STUDIES

FROM THE INSTITUTE OF MEDICAL CHEMISTRY UNIVERSITY SZEGED

VOL II.

(1942)

EDITED BY

A. SZENT-GYÖRGYI

(Sent to press on Dec. the 22nd 1942)

S. KARGER

BASEL, NEW YORK.

SZEGED VÁROSI NYOMDA RT. Felelős nyomdavezető : Klenóczky József

Actin.

by

F. B. Straub.

It has been shown by *Banga* and *Szent-Györgyi*[1] that myosin can be extracted from rabbit's muscle in two different forms.

Myosin A is obtained by extracting the muscle tissue with three volumes of *Weber*'s solution* for 20 minutes in the cold and centrifuged immediately thereafter. The solution of myosin A obtained in this way is viscous and threads may be prepared from it. Neither the viscosity of the solution, nor the threads prepared from it, show any significant change on adding adenyltriphosphate (ATP).

If the muscle is extracted in a similar way for 20 minutes with the same solution, but left to stand for 24 hours in the cold and centrifuged only thereafter, a turbid and very viscous solution is obtained. On addition of ATP the viscosity of such a myosin B solution is decreased to a great extent. Threads prepared from myosin B show a vigorous contraction on addition of ATP and in presence of definite amounts of salts such as KCl and $MgCl_2$.

There was another difference between the two myosin modifications. Whereas both of them would split adenyltriphosphate with the appearence of free posphate, this enzyme action was increased by Mg ions in the case of myosin B, and not increased but rather inhibited in the case of myosin A.

The problem of understanding the difference between these two modifications of myosin was taken up by studying

* This solution contains 0,6 M KCl, 0,04 M $NaHCO_3$ and 0,01 M Na_2CO_3. In previous communications from this Institute (Studies from the Institute of Medical Chemistry, Szeged, vol *1)* this solution was referred to as *„Edsall*'s solution". Since then it was brought to our attention that this solution was first used by *Weber* and *Meyer*[2].

the factors bringing about the transformation of myosin A into myosin B. These investigations led to the discovery of a new protein present in the muscle stroma. The name *actin* was given to this protein. In combination with myosin it gives the contractile protein of the muscle. As shown later in this paper, myosin B is formed if a certain amount of myosin A and actin are mixed. It follows that myosin A is what was termed by earlier investigators as myosin, myosin B on the other hand is a mixture of a definite amount of actin and myosin.

The ability of a myosin preparation to react with a decrease of viscosity on addition of ATP, was termed by *Banga* and *Szent-Györgyi* the „activity" of the respective myosin. It will be shown in this paper that apart from myosin B other mixtures of myosin and actin can be prepared, which show varying degrees of activity. Myosin B is only one of the possible combinations, its significance being only that if the muscle is extracted in the way described above, myosin B vill be invariably extracted. But muscle does not contain myosin B, instead it contains an actin-myosin complex with a higher activity than myosin B. We therefore think it advisable to modify the nomenclature put forward by *Szent-Györgyi*[3] in such a way that *actomyosin* is generally a mixture or compound of actin and myosin, there being many possible actomyosins. One of them is myosin B.

Methods.

The viscosimeters used in this work had the following measurements: capillary diameter 0,060 cm, length of capillary 210 mm, diameter of the cylindrical reservoir tube 1,65 cm, amount of outflowing fluid 1,2—1,7 ml. The viscosimeters have been placed in an icebath, which was vigorously stirred. The time of outflow of the solution was referred to the time of outflow of the solvent. No correction was taken for the change of specific weight by the presence of the proteins, as the protein content was maximally 3 mg/ml. All measurements have been performed using a buffered KCl solution of pH 7 as solvent. (Its composition see in the following paper of *Balenovič* and *Straub.)* 4 ml of myosin solution were placed in the viscosimeter. ATP was added in the form of its K salt, 0,1 ml of a 1,4% solution were added to 4 ml solution.

Determination of actin in solution.

Any specific property of actin which is in any way proportional to its quantity, may be utilized for its quantitative determination. The combination of actin with myosin to form an actomyosin is such a specific property. The activity of the resulting actomyosin is the higher, the more actin is added to the myosin. It remains to define the measure of the activity of actomyosin. This is complicated by the fact that the decrease of viscosity on addition of ATP depends not only on the actin content but also on the viscosity of the actomyosin. This is cleary brought out in Fig. 1. It shows the decrease of the specific viscosity ($\Delta\eta_{sp}$) on addition of ATP as the function of the specific viscosity in presence of ATP (η_{ATP}). The curve is valid for myosin B and it was constructed from the data of *Balenovič* and *Straub* (Fig. 1. of the following paper).

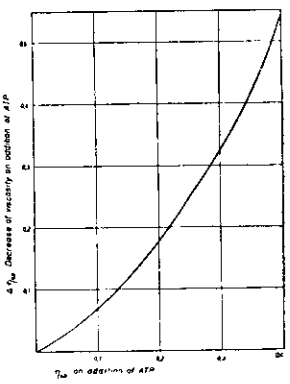

Fig. 1.

The activity of an unknown myosin solution is defined as the relation of its $\Delta\eta_{sp}$ to the $\Delta\eta_{sp}$ of a myosin B solution having the same η_{ATP} value.

As example let us take a myosin solution, which has the specific viscosity $(\eta) = 0{,}625$, in presence of ATP $(\eta_{ATP}) = 0{,}35$. Fig. 1. shows that in case of myosin B to this value of η_{ATP} corresponds a $\Delta\eta_{sp}$ of 0,41. After the above definition the activity is

$$\frac{0{,}625 - 0{,}35}{0{,}41} \cdot 100 = 67\,\%$$

To a myosin A solution,* which contains 6 mg of myosin, and has no activity (no decrease of viscosity on addition of ATP) different amounts of an actin solution are added, the solution is made up to 6 ml so as to have 0,6 M KCl concentration and pH 7, and the activity of the resulting mixture is

* All experiments described in this paper have been performed using crude myosin A solution, obtained by extracting the muscle tissue with the *Weber* solution.

determined in every case. The results are plotted against the amount of actin as in Fig. 2. From the curve it is easy to obtain the amount of actin which is necessary to transform the 6 mg of myosin present in the experiment into myosin B (100% activity). From the purest actin so far obtained 1 mg protein is needed to transform 6 mg of myosin into a 100% active myosin. The actin content of an unknown actin solution can be therefore evaluated if we determine the amount of the solution necessary to activate 6 mg myosin from 0 to 100%. In this way the element of arbitrariness introduced by the arbitrary measure of activity, is again eliminated.

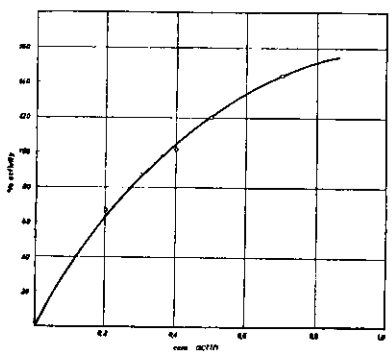

Fig. 2.

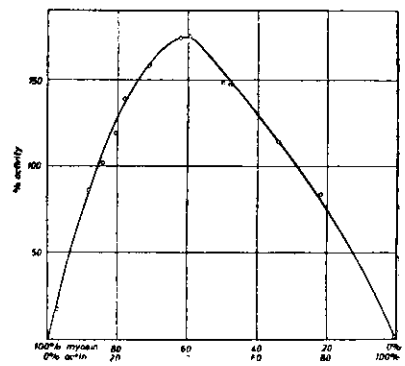

Fig. 3.

There are good reasons to believe that the actin preparations, of which 1 mg activates 6 mg myosin to a 100% active actomyosin, represent the pure actin. This means that myosin B is a compound of 6 mg myosin and 1 mg actin.

That myosin B is a compound of myosin and actin, is supported by the following experiment, in which actin was prepared from myosin B. In the usual way a myosin B was obtained, dissolved in *Weber*'s solution. It was diluted with 5 volumes of distilled water and centrifuged. The precipitate was then treated first with 4 and then with one volume of acetone and left to dry at room temperature. By extracting the dried myosin with distilled water a viscous solution of actin is obtained, which does not contain any myosin. $^1/_4$-$^1/_3$ of the estimated actin contents of myosin B can be extracted in this way.

It is seen from Fig. 2. that on addition of more actin than necessary to bring about 100% activation, actomyosins may be

obtained with more than 100% activity. Fig. 3. shows the extension of these studies. It is seen that addition of more and more actin, i. e. increasing the ratio actin: myosin, results first in an increase of activity and later in a decrease of it. Finally, actin alone like myosin alone does not show any change on addition of ATP.

The existence of actomyosins more active than myosin B is rather important. It will be described in the following paper that muscle contains an actomyosin with 170% activity. It is seen from Fig. 3. that this is the maximal activity to be achieved at pH 7.

Preparation of actin.

Rabbit's muscle from the legs and of the back are minced in a cooled Latapie mincer. To every 100 g are added 300 ml of *Weber*'s solution and stirred mechanically for 20 minutes at 0°. The mixture is centrifuged and the supernatant solution discarded. The residue is left to stand in the cold room for one or two days. It is then stirred up with 5 times its volume of distilled water and centrifuged again. The solution is again discarded and the residue mixed with 4 volumes of acetone. After 10 minutes the acetone is sucked off and replaced by a fresh lot of 1 volume of acetone. After 10 minutes this is sucked off again and the residue is spread over a filter paper and left to dry at room temperature over night.

The acetone dried muscle is extracted with 20 volumes of distilled water. The extraction can be carried out in either of the two following ways:

a) The dried muscle is mixed with the distilled water and then left to stand in itself without stirring for 4 hours at room temperature.

b) The dried muscle is ground with the distilled water in a mechanical mortar. In about $^1/_2$—1 hour, depending on the amount to be extracted, the mixture will become a rather rigid mass of foam. Further grinding is then inadvisable as the actin is denatured when present in the foam. During the subsequent centrifugation the foam is reduced to a viscous liquid.

After the extraction the undissolved muscle particles are centrifuged off; the resulting solution contains the actin. There

is no difference in the purity of the actin obtained by either of these procedures. But whereas by the thorough grinding nearly all of the actin is extracted, by procedure a) only about $^1/_6$-$^1/_4$ of the estimated actin of the muscle can be obtained in solution. On the other hand, actin obtained by a) is a clear solution, that obtained by b) is a stable milky suspension. Usually these solutions contain 3—4 mg protein per ml and the purity is such that 1,5—2 mg activate 6 mg myosin to 100%. From these solutions of the actin further purification can be achieved by precipitating it isoelectrically or by Ca ions.

The actin solution, obtained by procedure a) is diluted 8 times by distilled water and then an acetate buffer of pH 4,8 is added. The buffer concentration will be 0,01 M. The precipitate is centrifuged off at room temperature and dissolved by the addition of bicarbonate to neutral reaction.

Actin can be precipitated by adding $CaCl_2$ solution to the actin solution. The amount of Ca necessary to precipitate the actin however depends largely on the concentration of univalent ions already present. Thus about 0,02 M $CaCl_2$ is needed to precipitate the actin of the first extract (which still contains some of the KCl of the *Weber*'s solution), but 0,002 M or less is sufficient to give complete precipitation from isoelectrically purified actin solutions, which are poor in salt.

The Ca-precipitate of actin is inactive. Its activity will however return if KCl is added to the solution. At the same time it is observed that the Ca precipitate will form a stable suspension if KCl is added to it. To ensure this dissolution and reactivation, 40—50 times as much K^+ ion should be added as there was Ca^{++} ion present.

Obviously, the facts recorded here, can be described as a Ca : K antagonism. As the action of Ca is very pronounced and is observed with small, physiological concentrations, moreover the ratio Ca : K, where the effect of Ca is abolished is close to the physiological values, further, considering that actin is a part of the contractile element of the muscle, we may suppose that these observations are related to the Ca : K antagonism in muscle physiology. Further work is planned along this line.

It should be mentioned here that not only Ca^{++}, but also Mg^{++}, Mn^{++} or Sr^{++} ions can precipitate the actin in the

same way. On the other hand the effect of Ca^{++} can be overcome by Na^{+} ions just as well as by K^{+}.

It appears that the colloidal state of actin does not materially influence its activity. It was mentioned before that a clear solution obtained by extraction a) activates myosin to the same extent as the suspension obtained by extraction b), if both have an equal protein content. For this reason we prefer to prepare actin by the first method and such preparations have been used throughout this study. If the clear actin solution is treated with acid to precipitate it at pH 5—6, when dissolved at ph 7 the actin does not form a clear solution any more, but is more or less cloudy. When precipitated with Ca and redissolved with KCl, it is always a suspension.

The isoelectric precipitation results in removing 10—15% of the protein and the activity of the preparation is raised sometimes by more than 50%. Whether this is due to the removal of inhibitors or some other factor could not be decided. An actin, purified through the Ca-salt, has the maximal activity and all of its protein is precipitated at pH 6 or by new addition of Ca.

As other purification procedures, like precipitation with alcohol or salting out with KCl did not lead to further purification and because all of the protein was precipitated by minimal Ca concentrations, we think that such preparations are homogeneous and do not contain any significant amount of impurities.

Actin is easily destroyed by heat over 50°. It is stable in a narrow pH zone between pH 7—7,5 but is rapidly destroyed even at 0° by more acid and alkaline reactions. It is precipitated from the solution at pH 6. Dialysis at pH 7 does not diminish its activity. From the solution it can be precipitated by cold alcohol but it is completely destroyed if treated with cold acetone. (This is the more interesting as it is very resistent to acetone when still in the muscle tissue.) If precipitated without loss of activity, the precipitate is voluminous. After centrifuging for 20 minutes at 3000 r. p. m. it still contains more than 99,5% of water.

Thixotropy. The most remarkable property of actin solutions is their very strong thixotropy. A dilute solution of neutral actin containing as little as 3—5 mg protein per ml sets

to a gel if left alone at 0° for a few hours. A slight shaking however breaks up the gel immediately. As common with thixotropic gels, the viscosity of actin solutions cannot be determined with accuracy. So much can be said only that it is roughly the same as that of myosin. The time of outflow is determined largely by the shearing forces present in the capillary viscosimeter. For this reason successive determinations of the time of outflow show strongly decreasing values. If the solution is left to stand for a while before the next determination, again a higher value of viscosity will be found. A quantitative study of these phenomena is planned in a system, in which the pressure can be taken into consideration.

Strictly speaking it would be better to use the term resistance (Strömungswiederstand) instead of viscosity. The resistance of a solution results from the true viscosity, which can be measured at high shearing forces and from the elasticity of the solution. That indeed elasticity plays an important part is shown by the fact that the viscosity increases if the time of outflow is longer, i. e. the shearing forces are smaller. The resistance of actin solutions decreases assymptotically with higher shearing forces. It is interesting to point out two parallelisms between actin and thixotropic inorganic sols. One is the permanent double refraction to be dealt with later. The other is the great influence, which inorganic salts exercise on the resistance (apparent viscosity) of actin solutions. It is known from the experiment of *Freundlich*[4] and the theoretical treatment by *Szegvári*[5] that the elasticity but not the viscosity is influenced by the salt concentration.

Not only the viscosity of actin, but also the viscosity of actomyosin is influenced by the strong thixotropy of actin. Whereas myosin A solutions show no variation in viscosity during successive determinations, myosin B shows already such an effect, a second run in the viscosimeter gives always lower value than the first one. This results in some ambiguity in the determination of viscosities. The more active an actomyosin is, the less reliable is the determination of viscosity. Therefore we have accepted a certain routine, which would give comparable results. The actin solution was kept at 0° for at least 1 hour prior to use, then it was gently shaken up and the desired amount mixed with the myosin, the mixture imme-

diately put into the icebath and its viscosity determined 5 minutes later. Immediately thereafter two more readings were taken. After the addition of the ATP, two more determinations were made. For the calculation of the activity the first readings were always used. The others served only to see the extent of the thixotropic effect. The solutions are to some extent thixotropic even after the addition of ATP.

Whereas the difficulties caused by the thixotropic effect make the viscosity values uncertain, the determination of activity by this routine procedure can be carried out with satisfying reproducibility, except for actomyosins containing more actin than myosin.

A thixotropic effect with salt free myosin has been observed already by *Muralt* and *Edsall*.[6] It is clear that these authors dealt with a myosin A preparation, that is an actomyosin of a few per cent activity. This is shown by the very small dependence of the viscosity of their solutions on the shear rate. *Freundlich*[7] has suggested that the interior of the muscle cell is a thixotropic gel, as evidenced by the observation of *Kühne*.[8] Knowing that the myosin in the muscle fibril is not the myosin studied by *Muralt* and *Edsall*, but an actomyosin of about 170% activity, which is a rather strongly thixotropic substance, this assumption is now experimentally supported.

Thixotropic behaviour is an expression of certain properties of actomyosin, which might be of considerable significance in understanding the architecture and the mechanical properties of muscle.

If we assume that the most active actin, described in this paper, is the pure actin, it was calculated (see *Balenović* and *Straub*) that 1 gramm of muscle contains 25—30 mg actin. It is impossible to make such a concentrated solution of actin, which requires a very close packing. Actin reveals by its thixotropy and permanent double refraction (see later) very strong intermolecular forces. At the close packing present in muscle, these intermolecular forces must be very strong and must play an important rôle in determining the mechanical properties of muscle.

High viscosity and thixotropy are two phenomena each pointing to the fact that actin has elongated, rod-shaped molecules of very great assymetry. Double refraction of flow is

another typical characteristic of actin solutions, bearing evidence to the same point. The sign of double refraction is identical with that of myosin. Myosin already has a strong double refraction of flow, which appears at very low shearing forces. But its double refraction is far weaker than that of actin solutions. A slight movement of the actin solution gives rise to the appearence of doubly refracting patches, which persist long after the fluid had ceased to move. If there is so much actin in solution that it turns into a thixotropic gel, the double refraction is permanent. It is interesting to remember that according to *Freundlich* and *Schalek*,[4] thixotropy is observed among those inorganic sols (like aged V_2O_5 sols) which show a permanent double refraction.

Of the physicochemical properties touched upon in this paper, viscosity and double refraction of flow show a parallelism with the actin content of the solution.

The process of activation.

If actin is mixed with myosin in solution, an actomyosin will be formed instantaneously. When in contact with muscle, myosin will be activated at 0° only in the course of many hours. It has been pointed out by *Banga* and *Szent-Györgyi*[1] that there is no activation at all, until the ATP of the muscle is split. That the disappearence of ATP is not the only factor involved in determining the rate of activation, is demonstrated by the following experiment. Rabbit's muscle is extracted at 0° several times in succession with *Weber*'s salt solution. By 3—4 extractions, practically all of the myosin is extracted. On adding an ATP-free myosin solution to the residue, which is by now likewise free of ATP, activation of myosin takes again nearly 24 hours at 0°. The rate of activation can be, however, strongly increased by grinding the muscle residue with sand, prior to the incubation with myosin. In this case activation will be complete within several minutes. From these experiments it follows that the rate determining process of the activation in muscle is the extraction of the actin from the muscle residue.

In absence of myosin, the *Weber* solution will extract no actin from the muscle residue. The presence of myosin is

therefore essential for the extraction of actin. In absence of ATP, as shown by *Banga* and *Szent-Györgyi*,[7] neither myosin, nor actin are extracted.

To account for these phenomena, we must assume that the actomyosin present in the muscle, dissociates into its components in presence of ATP and the salts of the *Weber* solution. The myosin is dissolved but the actin is retained by the strong intermolecular forces. After the ATP has disappeared, the myosin already in solution forms a compound again with the actin. By forming this compound, the forces binding actin in its place are overcome and the dissolved myosin dissolves the actin.

The only other way, by which I succeeded to liberate actin is the acetone treatment described above. But even acetone is capable of breaking up the structure only if the latter has been loosened up by a prolonged treatment with the alkaline salt solution.

Actin is not extracted by the usual salt solutions which have been used to study the distribution of muscle proteins. Therefore it is clear that only a fraction of it goes into solution (this gives the few % activity of myosin A), whereas the greater bulk of it remains in the so called muscle stroma. This insoluble protein fraction has been estimated as 15—20% of the muscle proteins. We find (see *Balenovič* and *Straub*) that actin represents about 12—15% of the muscle protein. Making an allowance for some connective tissue and the nucleoproteids of the nuclei there is not much protein left unaccounted. We therefore find that there is no other protein left in the muscle stroma, to which the rôle of a structure-protein could be assigned. The possibility that actin is the main structure-protein, receives strong support by its properties, discussed above.

The formation of the myosin-ATP complex.

It has been shown by *Mommaerts*[9] that about one gramm molecule of ATP are needed to give a maximal effect with 100,000 gramm of myosin. From the experiments of the author[10] on myosin A at acid reaction, it became obvious that ATP is bound to the myosin part of the actomyosin complex. This

is supported also by the fact that myosin is the carrier of the adenyltriphosphatase activity.

On a 100% active actomyosin, prepared from myosin A and actin, I have reinvestigated this problem. As *Mommaerts'* experiments were performed at the alkaline reaction of the *Weber* solution, it was important to know the conditions at physiological pH. The viscosities have been determined at pH 7,0 in a solution containing 0,6 M K ions and veronal acetate buffer. The decrease of viscosity on addition of ATP was

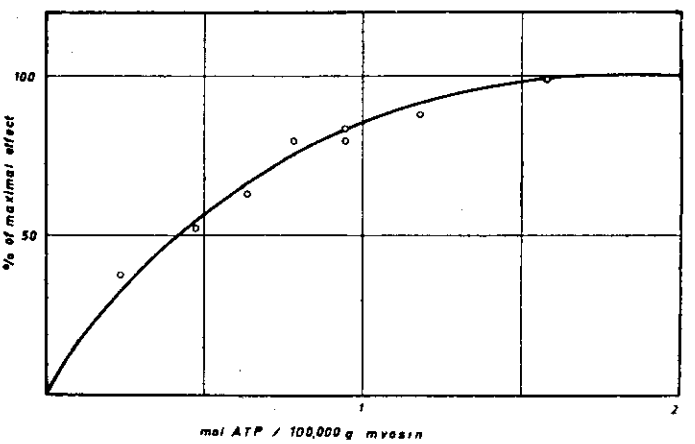

Fig. 4.

measured and the results are expressed as % maximal effect and plotted against the amount of ATP in Fig. 4. 0,1 ml ATP solution was added in each case to the 6 mg of actomyosin present in 4 ml salt solution. From four successive determinations after the addition of ATP, the viscosity of the solution at the moment of adding the ATP was determined by linear extrapolation. It is evident, from the nature of the curve that the bond between myosin and ATP is very strong, less than 2 g molecules of ATP being needed for 100,000 g of myosin B to give a maximal effect. There is some dissociation of the myosin-ATP complex, but the results are essentially in agreement with the assumption that 1 g molecule of ATP reacts with 100,000 weight of myosin.

It is quite convenient to work with 6 mg of myosin in the 4 ml of the solution placed in the viscosimeter. In this case about 0,015 mg ATP would give a 50% effect. In our experi-

ments such an amount of ATP caused the viscosity of the myosin to decrease from 1,83 to 1,61. Such a change in viscosity can be determined with satisfactory precision. Considering the absolute specificity of ATP in decreasing the viscosity of myosin, these facts may be utilized for determining very small amounts of ATP.

Summary.

A method for extracting a new protein, actin, from muscle tissue is described. Actin forms, together with myosin, actomyosin, the contractile protein of muscle. The most conspicuous properties of actin are its high viscosity, thixotropy and strong double refraction, all proving a great molecular assymetry.

The antagonistic effect of Ca and K ions on actin is described.

References:

1. *I. Banga* and *A. Szent-Györgyi,* Studies from the Institute of Medical Chemistry, Szeged, *1,* 5 (1942).

2. *H. H. Weber* and *K. Meyer,* Biochem. Z. *266,* 137 (1933).

3. *A. Szent-Györgyi,* Studies from the Institute of Medical Chemistry, Szeged, *1,* 67 (1942).

4. *H. Freundlich* and *E. Schalek,* Z. physik. Chem., *108,* 153 (1924).

5. *A. Szegvári,* Z .physik. Chem. *108,* 175 (1924).

6. *A. L. v. Muralt* and *J. T. Edsall,* J. biol. Chem., *89,* 315 (1930).

7. *H. Freundlich,* Protoplasma, *2,* 278 (1927).

8. *A. Kühne,* Virchow Arch. path. Anat., *26, 222* (1863).

9. *W. F. H. M. Mommaerts,* Studies from the Institute of Medical Chemistry, Szeged, *1,* 37 (1942).

10. *F. B. Straub,* ibid., *1,* 43 (1942).