepared plasmodial extracts have been shown to respond specifically to additions of ATP. The plasmodia exhibit increased streaming (2), whereas the extracts become less viscous (2, 3). The change in viscosity is transient; during the recovery phase ATP is destroyed and inorganic phosphate released. This cycle may be repeated several times as with actomyosin (15, 16).

The previously reported separation of the cytoplasmic extract into active and inactive fractions by ammonium sulfate precipitation (2) suggested that further effort might yield active concentrates suitable for studying the mechanism of the ATP-induced changes in viscosity, and the relation of this reaction to the mechanism of protoplasmic streaming. The present paper deals with the method of isolation of the active material and some of its physical and chemical characteristics.

## EXPERIMENTAL

### *Materials*

*Physarum polycephalum* was cultured and harvested by a modification (2) of a procedure described by Loewy (3). The dipotassium salt of adenosine triphosphate (ATP) obtained from Pabst Co. was used without further purification. Solutions were neutralized to pH 7.0 before use. All other chemicals were reagent grade.

### *Methods*

Protein concentration was determined by the biuret method described by Cornell, Bardawill, and David (4), and by precipitation by trichloroacetic acid (TCA) (2).

\* Report of work supported in part by a grant from the National Institutes of Health, United States Public Health Service.

† Contribution No. 2030.

Total phosphate was determined by a modification of the method of Allen (5) after digestion with perchloric acid. The method of Wyatt (6) was used to hydrolyze, chromatograph, and identify the nucleotide constituents of ribonucleic acid.

Moving boundary electrophoresis patterns were obtained with the Perkin Elmer Model 38 apparatus and the 2 ml. cell assembly. Prior to electrophoresis, samples were dialyzed with stirring for at least 10 hours. Buffer and solution conductances agreed to within 1 per cent.

Ultracentrifuge runs were performed in the direct electric drive apparatus constructed at the California Institute of Technology and described briefly by Singer and Campbell (7). The synthetic boundary cell described by Schachman and Harrington (8) was used. Centrifuge runs were performed at temperatures between 20 and 25°, constant to within 0.1°.

Preparative centrifugations were carried out in a No. 40 rotor with a Spinco model L ultracentrifuge. Chamber and rotor were refrigerated before and during use. Viscosities were measured at 24.5°C. in modified Ostwald viscometers with flow times of about 60 seconds and maximum shear gradients of about 1200 sec.<sup>-1</sup> for water. Kinetic energy corrections in these measurements were small and were neglected.

#### RESULTS

The procedure developed for concentrating the active material employed successive fractionation by ammonium sulfate and differential centrifugation. The preparative work from the beginning was complicated by difficulties in defining and measuring activity; *i.e.*, the rheological response to ATP. This activity is normally expressed as the drop in reduced viscosity caused by addition of an amount of ATP sufficient to produce a maximum effect (2). In any preparation the magnitude of the response depends upon the concentration of protein, the rate of shear, and in some upon the mechanical and thermal treatment of the sample prior to measurement (2). It was necessary therefore to consider a variety of rheological factors for each preparation before quantitative significance was attached to the viscosity results. These have been used semiquantitatively as a guide in the development of the fractionation procedure, and only similar kinds of preparations have been compared directly.

##### *Preparation of an ATP-Sensitive Concentrate*

In the preparative procedure four fractionations of the initial extract were made. In each step the response to ATP and the relative amount of the active component defined by electrophoresis and ultracentrifugation increased. The entire procedure was carried out at 0 to 3°.

I. 500 gm. of frozen plasmodia<sup>1</sup> were ground in a mortar and extracted for an hour with 700 ml. of 1.4 M KCl. The slurry was freed of particulate matter by centrifugation for 20 minutes at 14,000 *g* in a Sorval SS-1 centrifuge. Coalesced lipid material was removed by filtration through a glass wool pad

<sup>1</sup> *Cf.* reference 2 for methods of harvest and storage of the plasmodia.

and centrifugation repeated. The resulting crude extract, solution I, contained about 1.5 per cent trichloroacetic acid (TCA) precipitable material, and is yellow and opalescent.

II. The crude extract was fractionated with ammonium sulfate at pH 7.0. The fraction precipitated between 30 and 40 per cent saturation was recovered by low speed centrifugation and redissolved in 75 ml. of 0.2  $\mu$  potassium maleate to give solution II with a final volume of about 140 ml. and a concentration of 1.5 per cent TCA-precipitable material.

III. The solution from II was refractionated with ammonium sulfate at pH 7. The product precipitated between 25 and 36 per cent saturation was washed once with 36 per cent saturated ammonium sulfate (SAS) and redissolved in 80 ml. of 0.05  $\mu$  potassium maleate buffer, pH 7.0, to give 110 ml. of solution III. This solution contained about 1 per cent TCA-precipitable material.

IV. Solution III was clarified by centrifugation at 35,000 R.P.M. for 30 minutes and dialyzed against 0.05  $\mu$  buffer for 6 hours to remove ammonium sulfate. The dialyzed solution was diluted with buffer to contain 0.5 per cent TCA-precipitable material, and was centrifuged for 2.5 hours at 40,000 R.P.M. The pellets were taken up in 100 ml. of 0.05  $\mu$  buffer. Resolution of these gelatinous pellets was aided by maceration and by stirring with a slow speed magnetic stirrer for 10 hours. The solution, freed of suspended material by centrifugation at 30,000 R.P.M. for 12 minutes, was adjusted to a concentration of 0.5 per cent TCA-precipitable solids to give *ca.* 90 ml. of solution IV.

V. The differential centrifugation procedure described in IV was essentially repeated except that stirring time for resolution of the pellets in 20 ml. of buffer was reduced to 8 hours. After dialysis against 0.2  $\mu$  potassium maleate and clarification at 30,000 R.P.M. for 12 minutes, the solution contained 200 to 250 mg. of TCA solids in approximately 20 ml., and was retained as solution V.

The viscosity of the initial KCl extracts must be raised by orthophosphate or AMP (2) before it can be affected in a typical fashion by ATP. Once the viscosity has been raised, however, the reduced viscosity of a 1 per cent solution drops about 25 ml./gm. upon addition of 3  $\mu$ M of ATP/ml. The electrophoretic patterns of solution I, inactive as a result of dialysis against 0.1  $\mu$  maleate buffer, are given in Fig. 1 *a*.

In step II the fractions precipitated between 0 to 32 per cent SAS and the supernatant solution after 40 per cent saturation were inactive. Together these two fractions constituted 80 per cent of the TCA-precipitable dry weight in solution I. Solution II, in contrast to solution I, was active without added AMP or orthophosphate, and could be dialyzed against 0.2  $\mu$  potassium maleate without loss of activity (2). The electrophoretic pattern of solution II is given in Fig. 1 *b*.

These solutions are opalescent. A clear solution may be obtained by centrifugation at 30,000 R.P.M. for 30 minutes. This centrifugation results in the removal of a sharp immobile electrophoretic boundary (*cf.* Figs. 1 *b* and 1 *c*) and an increase in the activity. For instance, in a typical clarification experiment the change in reduced viscosity caused by 3  $\mu$ M of ATP/ml. increased from 39 to 47 ml./gm. even though the concentration decreased from 17.0 to 14.0 mg./ml.

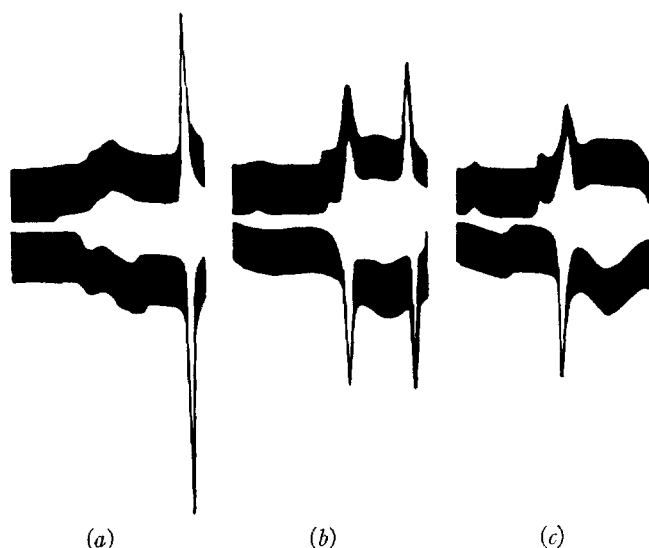


FIG. 1. Electrophoretic patterns of plasmodial extracts at pH 7.0. Migration proceeds from right to left. Upper patterns are from ascending limb. (a) Crude extract, 0.1  $\mu$  potassium maleate, 6 ma., 5500 sec., 1.5 per cent. (b) Solution II, 0.1  $\mu$  potassium maleate, 0.1 M KCl, 9 ma., 9500 seconds, 1.2 per cent. (c) Solution II clarified 30 minutes at 30,000 R.P.M. 0.1  $\mu$  potassium maleate, 0.1 M KCl, 9 ma., 9000 sec., 1.1 per cent.

In step III, the second fractionation with ammonium sulfate, little or no material precipitated below 25 per cent saturation. The material remaining after precipitation at 36 per cent SAS could be removed at 50 per cent SAS, but was entirely inactive. As shown in Table I, both the reduced viscosity and the response to ATP were enhanced in step III.

During experiments to clarify solution III, it was noticed that if the solution was centrifuged for 3 hours at 30,000 R.P.M., a clear pellet formed in the bottom of tube, and the supernatant solution contained regions of differing color and protein concentration. Analyses of samples from the centrifuge tube showed that activity concentrated in the pellet and in the lower part of the tube (Table II). From this it was evident that differential centrifugation procedures could

be used advantageously to concentrate the active material further, and that it might be possible to identify the active material in the ultracentrifuge. Electrophoresis and ultracentrifuge patterns for the three solutions obtained by incomplete sedimentation of solution III are shown in Fig. 2.

TABLE I  
*Fractionation of Cytoplasmic Extracts of Physarum by Ammonium Sulfate and by Differential Centrifugation*

Material	Concentration	Reduced viscosity*	ATP response‡
	<i>mg./ml.</i>	<i>ml./gm.</i>	<i>ml./gm.</i>
Solution II§	18.4	136.7	-35.2
Solution III§	18.2	171.5	-53.2
Solution V§	5.5	330.5	-113.5

\* Velocity gradient *ca.* 1200 sec.<sup>-1</sup>.

‡ Change in reduced viscosity caused by addition of 3  $\mu$ M ATP/ml.

§ *Cf.* text.

TABLE II  
*Distribution of ATP-Response in Centrifuge Tube after Sedimentation\* of a Salt-Fractionated Cytoplasmic Extract‡*

Solution	Concentration	Reduced viscosity	ATP response§
	<i>mg./ml.</i>	<i>ml./gm.</i>	<i>ml./gm.</i>
Top 2 ml.	3.5	35	-4
Bottom 2 ml.	4.3	110	-11
Pellet fraction	4.0	187	-55

\* 3 hours, 30,000 R.P.M.

‡ Solution III, clarified by preliminary centrifugation, 30 minutes, 30,000 R.P.M.

§ Change in reduced viscosity caused by addition of 3  $\mu$ M ATP/ml.

|| Solutions and redissolved pellets were dialyzed against buffer, 0.1 M KCl and 0.1  $\mu$  potassium maleate, prior to measurement.

In the ultracentrifuge, the redissolved pellet and the bottom fraction resolved into two boundaries having uncorrected sedimentation constants of 24 S and 3 to 4 S. The top fraction contained only the slow components. Since this top fraction was inactive, it was concluded that the slow, 3 to 4 S, components are also inactive. In addition, the activity appears to increase as the relative amount of the fast component increases, as shown by a comparison of the ultracentrifuge patterns of the bottom fraction and the redissolved pellet.

The electrophoretic patterns are more complex. Without now identifying the active materials, it can be stated that the slow moving substances in electrophoresis are inactive. The relative area of the broad zone originating at the

starting boundary and ending with materials having a mobility of about  $3 \times 10^{-5} \text{ cm.}^2 \text{ volt}^{-1} \text{ sec.}^{-1}$  decreased as the activity of the samples was increased by sedimentation. Thus the slow components in both electrophoresis and in the ultracentrifuge (these may or may not be the same materials) are

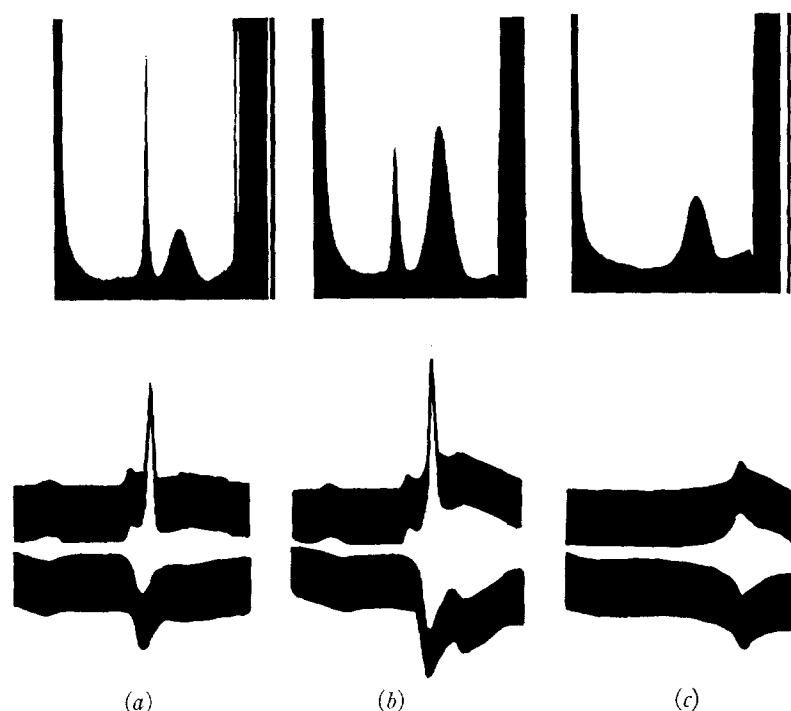


FIG. 2. Analyses of liquid layers from 10 ml. centrifuge tube after incomplete sedimentation of solution III. Migration proceeds from right to left. Upper centrifuge patterns obtained after 20 to 30 minutes at 850 R.P.S. Upper electrophoretic patterns from ascending limb. All solutions at pH 6.6 and in  $0.1 \mu$  potassium maleate and  $0.1 \text{ KCl}$ . Electrophoretic patterns at 9000 seconds and 9 ma. (a) Redissolved pellet, 0.4 per cent. (b) Bottom 2 ml., 0.86 per cent. (c) Top 2 ml., 0.35 per cent.

inactive. Further attempts to purify the active material were based upon removal of these components.

The enrichment of the fast component by differential centrifugation in steps IV and V is shown in the ultracentrifuge diagrams of Fig. 3. These diagrams also indicate that the conditions adopted for the differential sedimentation were adequate for the complete removal of the fast component and that the ratio of fast to slow components increased in the twice cycled product.

The solvent was changed to  $0.05 \mu$  maleate buffer prior to the differential centrifugation in step IV in order to redissolve the gelatinous pellet. In  $1.0 \text{ M}$

KCl the pellet was refractory, only 10 per cent going into solution. Approximately 25 per cent of the pellet redissolved in  $0.2 \mu$  maleate buffer; 75 per cent of the pellet was soluble in  $0.05 \mu$  maleate. If the ionic strength was reduced to  $0.01 \mu$ , all activity was lost. In step V, 85 per cent of the pellet redissolved in  $0.05 \mu$  buffer.

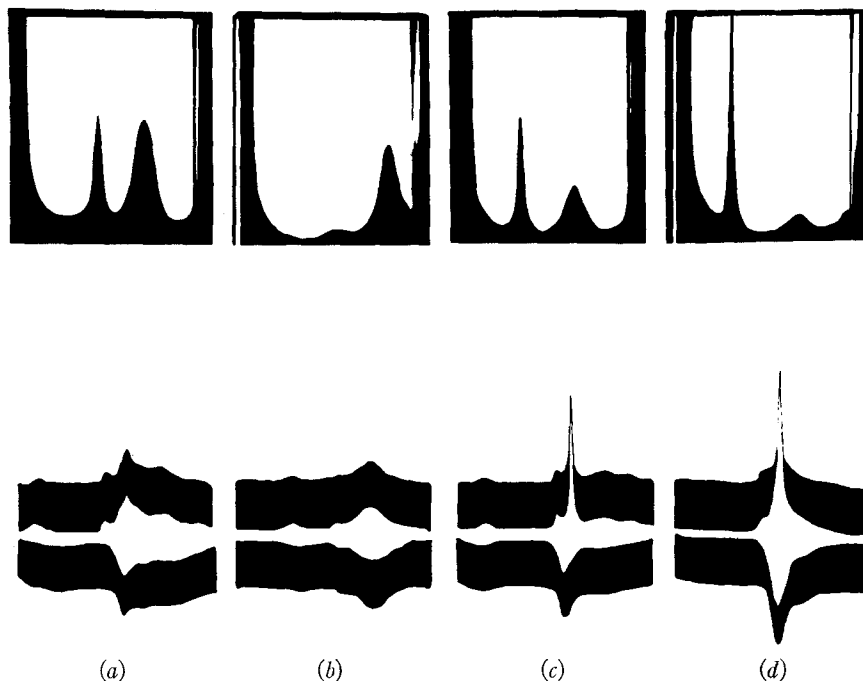


FIG. 3. Purification of myxomyosin by differential centrifugation. Migration proceeds from right to left. Upper centrifuge patterns obtained after 20 to 30 minutes at 850 R.P.S. Upper electrophoretic patterns from ascending limb. All solutions at pH 7.0, and in  $0.2 \mu$  potassium maleate. (a) Solution III, 9 ma., 6000 seconds, 0.59 per cent. (b) Supernatant solution from first differential centrifugation, 9 ma., 6000 seconds, 0.31 per cent. (c) Solution IV, 9 ma., 9,000 seconds 0.50 per cent. (d) Solution V, 6 ma., 10,000 seconds, 0.55 per cent.

Since it had been noted that the sedimentation rate of the fast sedimenting material decreased rapidly with concentration, the differential centrifugation steps were always performed at the same concentration, 0.5 per cent. At a concentration of 1.0 per cent in the centrifugation time employed in step V, all the active material was not removed from the supernatant solution.

Electrophoresis diagrams in Fig. 3 indicate that the relative amount of

slow material was reduced from 35 per cent in solution IV to 20 per cent in solution V.

*Composition and Properties of Solution V*

Typical electrophoretic and ultracentrifugal analyses of solution V preparations are illustrated in Fig. 3. All other solution V preparations have given substantially similar patterns. The activity in these solutions was high, and, as shown in Table I, has been substantially increased by the differential centrifugations. The viscosities in the three solutions listed in Table I were measured at similar levels of viscosity, and in a range of velocity gradients in which the dependence of viscosity on gradient is small.

TABLE III  
*Mobilities\* of Electrophoretic Boundaries in Myxomyosin Preparations*

Boundary		Solution†		
		III‡	IV	V
Ascending	A	14.5	14.1	14.7
	B	7.9	8.3	8.2
	C	6.9	6.5	7.0
Descending	A	13.6	13.5	13.9
	B + C	6.8	7.0	6.9

\*  $\text{Cm.}^2 \text{ volt}^{-1} \text{ sec.}^{-1} \times 10^5$ .

† See text for designation of solutions.

‡ In pH 7.0 buffer 0.1 M KCl and 0.1  $\mu$  potassium maleate.

|| In pH 7.0 buffer 0.2  $\mu$  potassium maleate.

The electrophoretic mobilities of the observed boundaries, except for the diffuse one starting at the initial boundary, are given in Table III for several preparations. The boundaries are identified in order of decreasing mobility as A, B, C, and D, in the ascending limb and A, B + C, and D, in the descending limb. The mobility of A corresponds to that measured for ATP,  $15.3 \times 10^{-5} \text{ cm.}^2 \text{ volt}^{-1} \text{ sec.}^{-1}$ , and for polynucleotides (9), suggesting the presence of nucleic acid.

In Table IV are summarized the relative concentrations of the components obtained by electrophoretic and ultracentrifugal analyses. The ultracentrifuge results have been corrected for radial dilution and Johnston-Ogston effects. The latter effect, 5 to 9 per cent, was calculated by the method of Trautman *et al.* (10). The same result is obtained with the Johnston-Ogston equation (11) if it is assumed that the sedimentation constant of the slow components in the mixture is 3.5 S. Results for all the solutions show that the rapidly sedimenting material and material under the B and C electrophoretic boundaries constitute 70 to 75 per cent of the preparation.



Biuret analyses of solution V were consistently 10 to 15 per cent lower than the TCA-precipitable dry weight, indicating that these preparations are largely but not exclusively protein.<sup>2</sup>

Approximately 0.8 to 1.0 per cent of the dry weight is TCA-precipitable phosphorus in the form of ribonucleic acid. This was shown by the ultraviolet spectrum and by chromatographic identification of the usual four RNA nucleotides in hydrolysates of a dried pellet obtained from solution IV. The ultraviolet absorption spectrum of solution V preparations showed a small

TABLE IV  
*Analyses of Solution V by Electrophoresis and Ultracentrifugation*

Ultracentrifuge analyses						
Component	Preparation					
	1	2	3			
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>			
Fast	70	73	77			
Slow	30	27	23			

Electrophoretic analyses						
Boundary	Limb					
	Ascending			Descending		
	Preparation			Preparation		
	1	2	3	1	2	3
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
B	6	9	6	—	—	—
C	66	67	65	—	—	—
B + C	—	—	—	80	76	76
A	3	4	5	5	4	5
D	24	20	23	15	20	19

minimum at *ca.* 245  $m\mu$ , the expected sharp maximum at 260  $m\mu$ , a minimum at 305 to 310  $m\mu$ , and a broad secondary maximum centered at *ca.* 350  $m\mu$ .

Solution V preparations are always yellow in color. The responsible pigment, as yet unidentified, travels with the rapidly sedimenting component. The pigment was recovered from solution V after precipitating the protein with acetone and concentrating the supernatant solution. In 0.01 M KCl it

<sup>2</sup> X-ray diffraction patterns of a poorly oriented fiber from the pellets, which are dissolved to give solution V, gave spacings of  $3.25 \pm 0.2$  A,  $4.5 \pm 0.1$  A, and  $10.5 \pm 1.0$  A. These spacings, similar to those of  $\alpha$ - and  $\beta$ -myosin patterns (12, 13), are additional evidence for the proteinaceous nature of the active material. Cf. Doctoral Thesis, P. O.P. Ts'o, California Institute of Technology, 1956.

fluoresces yellow when irradiated at 360  $m\mu$ . Its ultraviolet spectrum is nearly identical with that of solution V preparations in the region from 230 to 285  $m\mu$  except that the maximum is shifted slightly to 265  $m\mu$ .<sup>3</sup> At 350  $m\mu$  a secondary maximum occurs.

While solution V is much more stable than the starting material, some activity is lost during storage for 1 week at 0°. The ultracentrifuge diagram of the stored material changed in 1 week, the relative area of the fast boundary declining from 75 to 50 per cent with a corresponding increase in the slow boundary in two separate preparations. Storage in the frozen state at -10° for 1 week does not affect either the ultracentrifuge diagram or the activity. All work reported on solution V, however, was performed within 1 to 3 days of preparation.

#### DISCUSSION

The plasmodial extract and the successive concentrates contain a material that makes these solutions viscous and reacts specifically with ATP to lower the viscosity. This material is called *myxomyosin*, because of its origin and its similarity to actomyosin (15, 16) in ATP response.

Two types of experiments show that myxomyosin migrates in electrophoresis and upon centrifugation with characteristic rates, and that the activity of a given preparation depends upon the relative concentration of material migrating with these rates. The data for the successive concentrates (Figs. 1, 2, and 3, and Tables I, III, and IV) show that an increase in activity accompanies an increased relative concentration in the fast sedimenting and intermediate electrophoretic boundary. That the enhancement both of activity and of relative concentration in the boundaries is caused by the accumulation of the same material, myxomyosin, is supported by the second type of experiment in which liquid layers from a partially sedimented preparation, (solution III) were examined. These data, presented above, show that the slower migrating materials are inactive, and that activity corresponds to the relative amount of the fast moving material.

If we consider the viscosity of the solutions instead of the activity, the pattern of data and argument above may be repeated. The increased viscosity in the successive preparations is caused by the accumulation of myxomyosin. This conclusion is further supported by the pronounced dependence of the rate of sedimentation of the fast sedimenting material upon the concentration, a result which shows this material to have a large frictional coefficient and to be likely to form viscous solutions.

Viscosity and activity in these solutions are linked together by direct measurements, which, however, are complicated by the factors of shear dependence, concentration, and in some cases thermal and mechanical history. They are

<sup>3</sup> Since this pigment did not become red at pH 1, it cannot be identical with that described by Seifriz and Zetzmann (14).

linked also by common correlation with the relative concentrations in the characteristic boundaries. While we are not concerned here with the mechanism of the ATP effect, we observe that the viscosity of solutions made viscous by myxomyosin will be especially sensitive to intermolecular interaction of myxomyosin and to any agent, *e.g.*, ATP, mediating the interaction.

The results in Table IV show solution V to contain approximately 75 per cent of its electrophoretic and ultracentrifugal composition in single boundaries. These results alone mean that at least one-half of the resolvable material in solution V has a common identity and migrates in the above boundaries. If we recall, however, the results of Fig. 2, which show that the slowly sedimenting material also migrates slowly in an electric field, the limit of one-half can be revised to three-quarters for the part of solution V that moves in one boundary in the ultracentrifuge and in electrophoresis.

Myxomyosin has been isolated in combination with RNA, as have some other cytoplasmic proteins. It is not yet known whether the complexing of these constituents is an artifact of preparation or exists in the cell (17). Since RNA has a sedimentation constant of 3 to 4 S, its presence in solution V after two cycles of differential centrifugation indicates that it is at least in part bound to and sediments with the fast sedimenting component. The RNA-protein complex appears to dissociate slowly, for during the preparation of solution V, the amount of RNA in the pellet is decreased relative to that in the supernatant solution. As judged by phosphorus analyses, the amount of RNA contained in the TCA solids from solutions IV and V decreased from 10 to 9 per cent, respectively, while that in the supernatant from solution IV increased to 15 per cent. Electrophoretically, the RNA is complexed with material which constitutes the B + C boundary (75 per cent) in the descending limb and the B and C boundaries (10 and 65 per cent, respectively) in the ascending limb. Similar splitting of a protein-nucleic acid boundary in the ascending limb has been observed by Goldwasser and Putnam (9) in their electrophoretic studies of protein-nucleic acid interaction.

Efforts to remove the remaining slowly sedimenting and apparently inert material from solution V by further differential centrifugation have failed. The resulting solutions were no more active than solution V and the concentration of the fast material was not increased. Apparently at this stage of purification the degradative changes which are occurring counterbalance the improved fractionation expected from a third differential centrifugation.

#### SUMMARY

1. A procedure has been developed for the preparation of an active concentrate from the myxomycete, *Physarum polycephalum*. This concentrate responds with a lowered viscosity to the addition of small amounts of ATP. The preparation recovers in viscosity, and the process may be repeated.
2. In the most active concentrates, 75 per cent of the non-dialyzable mate-

rial moves as a single boundary both in the descending limb in electrophoresis and in the ultracentrifuge. It contains about 10 per cent ribonucleic acid, which is at least in part reversibly bound to the protein.

3. The active material has been designated myxomyosin because of its origin and its similarity to actomyosin in ATP response.

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