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MYOSIN AND MUSCULAR CONTRACTION

BY

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To the memory of

L. KAST,

president of the *Josiah Macy Jr.*

Foundation, *New York.*

Preparation and properties of myosin A and B.

by

I. Banga and A. Szent-Györgyi.

The contractile element of muscle is the muscle fibril and the chief building stone of the muscle fibril is myosin. It is evident, then, that we have to understand myosin if we want to understand contraction.

Myosin is defined, according to J. T. EDSALL (1) as the protein fraction of muscle which is extracted by a pH 8,5—9 mol 0,6 KCl solution and precipitates at pH 7 when the solution is diluted to 0,1 mol KCl.

It will be shown in this paper that myosin can be obtained from muscle in two different forms which we will call myosin A and B. The myosin obtained by EDSALL's method, and generally used by investigators is a solution of myosin A with a small admixture of B.

Material.

We used rabbit's muscle as material and EDSALL's salt solution for the extraction, which we will briefly call „salt solution." This contains 0,6 mol KCl, 0,01 mol Na_2CO_3 and 0,04 mol $NaHCO_3$. Only reagents of high purity were used.

As solvent and for other purposes (dilution, precipitation, etc.) we used distilled water exclusively which was redistilled twice from glass vessels. The importance of this will be evident later from the paper of M. GERENDÁS.

The adenyltriphosphate („ATP") used in this work was prepared in our laboratory as the Ca salt and was of 100% purity. Only in spcial cases, where absolute purity was immaterial, did we use preparations less pure (60%). The ATP was liberated from its Ca salt with HCl, then treated with the calculated amount of K-oxalate. The Ca-oxalate was separated on the centrifuge and the solution neutralised with

KOH. The solution obtained contained the K salt of ATP and about 0,3% KCl.[1]

The extraction of myosin A and B.

The animals head was cut off, its body rapidly skinned, then eviscerated and dipped, for a minute or so, in ice-water. Then the muscles of the hind legs, the belly and the back were quickly cut out, cooled for a short time by packing in ice, and minced in a cooled meat mincer with holes of 2 mm diameter.[2] 40 g of the mince were suspended in 120 ml salt solution of 0°C and stirred energetically for 20 minutes. The suspension was then quickly centrifuged in the cold room (0°C) and the supernatant thin fluid poured off. This contained about 20 mg of myosin per ml. It was stored over night at 0° during which time the adenyltriphosphate (ATP) present was split and the fluid became slightly more viscous. We will briefly call this fluid „the 20 min. extract".

If the suspension, after having been stirred for 20 min., is stored over night at 0° or for 6 hours at room temperature, without having been centrifuged, it turns into a semi-solid gel from which the muscle particles cannot be separated on the centrifuge any more.[3] If, however, a small quantity (0,014%) of ATP is added, the solution liquifies, assuming the appearance of a 20 min. extract and the muscle particles can be separated on the centrifuge. After a few minutes the ATP is split and the fluid gelatinises again. Thus, when the

[1] A preparation of equal properties is brought on the market by „Magyar Gyógyszer RT." Budapest.

[2] Mincing on a finer mincer like LATAPIE's did not improve the extraction.

[3] During the winter 1941—42 this transformation of myosin within the given time took place with absolute regularity. Simultaneously with the beginning of the hot season results began to be less regular and it was found repeatedly that the muscle suspension needed more, sometimes twice as much time for this transformation. The suspensions prepared from the muscle of animals that had been kept for 48 hours in the cold room, showed a tendency to imitate our winter results but the experience was not quite regular and could not always be reproduced.

Different species of animals may behave differently. For example the transformation of myosin in the pike is very rapid, taking place within the 20 min. of stirring.

extract stands in contact with muscle particles, a change takes place characterised by a greatly increased viscosity which can be lowered reversibly by the addition of small amounts of ATP.

This change in the qualities of the extract is not due to an increased myosin concentration since this latter rises only by 5—10% during storage. It is not due to the presence of some highly viscous new substance either but to a change of the qualities of the myosin itself, as can be shown by isolation of the myosin. If the myosin is precipitated by dilution and neutralisation, washed and redissolved in salt solution, it has the same qualities as the extract from which it was prepared.

We will call this highly viscous extract obtained by 24 hours storage of the stirred but uncentrifuged muscle suspension „the 24 h. extract".

Viscosity.

To compare the viscosity of a 20 min. and a 24 h. extract the latter must also be freed from the suspended muscle particles. This can be done on the centrifuge after dilution with salt solution. 80 vol. salt solution to 100 vol. of the suspension mostly suffices to allow centrifugation.

The extracts can be compared by plotting their viscosity against their myosin concentration.

The myosin concentration was determined as follows: 2 ml. of the extract was placed in a weighed centrifuge tube, 10 ml. of water added and the solution neutralized with 5% acetic acid. The myosin precipitate was then separated on the centrifuge, washed twice with 10 ml. of water, the tube dried at 120° C and weighed.

The viscosity was determined in OSTWALD's viscosimeter at 0°C. The relative viscosity is defined as t/t_0, t being the time required for the outflow of the extract, t_0 the time required for the outflow of a pure salt solution. Naturally such a crude salt extract of muscle contains other proteins besides myosin, like myoalbumin and globulin X. These proteins, being globular, have relatively little influence on the viscosity which is governed by the viscosity of the fibrous myosin. That other proteins present do not materially affect viscosity can be demonstrated by purifying the myosin. If this is precipitated, washed and redissolved in salt solution, it still has the same viscosity and gives the same reaction with ATP as the extract.

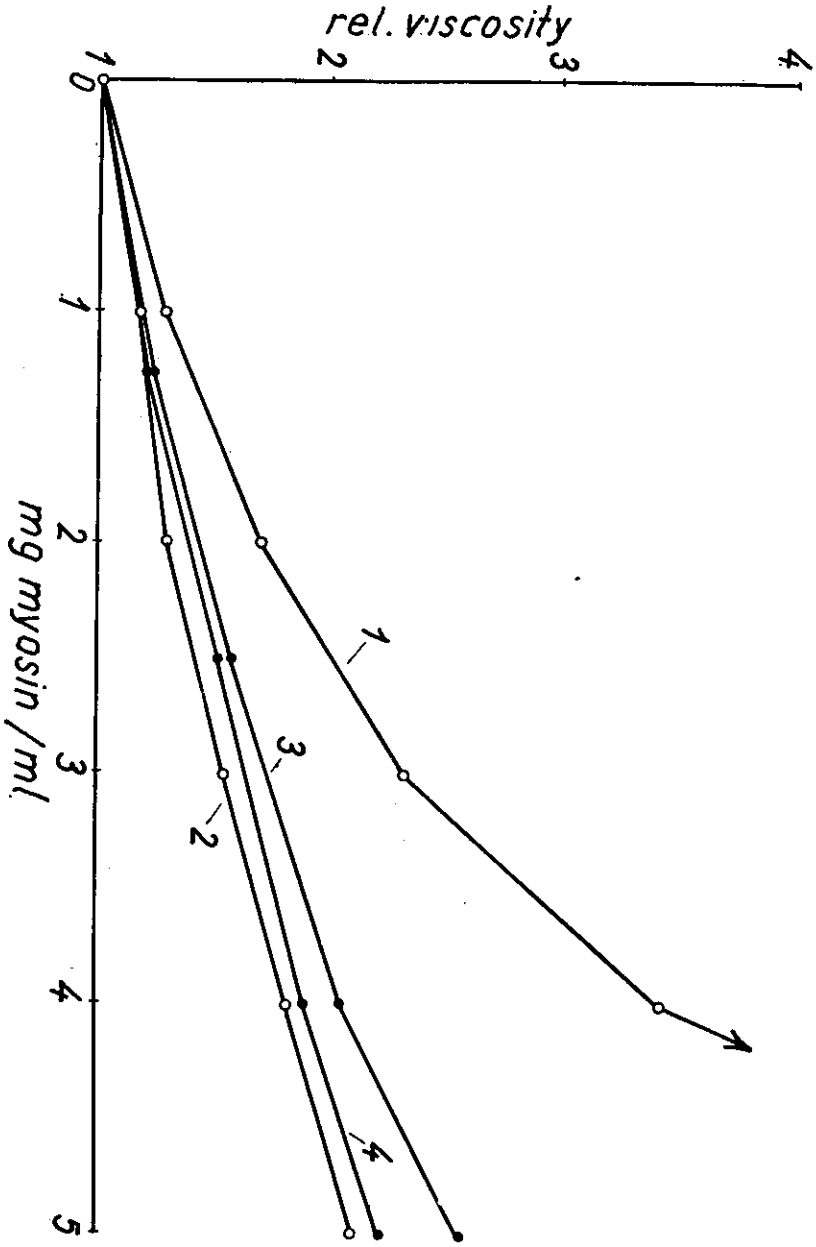

Fig. 1.

In Fig. 1 the relative viscosities of a 20 min. and a 24 h. extract are plotted against the myosin concentration, with and without the additon of ATP. Curve 1 gives the viscosity of a 24 h. extract; curve 2 the same after the addition of 0,014% ATP.; curve 3 gives the viscosity of a 20 min. extract; curve 4 the same after the addition of ATP. It will be seen that the viscosity of the 24 h. extract is much higher than that of the 20 min. extract and is anomalous even at low concentrations. The viscosity of a 20 min. extract is much lower and becomes anomalous only above 0,4%. The viscosity of both extracts falls on addition of ATP to about the same value, the viscosity of the 24 h. extract being somewhat lower. Both ATP curves are up to 0,5% roughly linear. By means of such a curve conclusions can be drawn on the myosin concentration of an extract.

By the „activity" of the myosin we will mean the fall of viscosity on addition of ATP.[4]

The myosin prevailing in the 20 min. extract and characterised by its low viscosity, will be called „myosin A"; the predominant myosin of the 24 h. extract, characterised by its relatively high viscosity and great „activity" will be called „myosin B."

It seemed likely that the low activity of the 20 min. extract is not due to myosin A but to the admixture of smaller quantities of myosin B. This supposition was verified by experiment. It was found that the myosin of the 24 h. extract is retained by the SEITZ K filter up to 90% while the 20 min. extract can be filtered without apreciable loss. Myosin B is thus retained by the filter to a much higher degree than myosin A. If the 20 min. extract is filtered repeatedly its „activity" disappears completely while its viscosity comes down to the level of curve 4. in fig. 1.

We cannot exclude the possibility that just as the 20 min. extract contains some myosin B so the 24 h. extract may contain some A. But in spite of this uncertainty there is no doubt that there is a distinct difference between myosin

[4] The relative viscosity itself also gives some indication of the „activity" but is not a reliable guide for it has been found that on incubation at 37° C the myosin solution becomes more viscous while the fall obtained on addition of ATP remains unchanged.

A and B. The latter is characterised by its higher viscosity. According to physical chemistry increased viscosity must be explained by an aggregation, and thus the lowering effect of ATP, by a disaggregation.. This is supported also by the lesser filterability of myosin B. On addition of ATP the latter also becomes more filterable, about 40% passing the filter.

The higher aggregation of myosin B must be due to increased cohesive forces and we can thus expect myosin B to be less soluble, and in this gel-form more solid than. A. This lesser solubility of B can be noticed at its preparation. While the myosin of the 24 h. extract precipitates already on dilution, the myosin of the 20 min. extract will come down only after the diluted extract has been neutralised.

It is most convenient to study the solubility of myosin on myosin gels, that is, on myosin threads which contain the myosin in the gel-form. Experiment shows that threads prepared from the 20 min. extract are very loose and dissolve even in a neutral 0,6 mol KCl solution. In fact it is quite difficult to obtain well formed threads from this extract. The 24 h. extract gives relatively strong, solid threads which do not dissolve even in mol. KCl or EDSALL's fluid, especially after the threads have been standing for some time and new links have developed between micells.

Turbidity.

It seems desirable to characterise the difference of A and B myosin with as many physical factors as possible. One of the physical factors easily measured, though less easily interpreted, is turbidity. If a 20 min. and a 24 h. extract of equal myosin concentration are compared, the latter will be found to be much more turbid. A small fraction of this turbidity is due to suspended particles but the major part is due to the turbidity of the myosin itself.

The turbidity was measured in the Stupho-nephelometer and the values obtained plotted against the myosin concentration. The 24 h. myosin was found to be 4 times more turbid than the 20 min. myosin.

A similar difference can be observed in a precipitated and redissolved myosin preparation. 0,1—0,5 mg. (per ml)

myosin of the 20 min. extract gives an almost limpid solution while the equally concentrated solution of 24 h. myosin is turbid. Almost limpid 1% solutions of myosin can be obtained if the 20 min. extract is passed through a Seitz K filter, precipitated and redissolved.

Addition of ATP greatly dicreases the turbidity of myosin B though it does not bring it down to the values of myosin A. (see MOMMAERST).

Double refraction of flow (DRF).

As known from EDSALL and v. MURALT's (2) publication, myosin shows a strong double refraction of flow. We have compared the DRF of a 20 min. and 24 h. extract at different dilutions, *i. e.* at different myosin and KCl concentrations. The results are given in table I. The intensity of the DRF is marked with crosses. We used the chamber of GERENDÁS. (3)

Myosin content	Mol. KCl	Double refractiõn of flow without ATP	with 0.5 mg ATP/ml.
	20 min. extract		
8 mg/ccm	0.24	++++	++++
10 mg/ccm	0.30	++++	++++
4 mg/ccm	0.12	++++	++++
2 mg/ccm	0,05	++	+++
1 mg/ccm	0.02	+	++
	24 h. extract.		
4 mg/ccm	0.24	++++	++++
5 mg/ccm	0.30	+	++++
2 mg/ccm	0.12	0	+
0.8 mg/ccm	0.06	0	0
0.4 mg/ccm	0.03	0	0

The table shows that both extracts give a strong DRF but on dilution the DRF of the 24 h. extract is more readily lost, probably owing to the lesser solubility and the precipitation of the myosin. At 0,24 mol KCl the ATP brings the disappearing DRF back. This effect is reversible: if the extract is kept at room temperature the ATP is split in 20—30 minutes and the DRF disappears but can be brought back again by the addition of ATP. Precipitated and redissolved myosin behaves, on the whole, in a similar way.

The results given above are not quite constant. In some

experiments we observed a decreased DRF on addition of ATP in a 0,6 mol KCl solution (20 min. extract.) We also observed a decrease or disappearance of the DRF on addition of ATP at a lower KCl concentration (0,12—0,03 mol. KCl.) in 24 h. myosin solutions.

Miscellaneous observations.

ATP and Extraction. The muscle was minced and suspended in 0,1 mol KCl (2 ml per g. of muscle). After 0,20,40,80 and 160 minutes samples were taken. To these samples a salt solution was added (1 ml per g. of muscle) which contained the salts in a three times higher concentration than the EDSALL solution. The final salt concentration of our samples corresponded thus to Edsall's solution. After 20 min. of stirring at 0°C the samples were centrifuged and the ATP and myosin in the fluid estimated. The sample taken at 0 min. contained 5,4 mg. ATP per g of muscle and 19 mg myosin per ml. That taken at 20 min. contained 3 mg. ATP and 21 mg. myosin. In the 40 min. sample we found only traces of ATP which could not be estimated with accuracy any more and the myosin concentration dropped to 4 mg. per ml. In the 80 min. sample the myosin equalled 2 mg. per ml. and in the 160 min. we found no myosin at all. After 160 min. the original ATP concentration was restored by the addion of this substanc. The extract now contained 10 mg. myosin per ml.

This experiment shows that the ATP present in fresh muscle has a deciding influence on the solubility of myosin and that the dissolution of the myosin of fresh muscle in EDSALL's solution is due to a combined effect of the salts and the ATP present.

The A—B transformation and ATP. Several experiments were performed to learn at what rate the A—B transformation of myosin takes place in muscle suspension and how this rate depends on the concentration of the ATP present. In these experiments the muscle was suspended in 0° salt solution and stirred for twenty minutes. Then a sample was taken, quickly centrifuged at 0° and the liquid poured off. The ATP and myosin were immediately estimated. The

liquid was then stored over night at 0°C and after the ATP had disappeared its „activity" was determined. The muscle suspension itself was kept at different temperatures and samples were taken and treated in the same way at different intervals.

The result of such an experiment is illustrated by fig 2. The muscle suspension was kept at room temperature and samples were taken at 0, 30, 60, 90, 120, 180, 240, 360, 480 min... The abscissa gives the time in hours. The ordinate

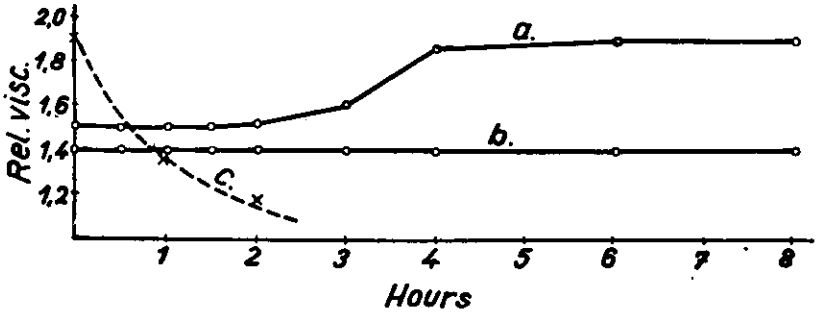

Fig. 2.

gives the relative viscosity. Curve a gives the viscosity without and curvee b the viscosity with 14 mg % ATP. Curve c gives the ATP concentration on a voluntary scale. The highest point of this curve corresponds to 1 mg ATP per ml of the extract. (3.5 mg ATP per g of muscle) For technical reasons the estimation of the last, smallest quantities of ATP is rather doubtful.

In agreement with other experiments this fig. shows that while there is ATP present in appreciable concentration there is no increase in „activity" at all. The activity begins to rise and becomes maximal within a fairly short time after the ATP has sunk to low values or has disappeared altogether.

At 0°C ATP needs about 8 hours to disappear during which time there is no activation. Then only the activation begins.

Phosphorylation of myosin. It has been shown that, in the presence of 0,6 mol KCl, ATP disaggregates myosin B. Later MOMMAERTS will show that 1 molecule of ATP for every molecule of myosin (MW 100.000) suffices

for this action. It could bee thought that ATP acts either as a whole or by transferring its phosphate on to the myosin. Our experiments permit to exclude this latter possibility and there is no transference of one molecule of phosphoric acid per 100,000 g of myosin. We cannot exclude the possibility that one phosphate is transferred to some higher unit.

Such experiments were made possible by the circumstance that the reaction between myosin and ATP is instantaneous and ATP, as shown by MOMMAERTS, need not be present in excess to cause maximal effect.

We added 1,5 mg. ATP to 300 mg. 24 h. myosin dissolved in salt solution *i. e.* one mol. ATP for every 100,000 g myosin. The fluid was thoroughly mixed. 15 seconds after the addition of the ATP the myosin was inactivated by the addition of acetic acid which brought the pH to 4—4,5. At this pH the myosin coagulated and could be separated on a cloth by filtration. Only about 20% of the myosin remained in solution and was precipitated by the addition of trichloroacetic acid. In the control experiment we added the acetic acid first, the ATP afterwards.

We estimated the free phosphate and the ATP in the filtrates (+ free phosphate after 7 min. hydrolysis at 100° in N HCl.)

Out of the four experiments performed we found the ATP quantitatively in the fluid of two while in the two others we found the ATP with the loss of 0,11 and 0,25 mg.

Adenylic acid. If ATP looses its pyrophosphate, adenylic acid is formed and it is of considerable interest to know how this adenylic acid behaves with regard to myosin. Adenylic acid was found to be completely inactive and had no influence on the viscosity of myosin.

Summary.

It has been shown that myosin can be obtained from muscle in two different forms which were called myosin A and B.

Myosin A has, dissolved in Edsall's fluid, a relatively low viscosity which is not influenced by ATP (adenyltriphosphate).

Myosin B has, under the same conditions, a relatively high viscosity which is brought down to the level of the viscosity of myosin A by the addition of small amounts of ATP.

Myosin B is less soluble and gives more solid gels than myosin A and also has a greater turbidity which is descreased by ATP.

The behaviour of myosin under different conditions of extraction has been discussed.

No phosphorylation of the myosin could be detected.

Adenylic acid was found to be inactive.

Literature.

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3. *M. Gerendás.* Enzymologia *9*, 123, 1940.