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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.

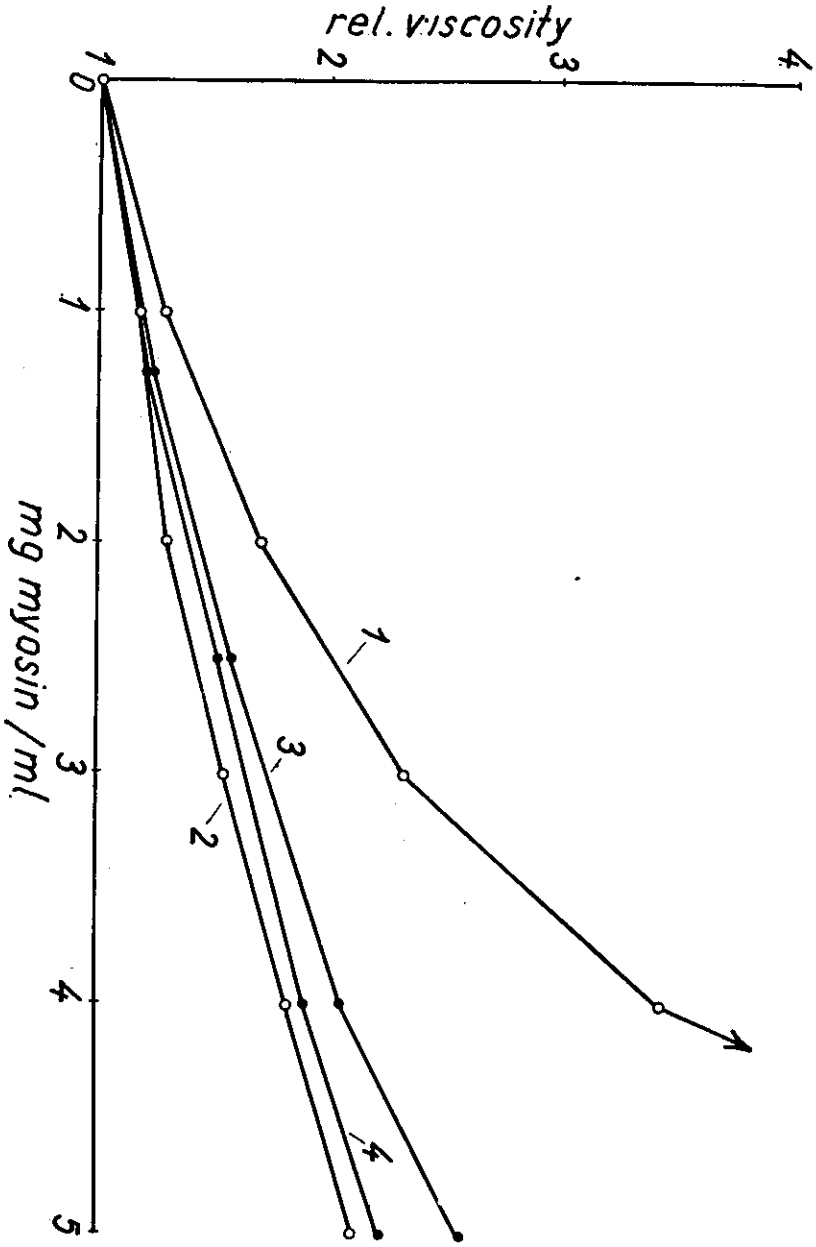

Fig. 1.

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

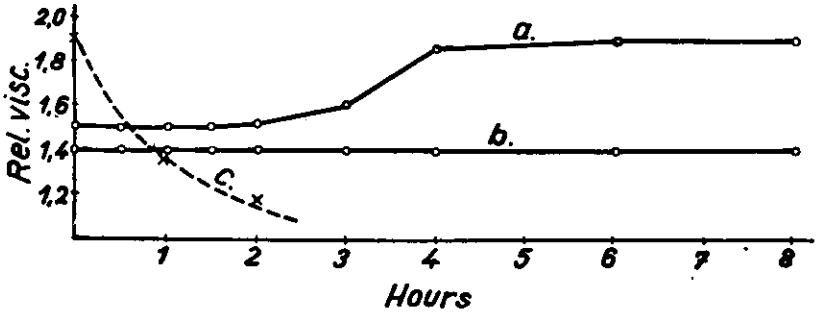

Fig. 2.

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.

1. *J. T. Edsall.* Jl. biol. Chem. *89*, 289, 1930.
2. *J. T. Edsall* and *A. v. Muralt.* Jl. biol. Chem. *89*, 315, 1930.
3. *M. Gerendás.* Enzymologia *9*, 123, 1940.

The contraction of myosin threads.

by

A. Szent-Györgyi.

It has been shown be H. H. WEBER, (1) that a myosin solution, if squirted in a thin jet into water, solidifies in the form of a thread. In this way the myosin can be brought into a form which resembles the muscle fibril in some respects. The myosin thread is an elongated piece of myosin gel.

It has been shown in the preceding paper by BANGA and myself that myosin can be obtained from muscle in two different forms which were called myosin A and B. Threads can be prepared from both. For the sake of convenience I will call the threads prepared from the 20 min. extract (see BANGA and Sz.) „myosin A threads" while the threads prepared from the 24 h. extract will be called „myosin B threads".

The threads used by previous investigators correspond, in all probability, to our myosin A threads.

The technique of the preparation and observation of threads will be described by M. GERENDÁS.

A watery extract of muscle was made in the following way) the rabbits muscle was cut out and minced (as described in the previous paper), suspended in water (1 ml per g of muscle), stirred for 5 minutes at 0°C and squeezed through a cloth. The fluid was then filtered through paper at 0°C.

If a myosin B thread is suspended in this extract and observed under the microscope, a violent contraction will be seen. The thread contracts within 30 seconds to less than half of its length and within 2—3 minutes it reaches a maximum contraction of 66%, $^1/_3$ of its original length. (Fig. 1.) At the same time the thread becomes proportionately thinner, and is seen to become quite dark.[1] Watched in lateral illumina-

[1] Frequently the contraction is so violent that the interior of the thread cannot keep pace with the contraction of the outer sheets, so that these latter break up, like a crocodile's skin. Fig. 2. For the same reason

tion the transparent thread is seen to turn white, opaque. The rate of contraction depends on the diameter of the thread. Fig. 3. curve a gives the time-curve of the contraction of a relatively thick thread of 0,3 mm diameter. Thinner threads, like those of 0,1 diameter, contract faster but their rate of contraction cannot be measured by the same method because it is too fast. Furthermore thin threads mostly curl up and stretch out again only after they have reached maximum contraction.

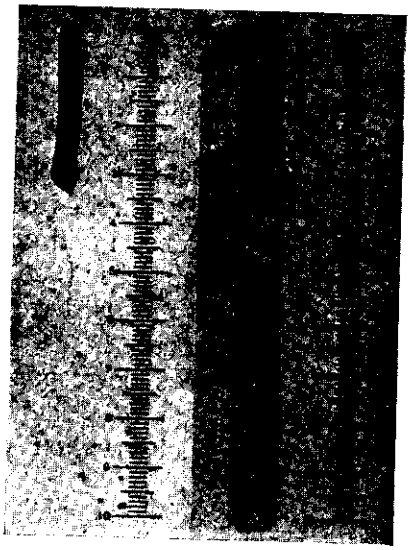

Fig. 1.

Fig. 2.

If a myosin A thread is suspended in the same fluid no striking change will be observed. (Fig. 4.) Measurement by the ocular micrometer will reveal a weak and slow contraction. (Fig. 3 curve b). If the myosin solution is filtered twice through a Seitz K filter before the thread has beene pulled, the contraction becomes still weaker (Fig. 3 curve c). As shown in the previous paper, the Seitz filter retains the myosin B present in our myosin A preparations as an impurity.

The frest, watery extract of thee muscle contains thus

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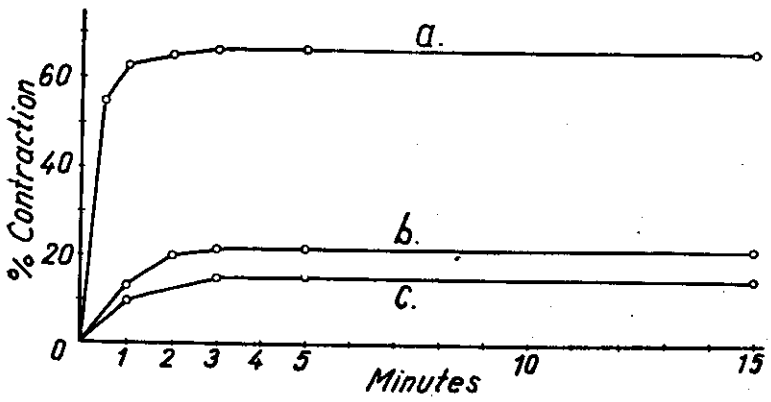
the whole thread sometimes breaks up into dark, solid lumps of myosin instead of giving a contraction.

something which causes a violent contraction in myosin B threads but has little influence on myosin A. The active agent seems to be present in excess for the extract can be diluted to 1: 4 with water and will still give the same contraction with thinner threads.

If the muscle suspension is stored over night at 0°C and filtered only the next day, the extract obtained will found to be entirely inactive. The myosin B thread, suspended in this extract, will show no change at all.

Rabbits muscle contains on the average 3,5 mg adenyl-



Fig, 3.

triphosphate („ATP") per g., thus the fresh extract contains ATP in about $^1/_2$ of this concentration. This ATP is split during storage by the phosphatase present. If the original ATP concentration is restored to the inactivated extract, again the same violent contraction will be obtained as in the fresh extract. Even half of this ATP concentration (0,9 mg per ml) is sufficient to give a maximum effect. This shows that ATP is involved in the observed contraction.

If the same quantity of ATP (0,09%) is dissolved in water and the myosin B thread suspended herein, no contraction will occur and the thread remains entirely unchanged. If we dissolve our ATP in the boiled extract instead of water we obtain a violent contraction again. Even incinerated juice will produce contraction with ATP. This makes it evident that, apart from ATP, inorganic constituents of the extract are also involved in the reaction.

If we use a 0,1 mol KCl solution as solvent for our ATP instead of water the contraction will be much slower. (see Fig. 5 curve b.)

Muscle contains 0,01 mol Mg. If we add 0,01—0,001 mol $MgCl_2$ to our 0,1 mol KCl and dissolve the ATP in this, the myosin thread suspended in this fluid will give the same violent contraction as in the fresh, watery extract (Fig. 5 curve a). It is evident thus that three factors were involved

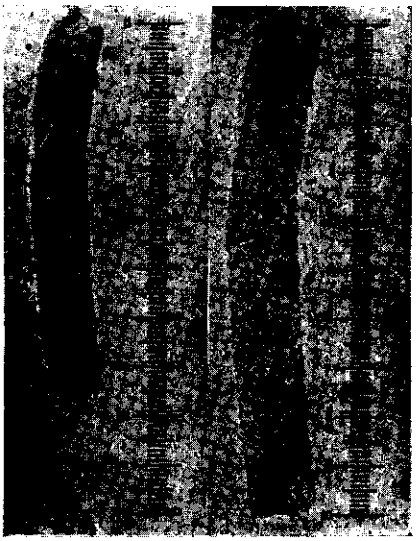


Fig. 4.

in the production of the contraction of our myosin B thread: ATP, K and Mg.

The myosin A thread will give the same weak and sluggish contraction (Fig. 5 curve c) with ATP in pure KCl or KCl plus $MgCl_2$. If the myosin solution is filtered twice through a Seitz K filter, the thread prepared from this solution will give a somewhat weaker contraction in KCl (Fig. 5. curve d). If, in addition to the 0,1 mol. KCl, 0,001 Mg is also present there will be practically no contraction at all (Fig. curve e). The contraction of myosin A is not only not enhanced but is almost completely inhibited by $MgCl_2$. If the thread, prepared from unfiltered myosin, gave the same contraction in KCl and $MgCl_2$ (curve c) this was due to the myosin B present as

an impurity: the contraction of this myosin B was enhanced and thus compensated the inhibition caused by Mg in the contraction of myosin A.

Myosin B gives thus a strong contraction with 0,1 mol. KCl and ATP and the contraction is greathy enhanced by Mg. Myosin A gives a weak and sluggish contraction with KCl and ATP and the contraction is suppressed by Mg.

If the concentration of KCl is increased, at 0,2 mol. the same reactions are still obtained. But if the concentration is raised to 0,04 mol., the thread, instead of giving a contraction,

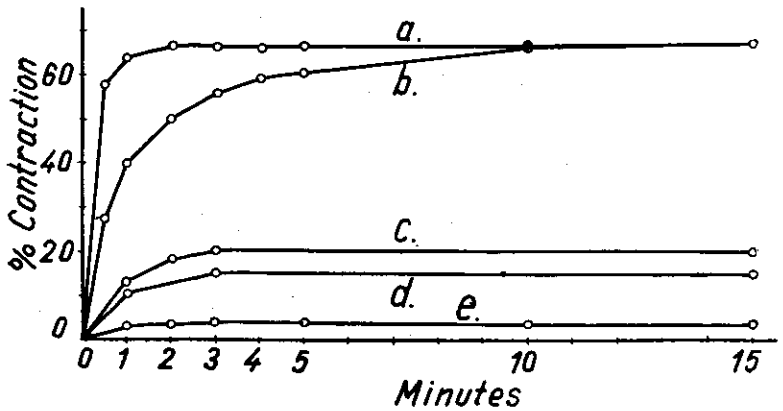


Fig. 5.

will dissolve. The action of our ATP—KCl—$MgCl_2$ mixture will depend on the concentration of the KCl present and at higher concentrations the action will be reverted; instead of contraction we will obtain dissolution and instead of aggregation, disaggregation. KCl without ATP will not dissolve the myosin B thread not even in a molar concentration.

Threads prepared from the precipitated, washed and redissolved myosin of these extracts gave identical results.

Adenylic acid, if employed instead of its pyrophosphate ester, ATP, was found to be entirely inactive. It does not give contraction or dissolution.

Neither the effect of KCl, nor that of $MgCl_2$ is specific and can be reproduced by other ions.

In fig. 6 the effect of KCl is compared with the effect of other halogen salts of K. The abscissa gives the log of the

molar concentration of the salt, the ordinate the % of shortening. By bringing the curve under the abscissa I wanted to express dissolution. The broken line means that the dissolution already takes place wihout the addition of ATP. The threads were prepared from precipitated and washed myosin B and were placed for 5 min. into the salt solution before the addition of ATP (0,018%). No value should be attached to the relative hight of the curves, which, in this respect, are not strictly comparable because the results were obtained with different myosin preparations. Readings ware made 5 min. after the addition of ATP.

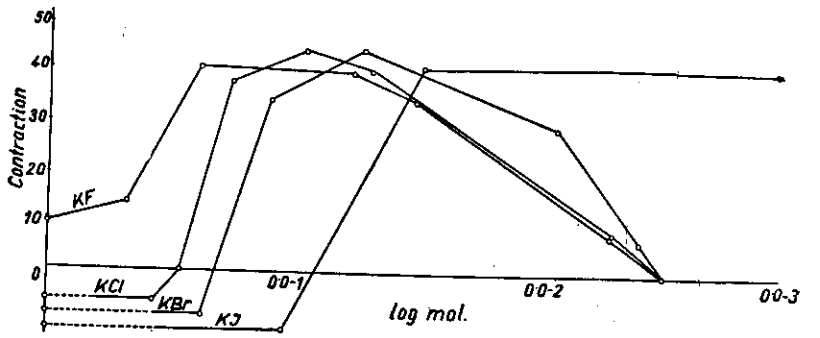


Fig. 6.

It will be seen that the effect of KCl is not specific and that the effect of a salt is not dependent on the kation only but depends on the anion too. With the increasing weight of the anion the curves are shifted more and more to the right. KJ has a very strong effect at relatively high dilutions.

In fig. 7. the anion (Cl) is kept constant and the kation varied. The differences are not very marked but there is a tendency to the opposite effect, a shift to the right with the decreasing weight of the kation. With Li the effect is distinct. In this curve NH_4Cl (neutralised with NH_4OH) and potassium phosphate (pH 7) are also given. This latter is effective at very high dilutions.

The enhancing effect of Mg can be reproduced by Co and Mn as will be shown by M. GERENDÁS. A detailed study on the effect of varied concentrations and pH will be given by T. ERDŐS.

Reversibility. The question presents itself whether

the contraction observed is reversible or whether it is connected with an irrevesible change of the myosin.

If the contracted myosin B thread is transferred into pure water it will remeain contracted. This naturally does not mean that the change is irreversible, for the contracted muscle has no reason to relax. Conditions in the muscle, where every myosin micell is fixed within a certain pattern, are different.

If the contracted thread is transferred into E d s a l l 's salt solution, it swells up again. When the thread has reached its

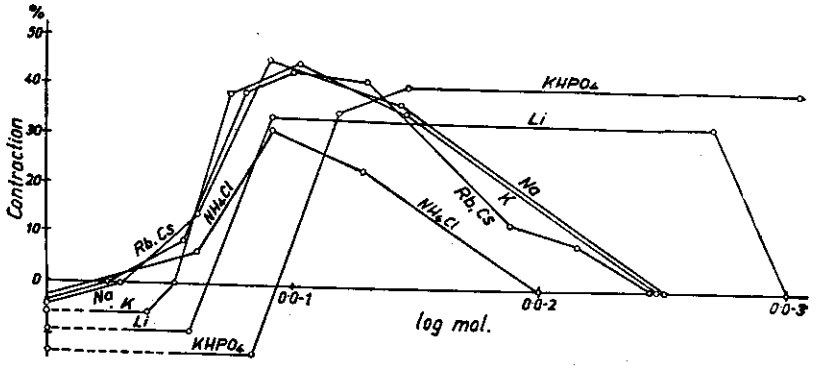


Fig. 7.

original dimensions the swelling can be stopped by transferring it into 0,1 mol. KCl. Such a thread has a perfectly normal appearance and does not contract spontaneously. If ATP is added it contracts again in the same way, as it did the first time. This shows that the contraction is reversible.

E x p e r i m e n t s w i t h m y o s i n *in stitu*. We may ask whether the contraction obtained with myosin threads can have any bearing on muscular contraction at all and whether in muscle myosin may behave also as myosin B. It has been shown that myosin gives a contraction even after it has been dissolved and precipitated, once the necessary ions and ATP are present. The contractility of muscle is a very sensitive process and seems to be thus the expression of a subtle organisation. Naturally it is just as possible that a higher organisation is not needed for the contraction itself, but for those changes that elicit this contraction or bring the muscle back to rest again.

To obtain some information on this question, I tried to destroy the finer structure of the muscle as far as possible without destroying the myosin. It is known that the excitability of the muscle is lost in distilled water and also by feezing. Neither of these destroy myosin.

The broad neck muscle of the rabbit was cut into 2 mm. wide strips parallel to the muscle fibres. The strips were placed into distiled water. After one to several hours the strips, if contracted, were stretched to their original length, frozen in solid CO_2 and cut into slices on the freezing microtome. The slices were made parallel to the musc'e fibres and were about one fibre thick. Thus they contained one sheet of muscle fibres running through the whole length of the preparation. The slices were put into distilled water and transferred after one to several hours into 0,1 mol. KCl, then placed on a slide under the microscope. After their length had been measured a drop of 0,14% ATP vas dropped on them. Immediately a strong contraction began, which reached a maximum within 15—120 seconds and shortened the fibres by 50—60%. The myosin behaved thus as myosin B.

Experiments with myosin suspensions. The last question I want to touch in this paper, is, whether myosin suspensions give changes which are analogous to the contraction of threads.

The muscle extract containing myosin was neutralised and diluted till the KCl concentration went down to 0,1 mol. The myosin precipitate was centrifuged, washed and reddissolved in Edsall's fluid, precipitated and washed thoroughly again.

The myosin obtained in this way is a fairly stable suspension which settles slowly. Salts is smaller concentration cause precipitation and the suspension will settle somewhat faster. Salt in higher contcentration will tend to dissolve the mysin. There is great difference in the behaviour of myosin A and B. The former is much less turbid and has a greater tendency for dissolution.

If, in addition to 0,1 KCl, a small quantity, say 14 mg % of ATP is also added to the myosin B suspension, the precipitation, will be greatly intensified. The precipitate immediately becomes roughly granular and settles quickly leaving a clear

fluid behind. The effect is very striking. We may call it a „superprecipitation", contrary to the precipitation caused by KCl alone. Mg still enhances the reaction.

4 mol. KCl has no appreciable dissolving action on the myosin B suspension. If ATP is added in addition to this KCl., the myosin dissolves. We can thus say that ATP greatly enhances the effect of salts, bringing about dissolution at concentrations at wich the salt by itself is inactive and it also greatly intensifies precipitation.

The phenomena seen in the myosin B suspension are analogous to the phenomena observed on myosin B threads. ATP and higher KCl concentrations dissolve both. ATP and lower salt concentrations, which produce a contraction in the thread, cause a superprecipitation in the suspension.

The analogy is lacking in one point. While smaller salt-concentrations cause by themselves a precipitation in the suspension, salts without ATP never give a contraction in threads. There seems to be a qualitative difference between the precipitating action of salts alone and the precipitation observed in the presence of ATP. Salts alone seem only to cause an aggregation of the myosin micells, while in the presence of ATP they seem to cause some deeper change within the single units, which change expresses itself in the superprecipitation of suspensions and the contraction of threads. GERENDÁS has found that while salts by themselves have no influence on the double refraction of oriented threads, salts + ATP cause, besides contraction, a complete disappearance of double refraction. Double refraction disappears in musculur contraction also.

Myosin A suspensions behave in an analogous way to myosin A threads. They dissolve without ATP at lower salt concentrations (0,4 mol. KCl) and ATP has only a very slight precipitating action which is not enhanced by Mg.

Summary.

It is shown that a myosin B thread, if suspended in a fresh, watery extract of muscle, gives a violent contraction. Myosin A is relatively inactive.

It is shown that three factors are involved in the contraction of myosin: ATP, K and Mg.

At higher salt concentrations, in the presence of ATP, dissolution is obtained.

The action of ions is not specific.

Under the same conditions which cause the myosin thread to contract, the myosin suspensions give a precipitate.

Literature.

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The phosphatase activity of myosin.

by

I. Banga.

ENGELHARDT and LJUBIMOVA (1,2) found close correspodence between phosphatase activity and the myosin content of muscle extracts. They concluded that myosin itself is the phosphatase which splits, according to K. LOHMANS (3) equation, adenyltriphosphate (ATP) into adenylic acid and two molecules of o phosphate. Two years ago I undertook to control this statement and could fully corroborate it. My myosin preparation showed different enzymic activities like that of a succinodehydrase, cytochromoxydase, desamidase, lactic acid formation etc., but in agreement with the russian authors I also found that on fractionation only the phosphatase activity went parallel to the myosin content and the myosin could be separated eventually from other enzymic activity.

By repeated extraction with EDSALL's salt solution the myosin can be extracted exhaustively. On the first extraction one obtains 2—2,2% myosin. The second extraction gives a solution containing about 0,7%, the third extraction about 0,3% myosin. Further extractions are practically free of myosin and the residue contains only traces of it. The phosphatase activity of these fractions was found to be proportional to the myosin content.

The desamidase can be separated from myosin by precipitation with half saturated ammonium suphate which leaves the desamidase in solution. Desamidase can be extracted from muscle, contrary to myosin, by isotonic NaCl or KCl.

The enzymes belonging to lactic acid fermentation can be separated from myosin by repeated precipitation with water at ph 7.

The succinodehydrogenase and cytochromoxydase are dissolved by the salt solution only to a small extent. The first extraction will bring out about $^1/_6$ of these enzymes, further extracts will have no activity at all. It is doubtful whether this small quantity of the enzymes is really dissolved or only suspended. Both enzymes seem to be bound to the less soluble fraction of the muscle.

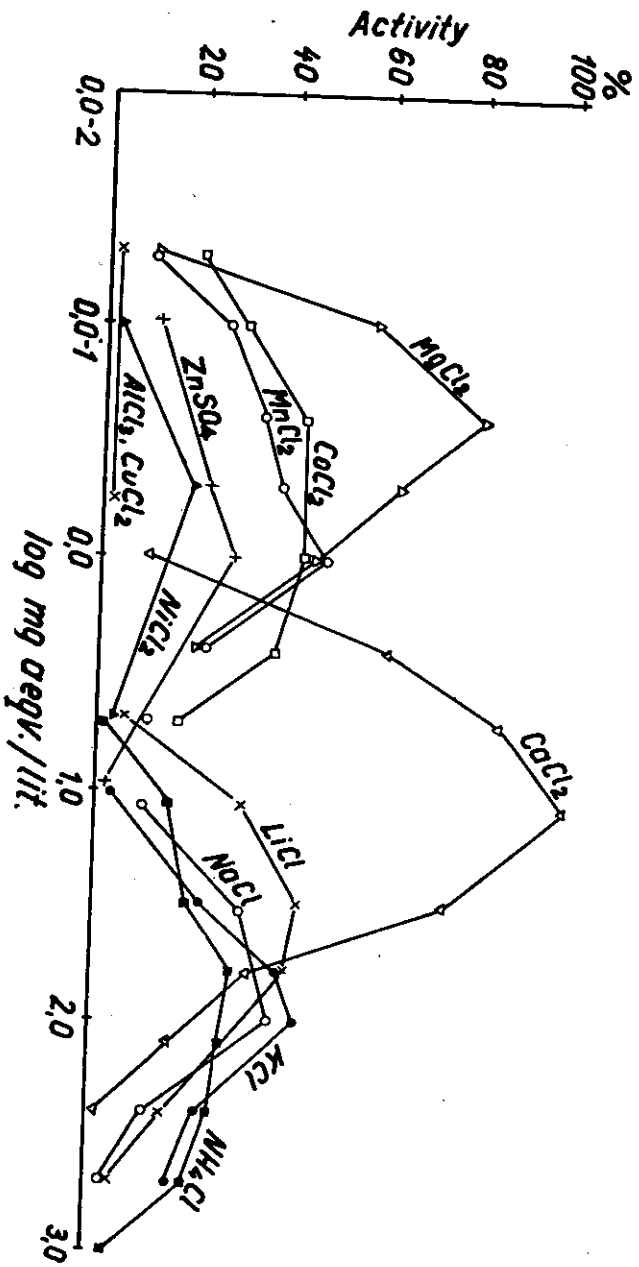
Activity
20
40
60
80
100
%
0·0-2
0·0-1
0·0
0·1
0·2
0·3
log mg equiv./lit.
MgCl2
ZnSO4
MnCl2
CoCl2
AlCl3, CuCl2
NiCl2
CaCl2
LiCl
NaCl
KCl
NH4Cl

Fig. 1.

In these experiments I found that by repeated precipitation and washing with water, or by protracted dialysis, the phosphatase activity of myosin was reduced to about 10% of its original value. This activity could be restored by muscle extract. Boiling or incineration did not annihilate this reaction and it was evident that by the purification I had removed some inorganic constituent which served as an activator to the myosin.

Further experiments showed that the action of salts is not specific and that a great number of different ions are capable of this reactivation.[1]

Our earlier experiments were made with rabbits muscle minced on LATAPIES mincer and extracted for 30 min. according to EDSALL. Later we used 24 and 20 min. extracts as described in the preceding paper of BANGA and SZENT-GYÖRGYI. The extracts were precipitated by dilution and neutralisation, washed with water, redissolved and precipitated and washed again. Then the myosin content of the suspension was determined. My original experiments gave the same results as the later ones on the whole and so some of these original observations will be quoted too at the end of this paper.

The reaction mixture had a final volume of 3 ml and contained 1 mg of mysoin, 4.6 of ATP and the salt in question. The mixture was placed into small flasks of 50 ml and incubated in the waterbath under constant shaking at 38° for 5 min. Then 1 ml. of 20% trichloroacetic acid was added and the inorganic phosphate estimated after FISKE and SUBBAROW. We used no buffer, the ATP itself buffers to some extent. Salts, which dissociate hydrolitically, were neutralised.

My results obtained with 24 h. myosin are summed up in Fig. 1. As will be seen, analogous to contraction, the phosphatase activity of myosin is also activated by a great variety of ions: most salts studied had a definite activity with distinct optima. White the salt is not active below a certain concentration, its excess inhibits the reaction. Activation was

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[1] A short note about these results was sent to SCIENCE but I am unaware whether our letter reached the editor. Definite publication was delayed by external circumstances and retained later because of complications which culminated in the discovery of the two different myosins.

In the mean time R. CLOETENS found that the alcaline phosphatase of kidney was activated by different metals, like Ca, Mg, Mn, Co, Ni, Zn, Hg. (Enzymologia *7*, 157, 1939. Naturwiss. *28*, 252, 1940. Biochem. Z. *307*, 352. 1941. Ibid. *308*, 37, and *310*, 400. 1941.)

hardly detectable with $AlCl_3$ and $CuCl_2$ which salts, as well as Ni, inactivate myosin irreversibly.

The great variety of ions which were found to be active allow to axclude the possibility that the metal acts as a prosthetic group of the enzyme.

It should be noted that I always found some free phosphate without the addition of salts, which is subtracted in Fig. 1. It is, to a small extent, due to the free phosphate present at the beginning of the experiment but mainly to enzymic activity. It seemed likely that this activity was due to the activating effect of the K ions introduced with the ATP partly as its cation partly as its KCl impurity. To decide this question the phosphatase activity was measured in the presence of varied ATP concentrations. Results of such an experiment are given in Tab. I. The experiment was made with 24 h. myosin precipitated once and washed twice. The numbers give the P split off from ATP in mg.

Tab. I.

Added mg ATP	no salt added	0.1 mol. KCl	0.001 mol. $MgCl_2$	0.01 mol. $CaCl_2$	0.01 mol. KCl. 0.001 mol. $MgCl_2$
1.4	0.003	0.037	0.074	0.055	0.074
2.8	0.006	0.078	0.104	0.100	0.120
4.2	0.019	0.104	0.162	0.140	0.134
5.6	0.038	0.134	0.168	0.162	0.180

As will be seen from col. II. the quantity of P rapidly falls with the decreasing quantity of ATP added in the absence of added salt. With 2.8 mg of ATP the activity is very small but the next column shows that this is not due to the lack of ATP but to the lack of ions, since in the presence of KCl the activity is still considerable. Thus the higher activity in presence of 5.6 mg of ATP must have been conditioned by the K ions introduced with the ATP. In this experiment the O value i. e. the value obtained without addition of salt, was fairly low. In other experiments the O value was found to be as high as 0.050 and it seems likely that here also other ions absorbed on the myosin play some role.

Tab. II gives the results of the experiment performed with the myosin of the 20 min. extract of the same muscle which was used in Tab. I. The columns and numbers have the same meaning as before.

Tab. II.

Added mg ATP	no salt added	0.1 mol. KCl	0 001 mol. $MgCl_2$	0.01 mol. $CaCl_2$	0.01 mol. KCl 0.001 mol. $MgCl_2$
1.4	0.002	0.023	0.004	0.064	0.028
2.8	0.004	0.044	0.008	0.109	—0.037
4.2	0.022	0.066	0.026	0.165	0.044

As can be seen the differences between A and B myosin (See BANGA and SZENT-GYÖRGYI) are the following: the O value of myosin A is somewhat lower than that of myosin B. Myosin A is activated by KCl to a lesser extent than myosin B. $MgCl_2$ which has a maximum effect on myosin B, has no activating affect on myosin A at all. Both A and B myosins are equally activated by $CaCl_2$. The KCl-activation is enhanced by Mg in myosin B and is inhibited in myosin A.[2]

This experiment shows the difference in the reaction of myosin A and B found in the majority of cases. There is, however, a certain variation in the results obtained with different preparations. Sometimes the K activation is equally strong in the 20 min. and in the 24 h. myosin. Sometimes the K activation of myosin A is depressed by Mg to a greater, sometimes to a lesser extent than was the case in the quoted experiment. In none of the experiments, however, had Mg any activiting effect on the 20 min. myosin while it always strongly activated the 24 h. preparation.

On the whole the results obtained with the phosphatase activity show a close analogy to the results obtained in the contraction of myosin threads. The analogy between phosphatase activity and contraction breaks down in the case of $CaCl_2$. Ca has no activating effect on contraction but activates phosphorilysis maximally in both A and B myosin. Ca even inhibits the contraction caused by ATP and other ions (see FRDŐS).

[2] The inhibition of KCl activation by $MgCl_2$ in myosin A becomes stronger if the myosin is sent twice through a SEITZ K filter. As has been shown 20 min. myosin always contains some myosin B, the activity of which is enhanced by Mg, while the activity of myosin A is inhibited. These two effects may compensate each others. (The Seitz filter retains myosin B.)

Repeated filtration through the SEITZ K filter reduced the KCl activation of the myosin of the 20 min. extract to one third of its original value as it also reduced the contractility and „activity" of this preparation (for definition of „activity" see BANGA and SZENT-GYÖRGYI.)

Apparently Ca induces some change in the myosin which renders its contraction impossible. This Ca activation of phosphorilysis is important because it proves that phosphatase activity is inherent to that part of the myosin which is the basic constituent of both A and B forms.

Apart from the case of Ca there are many and close analogies between the phosphatase activity and the contraction of myosin. My next question was thus, how far this analogy can

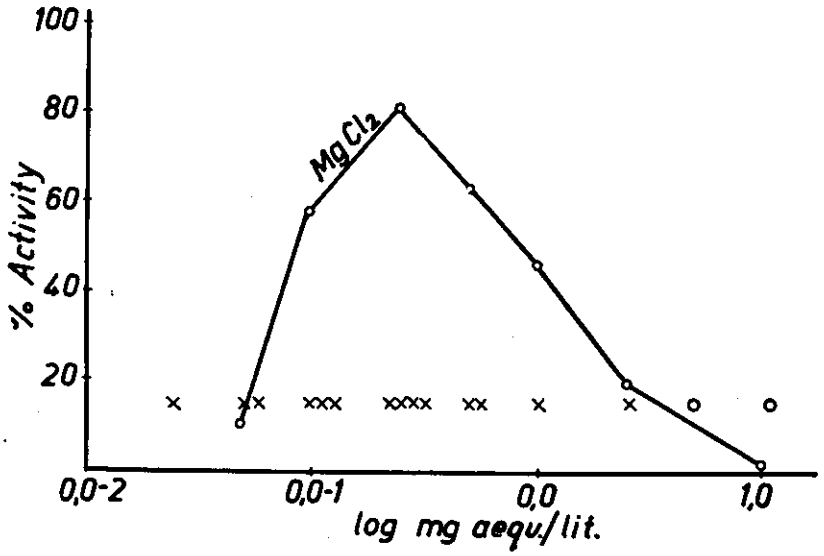

Fig. 2.

be drawn and whether there is not an even more intimate relation between both phenomena. As has been shown (SZENT-GYÖRGYI) contraction of threads and precipitation of myosin suspensions are but different expressions of the same process. For technical reasons it seemed more convenient to compare phosphorilysis with precipitation than with contraction. Maxima can be judged more easily in precipitation since contraction is very fast, its extent depends on time, and diffusion complicates the situation.

I therefore compared precipitation and phosphatase action. The results of one experiment with $MgCl_2$ (fig. 2.) and KCl (fig. 3.) is summed up in the curves which are analogous to fig. 1. Two identical sets of dilutions were prepared with both salts. One of the sets was prepared in flasks and served to

measure the phosphorilysis, while the other set was prepared in reagent tubes and served to observe the precipitation. Maximum precipitation is denoted with four crosses, 1—3 crosses mean weaker precipitation while O means the dissolution of the myosin. (24 h. extract).

As the figures show there is close connection between the physical state of the myosin and phosphorilysis: while the maximum of precipitation coincides with maximal

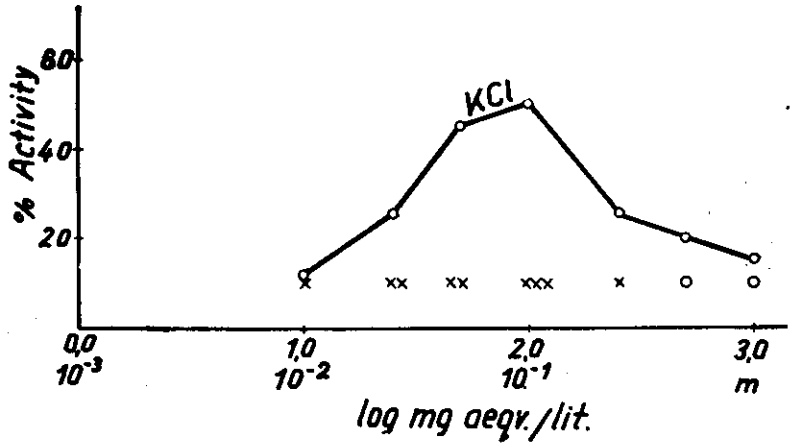

Fig. 3.

enzymic activity, dissolved myosin is inactive. Myosin is thus fully active in its most contracted state only.

The dependence on pH of the phosphatase activity of myosin B (24 h.) is shown in fig. 4. K and Mg were used in optimal concentration as activator. There is a sharp optimum at pH 6, the isoelectric point of myosin, which is additional evidence for the identity of myosin and phosphatase. Unfortunately the curve is not free of criticism. I did not dispose of a suitable buffer between pH 6 and 7, as the use of phosphate was excluded, since it interfered with the P estimations. Furthermore buffers are salts themselves and have their own activating effect. Up to pH 7 I used an acetate buffer, between Ph 7.60—10 a $NaHCO_3$—Na_2CO_3 buffer, both in 0.05 mol concentration. The buffers were controlled electrometrically. The ATP solution was adjusted colorimetrically, and did not materially influence the pH of the buffer.

I want to close this chapter with a few observations taken from my unpublished paper of two years ago.

Phosphatase activity at varied temperatures. Results are given in fig. 5. The abscissa gives the time, the ordinate the log. of the ATP still present. As will be seen

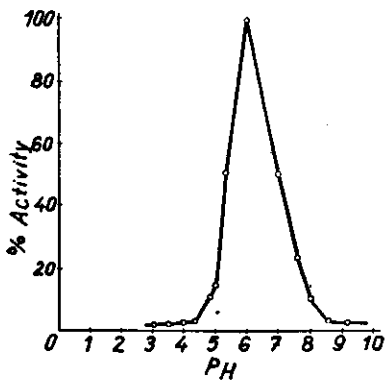

Fig. 4.

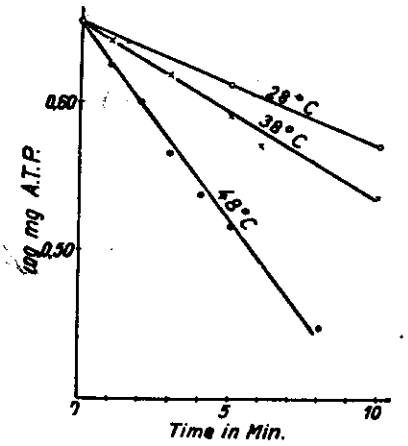

Fig. 5.

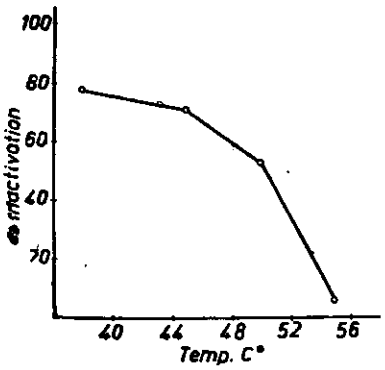

Fig. 6.

the curves are straight which is in agreement with a monomolecular type of reaction.

Inactivation of myosin at elevated temperatures. The results are given in fig 6. Incubation for 10 minutes. pH 7. The activity measured at 38°C.

Reversibility. If our myosin contains no other en-

zyme which acts on ATP or its splitting-product we can study the kinetics of its phosphatase activity. According to Lohmann[3], ATP is split by muscle into adenylic acid and two molecules of o-phosphate. If this reaction would be reversible we would always have to arrive at the same equilibrium mixture at varied ATP concentrations. On the other side addition of adenylic acid or phosphate should inhibit the reaction and ATP should be formed from the mixture of both, provided the equilibrium is not too far on the side of the splitting products. I found that the % of the ATP split varies at varied ATP concentrations (the myosin concentration remaining constant). The more ATP I added the higher was the % of it that was split, as shown in Tab. III.

Tab. III.

mg. ATP added	mg. ATP split	% AT Psplit
1.15	0.36	31.2
2.30	0.91	39.2
3.45	1.34	39.0
4.60	1.95	42.0
5.75	2.60	45.0
6.90	3.20	46.2

On the other hand addition of adenylic acid or phosphate had no influence on the splitting of ATP and no ATP could be detected in the mixture of the two substances. (Tab. IV.)

Tab. IV.

Added mg. ATP	Adddd mg Aden. acid	Added mg. P (as PO_4)	mg. ATP split	mg ATP formed
4.6	—	—	2.65	—
4.6	—	0.148	2.73	—
4.6	4.6	—	2.65	—
—	4.0	0.104	—	0.0

A few experiments were made on the accelerating or inhibiting effect of pharmacologically active substances (in the presence of 0.1 mol. KCl or 0.01 mol. $CaCl_2$). Aconitin, veratrin, acetylcholin (in presence of phisostigmin), adrenalin, coffein, chloroform, urethan, guanidin-sulfate were found to be inactive at a dilution of 1:1000 and inhibited at higher concentrations. Quinine's inhibition was 50% at 1:10,000 dilu-

tion. Higher concentrations could not be studied since the alcaloid interfered with the P estimation. Nicotine inhibits in 1:1000 completely, in 10^{-4} concentration to 25%. NaF does not inhibit, not even at a mol/30 concentration, in which concenration it inhibitis glycolysis completely. Mol/500 Na-oxalate inhibitis 100%. Monojodacetate and maleinic acid were inactive.

I also tried to find some connection between the functional states of myosin and its activity. I hoped to be able to increase the enzymic activity by a tetanisation of the muscle before the extraction. Frog-muscle was subjected to a short, in other experiments to an exhaustive tetanization (indirect electrical stimulation), then frozen in CO_2, minced in the frozen condition and extracted. The best results i. e. the highest phosphatase activity, could be obtained with resting muscle cooled to 0°C, minced and treated carefully at a low temperature.

Summary.

ENGELHARDT and LJUBIMOWA are corroborated.

It is shown that the phosphatase activity of myosin depends on the presence of ions. Differences between the activation of myosin A and B are described.

Myosin activated by K and Mg is most active at the maximum of its precipitation or contraction.

Dependence of the phosphatase activity of myosin on pH and temperature are described and the reversibility of phosphatase action is discussed.

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2. *M. N. Ljubimowa* and *U. A. Engelhardt:* Biokhimija *4,* 716, 1939.
3. *K. Lohmann:* Biochem. Z. *271,* 264, 1934.

Quantitative Studies on some Effects of Adenyltriphosphate on Myosin B.

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The question discussed in this paper is the following: which is the minimal quality of adenyltriposphate („ATP") which still gives rise to a maximal effect on myosin?

The effects of ATP on viscosity, on light scattering and on precipitation have been studied.

1. The viscosity-lowering effect. The relative viscosity of a solution of myosin B is high; addition of ATP causes a marked fall. It is, however, not possible to determine the quantitative relations between ATP and myosin simply from this, because the viscosity-lowering effect of very small quantities of ATP decreases with time, apparently due to the splitting of ATP by myosin. The influence of time has therefore to be taken into account.

0,2—0,3% solution of myosin in Edsall's fluid were used and measurements taken with a viscosimeter of the Ostwald type, which was immersed in ice-water.

Results are given as specific viscosity; the differences in density of the solution and the solvent have not been taken into account in the calculations, this correction being of no importance, as all determinations of one series of experiments were made with solutions of the same concentration.

First the viscosity of the myosin solution was determined. The fluid was then poured out of the viscosimeter into a tube, likewise suspended in ice-water and the viscosimeter immediately put back in its place. After a short time dissolved ATP was added in an amount which did not cause dilution of the myosin solution by more than 0.1%. The time of complete mixing was noted. The solution was then put back

into the viscosimeter, with a pipette surrounded by a mantle of ice-water. If care is taken to avoid warming of the solution or instruments, the first reading can be made immediately, without error due to a rise in temperature. Measurements were repeated every 3 or 4 minutes, usually for 20 to 40 minutes.

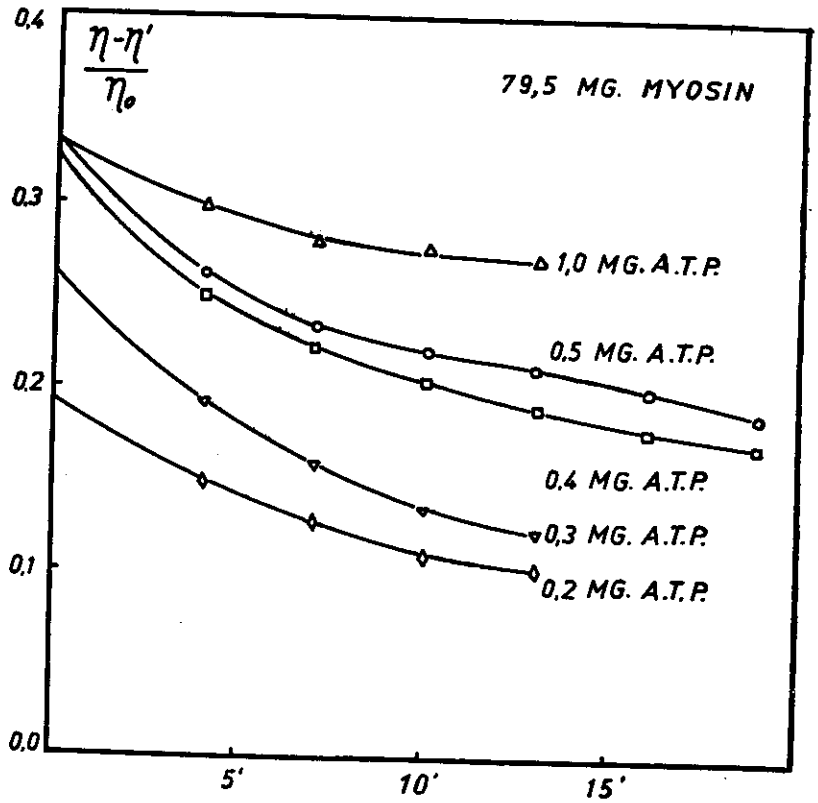

Fig. 1.

The graphs show the viscosity-lowering effect as the difference between the specific or relative viscosities of the solution with and without ATP at different moments after mixing.

An example of a series of experiments is shown in fig. I. As will be seen, the viscosity-lowering effect decreases regulary with time, an end-value usually being reached within 15 to 45 minutes. Extrapolation of the curves towards

zero time gives the magnitude of the initial effects. The figures in this example show that 1,0 and 0,5 mg ATP had the same effect; 0,4 mg acts slightly less and still smaller doses of ATP are clearly submaximal.

In Fig. 2 the results of a number of series of experiments have been plotted in the following way: the ordinate shows the effect of each ATP quantity as the percentage of the maximal effect in the same series; the abscissa gives the ATP quantity present per 100 mg of myosin.

In the range of lower concentrations the spreading of the

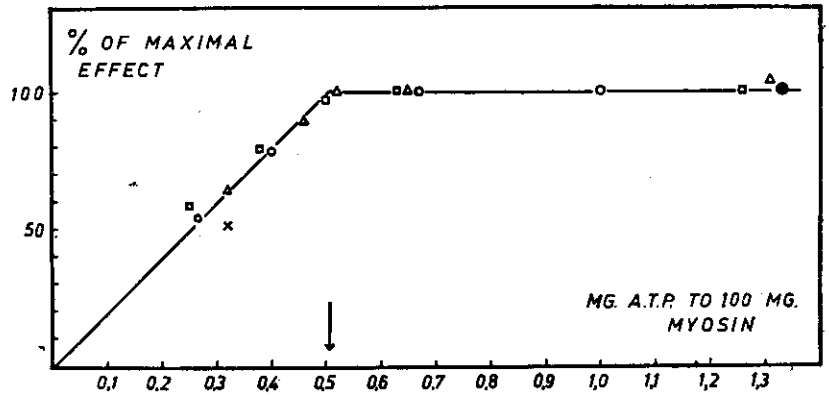

Fig. 2.

points is considerable owing to the fact that in the case of actions far below the maximum the extrapolation of the time-curves is difficult. In the neighbourhood of the maximal effect it can be made with sufficient accuracy.

The relation between the ATP quantity and the magnitude of the effect in the submaximal range seems to be a linear one: the transition towards the horizontal part of the curve is very sharp; the transition-point, which indicates the minimal fully effective ATP quantity, seems to lie between 0,50 and 0,51 mg ATP per 100 g myosin. From this the weight of the reacting myosin-unit may be calculated to be about 100,000.

2. The effect on light-scattering. As has been decribed in another paper of this series (BANGA and SZ.) the turbidity of myosin B solutions is higher than the turbidity of solutions of myosin A. Addition of ATP in sufficient

quantity to the former causes a decrease of turbidity by 20—30%.

Measurements were made with the Zeiss Stupho-nephelometer using the S. 53 greenfilter. The lightscattering of different samples of myosin solution was measured, before and after the addition of a known amount of ATP. Like in the case of viscosity, the ATP-effect depends on time and the real value can be found by the extrapolation of the time-curve towards zero; however, the mean value of 5 readings made

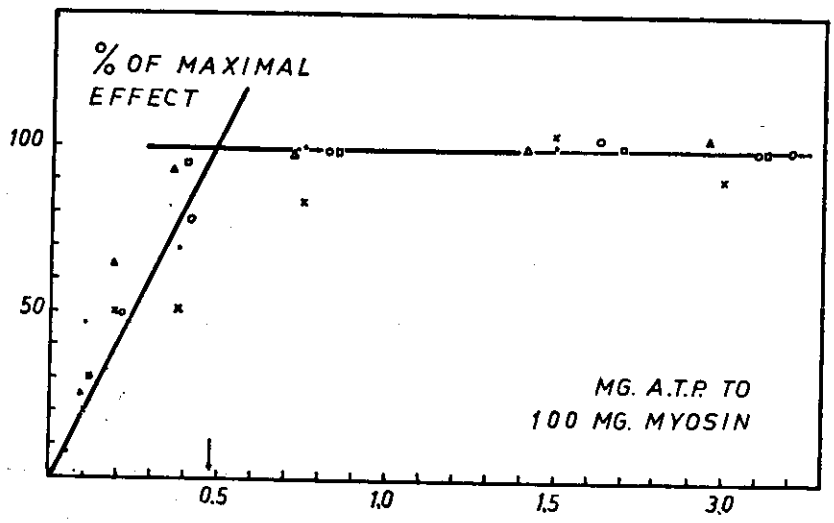

Fig. 3.

within 1,5 minutes after the addition of ATP, can be used as a base of comparison just as well as the extrapolated value. All measurements were made at room temperature (30°C.)

The results of a number of series of experiments, made with different preparations containing 0,4—2,0 mg myosin per ml are given in fig. 3. The spreading of points is considerable owing to the smallness of the effects; it is even difficult to decide about the exact form and situation of the curve. The line which has been chosen as the most probable one points to 0,47 mg ATP per 100 mg of myosin as the minimal quantity giving full effect; this value is almost the same as that got with the viscosimentric method (0,51).

It may, therefore, be concluded that the study of the

effect of ATP on light-scattering in myosin solutions is not in disagreement with the results of the viscosity measurements.

3. The effect on gel-volume. If a myosin preparation, dissolved in Edsall's fluid, is diluted with a five-fold volume of distilled water and neutralised with acetic acid, a precipitate is formed. The precipitation becomes more intense on addition of ATP and correspondingly the volume of the precipitate becomes smaller.

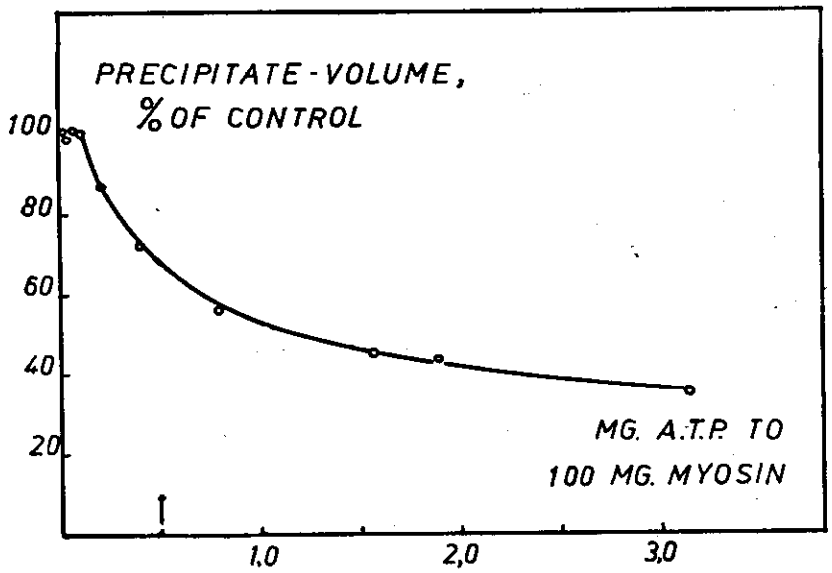

Fig. 4.

For the determination of the volume of the precipitate, 1 ml of myosin solution (in most cases containing 12 mg per ml) was mixed with 5 ml of distilled water and different amounts of ATP in a weighed cetrifuge tube of 10 ml. The volume of the precipitate was determined by weighing it in the wet state, the tube having been dried carefully with filter paper. Neutralisation was found not to be essential for precipitation, although the gel settled more easily and with a smaller volume after neutralisation; this is in agreement with the results of Edsall (1). Common distilled water had to be used because no well-formed gel could be obtained in glass-distilled water. The most regular results were obtained by precipitating the myosin first and then adding the ATP; similar results, only

with somewhat scattered data, were obtained if the myosin solution was diluted with water already containing ATP. If first the ATP was added to the myosin and dilution carried out after the elapse of a few seconds or minutes, higher ATP concentrations were needed in order to get the same effect, which was doubtless due to the splitting of the ATP in the concentrated myosin solution. This method seemed, therefore, to be less suited for the present purposes.

Fig. 4 shows the mean values of the results of six series of experiments. As will be seen, a clear maximal effect was not reached, not even in the case of the highest ATP doses. For the explanation of this fact it should be mentioned that between the addition of ATP and the determination of the gel-volume a time of 20 to 25 minutes elapses. Therefore the ATP added will in greater part be split during this time, thus making addition of a larger amount necessary and smoothening the sharp curve. It will be seen, however that at 0,5 mg ATP per 100 mg of myosin the effect is considerable and differs from that of the higher doses by an amount which can be expected to be caused by the splitting of ATP during the experiment. It can be concluded therefore, that the results of these experiments agree in order of magnitude with the results obtained by other methods (The arrow marks the value corresponding to 1 mol. ATP per 100,000 g myosin).

4. Conclusion. From the quantitative study of the effects of ATP on the physical properties of myosin one can conclude that myosin reacts in units of 100.000, a number which seems to be related to the molecular weight in urea solution as found by WEBER and STÖVER (*2*). Every single unit reacts with one molecule of ATP. This result is based on three entirely different methods of investigation in which myosin was studied at O°C and at room temperature, in solution and as a precipitate, in the streaming as well as the resting condition. From the sharpness of the transition point of curve *2* it must be concluded that the myosin-ATP-compound is, at least at O°C, not dissociated to a marked degree.

Literature.

1. *J. T. Edsall:* Jl. of Biol. Chem. *89*, 289, 1930.
2. *H. H. Weber* and *R. Stöver:* Biochem. Z. *259*, 269, 1933.

Reaction of Adenyltriphosphate with Myosin A.

by

F. B. Straub.

It has been described by BANGA and SZENT-GYÖRGYI that the viscosity[1] of a 24 h. extract of muscle, containing myosin chiefly in the B form, decreases very strongly on addition of adenyltriphosphate (ATP). Under similar conditions the viscosity of a 20 min. extract, containing mainly the A form, decreases but slightly, the cange being only about 10% of the change observed in the 24 h. extract. It has also been shown that this slight decrease of viscosity is not due to myosin A, but to the small quantitities of myosin B present as an impurity.

These experiments have been done with extracts of muscle. Edsall's fluid was used as solvent which is an alkaline 0,6 mol. KCl solution. If the 24 h. extract, which I will call for the sake of convenience „myosin B", is tested in a solution of lower pH and KCl concentration, the viscosity will show an even more pronounced decrease (40% more) on addition of ATP.

Myosin A behaves in an interesting way if the pH and salt concentration are varied. If e. g. myosin A is dissolved in a veronal-acetate buffer of pH 5,3 and the potassium ion concentration is varied by adding different amounts of KCl, ATP will cause a great decrease of viscosity, the magnitude of this effect varying with the salt concentration. Such results are shown in Fig. 1. The maximal value of this ATP effect is quantitatively the same as that which would be obtained with a myosin B solution having the same viscosity. The same

[1] The term viscosity is used in this paper knowing that, in the type of viscometer used in these experiments, deviations from POISEUILLE's law are perceptible. The effects described here, however, are not materially influenced by possible corrections in the viscosity values.

type of curve is obtained if the potassium ion concentration is kept constant and the pH varied.

While myosin A gives a clear solution in EDSALL's alkaline KCl, in solvents in which the ATP effect in increased,

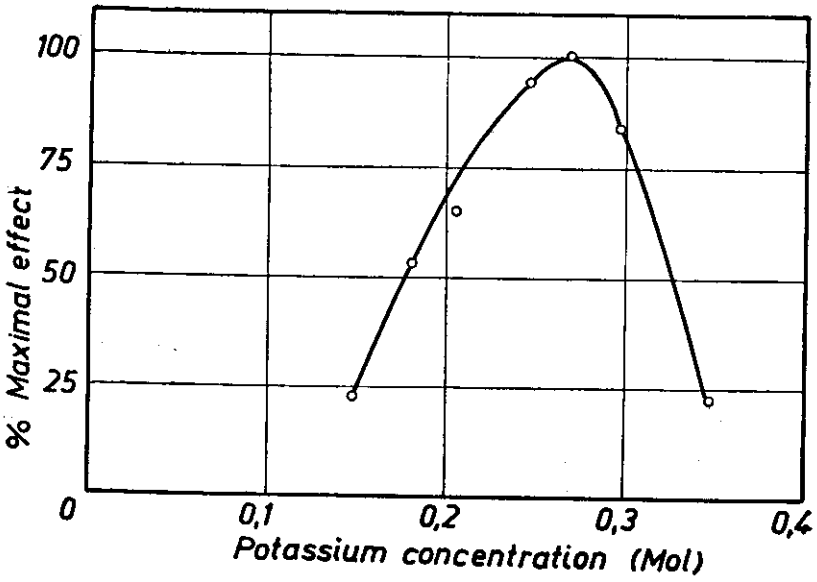

Fig. 1.

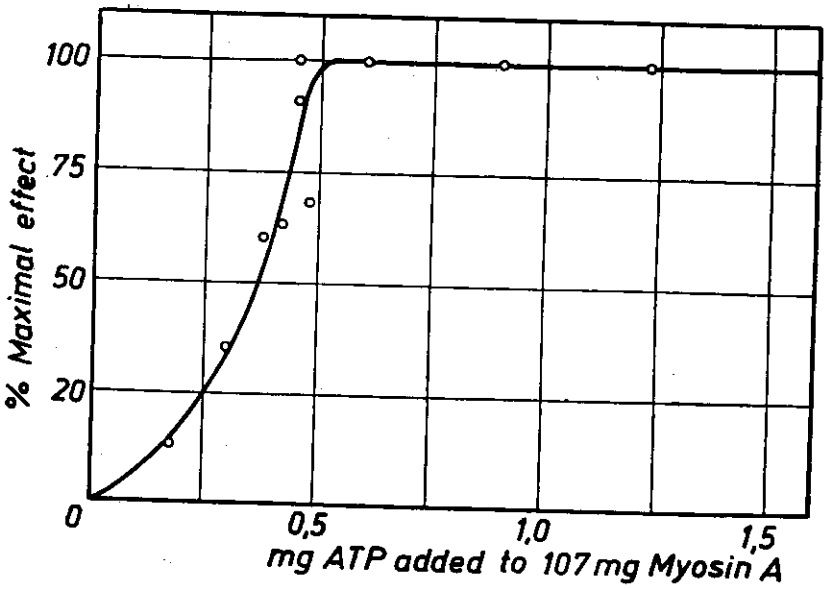

Fig. 2.

the opalescence increases roughly in proportion. Finally, decreasing the potassium ion concentration below its optimum (which gave maximal ATP effect) will result in inreasing flockulation. Considring the combined action of pH and salt concentration, one is led to suppose. that the ATP effect is the function of a particular colloidal state and not of the difference between the two myosin.

It was interesting to see what concentration of ATP is required to give a maximal effect (maximal decrease of viscosity) in these experiments with myosin A. It will be seen from Fig. 2. that 0,5 mg ATP in 30 ml are needed to give a maximal decrease of viscosity with 107 mg myosin A. The combination between ATP and myosin A is therefore just as strong as the combination between ATP and B. Moreover it should be pointed out that in spite of a difference in solubility and viscosity, suggesting a difference in size of the myosin A and B particles, the equivalent reacting weight of myosin with ATP is the same in both cases.

Experiments.

In the experiments recorded in Fig. 1., 5 ml of myosin A solution (100 mg myosin in 0,6 mol. KCl) were added to 25 ml of a buffer solution and the viscosity determined in a capillary vicometer at O°C. The buffer solution consisted of 2,5 ml of 0,28 mol. veronal-K, 2,5 ml of potassium acetate of the same concentration, 8,0 ml n/10 HCl, varying amounts of KCl and distilled water to make up 25 ml. If no KCl was added, the potassium concentration in the final volume of 30 ml was 0,147 mol, resulting from the potassium of the buffer and myosin solutions.

A similar mixture was used in the experiment from which Fig. 2. was constructed. The 30 ml of mixture, analysed in the viscometer, contained the same buffer and 5 ml of a myosin A solution (107 mg myosin), together with so much KCl, as to bring the concentration of potassium ions to 0,25 mol. When the amount of ATP was too small, successive readings in the viscometer did not agree but showed an ever decreasing ATP effect. This is due to the splitting of ATP by myosin. In these cases the viscosity at zero time (at the moment

of adding the ATP) was evaluated by linear extrapolation from the values of 3—4 successive determinations. The errors inherent in this extrapolation may account for the fact that the rising part of the curve is not linear, as could be expected.

Summary.

It is shown that, at a lower pH and a lower salt concentration, myosin A also has a high viscosity which is decreased by ATP. ATP reacts with myosin A in the same proportion as with myosin B (one g. mol. ATP per 100,000 g of myosin).

Technisches über Myosinfäden nebst einigen Beobachtungen über ihre Kontraktion.

von

M. Gerendás.

H. H. WEBER[1] zeigte, dass wenn eine genügend konzentrierte Myosinlösung durch eine Kapillare in Wasser eingelassen wird, das ausströmende Myosin in Form eines fadenförmigen Gels erstarrt. Nach WEBER sind diese Fäden elastisch und 100—300% dehnbar.

Ich habe mich bereits früher 2.) 3.) mit den Eigenschaften solcher Fäden beschäftigt und konnte WEBER's Angaben vollauf bestätigen. Bei der Wiederaufnahme des Problemes hatten wir Fäden nötig die ATP. spalteten. Obwohl die Phosphatase-Aktivität des Myosins äusseren Eingriffen gegenüber nicht empfindlich ist und Myosin bei 0° ohne Verlust der Fermentaktivität wiederholt pezipitiert werden kann, fand ich unsere Fäden fermentativ unwirksam. Die Analyse zeigte, dass unser Myosin durch die, im destillierten Wasser anwesenden Metalle, in erster Linie durch das Kupfer inaktiviert wurde. Cu wird durch das Myosin gebunden und angereichert. Aus diesem Grunde wurden die Versuche derart wiederholt, dass für alle Zwecke nur destilliertes Wasser zur Verwendung kam, das aus Glasgefässen zweimal umdestilliert wurde. Diese Fäden, auf die sich alle Angaben dieses Bandes beziehen, sind fermentativ aktiv, kontrahieren mit ATP in Gegenwart von Salzen, sind aber locker, brüchig, unelastisch, können nicht mehr als 10—15% gestreckt werden ohne zu zerreissen.

Wird dem Wasser, in dem man die Fäden zieht, 0,001 mol $CuCl_2$ zugefügt, so erhält man wieder Fäden, die elastisch, 100—300% streckbar, aber fermentativ unwirksam sind.

Methodisches über die Herstellung von Myosinfäden.

Myosinfäden können am einfachsten derart hergesstellt werden, dass man die Myosinlösung in ein Glassrohr aufsaugt dessen eines Ende zur Kapillare ausgezogen ist. Man lässt nun das Myosin in einer flachen Schale in Wasser oder in verdünnte Salzlösung einlaufen und bewegt das Glasrohr hin her. Bei visköseren Lösungen muss noch ein Druck aufgesetzt werden: am einfachsten verbindet man das dicke Ende des Rohres mit einem Gummischlauch, in den man einbläst. Mit einiger Übung

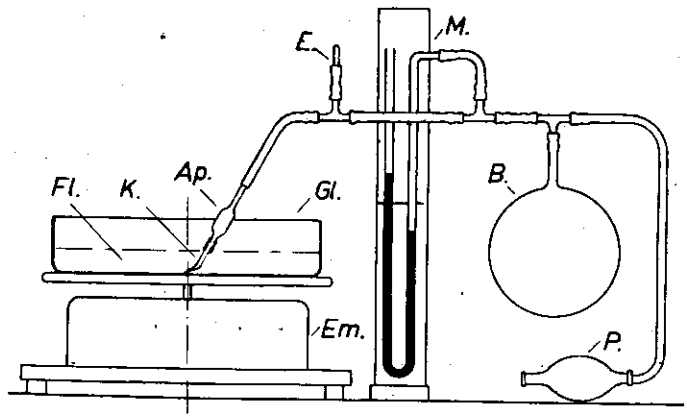

Fig. 1.

kann man in dieser Weise ziemlich gerade und gleichmässige Fäden herstellen. Die Dicke der Fäden hängt vom Diameter der Kapillare, der Viscosität der Lösung, der Auslaufsgeschwindigkeit und von der Geschwindigkeit ab, mit der man das Rohr bewegt. Am bessten eigneten sich für unsere Zwecke Fäden von etwa 0,2—0,3 mm. Diameter.

Für viele Fragen sind aber die, in dieser Weise hergestellten Fäden nicht geichmässig genug. In solchen Fällen müssen die Fäden mechanisch gezogen werden. Die von uns gebrauchte Einrichtung ist in beistehender Figur 1 abgebildet. Diese besteht aus einem drehbaren Tisch der durch einen Motor (Em) langsam ca 3 Rev. per Min. gedreht wird. Die Geschwindigkeit ist regulierebar. Auf diesen Tisch wird, wohl zentriert, das Wasser oder die vedünnte Salzlösung in einer weiten, flachen Schale (Ge) gelegt. Die Auslaufskapillare (K) wird mit

einem kleinen Reservoir verbunden (Ap), das mit der Myosinlösung gefüllt wird. Der Druck über der Myosinlösung wird mit einer kleinen Gummipumpe (P) hergestellt, am eingeschalteten Manometer (M) kontrolliert und mit Hilfe eines eingeschalteten Gummiballes (B, innere eines Fussballen) stabilisiert. Der Druck konnte durch öffnen bei E aufgehoben werden. Die Kapillare und das Reservoir sind verschiebbar auf einen Querbalken montiert so, dass sie nach einen jeden Umlauf des Tisches verschoben werden können. Der Druck wird erst aufgesetzt und das Fadenziehen begonnen nachdem die Flüssigkeit im Gefäss die Bewegung des Tisches aufgenommen hat. Die Kapillare ist schräge gestellt, es ist aber auch vorteilhaft ihr Ende etwas umzubiegen, um dieses noch mehr in die Auslaufsrichtung zu bringen. Das Ende der Kapillare ist etwa 1—2 cm. vom Boden der Glasschale entfernt. Der Faden setzt sich in Form einer Spirale auf den Boden des Gefässes und kann von diesem, nachdem er genügend erstarrt is, leicht abgetrennt werden.

Je nach Natur des Myosins können Fäden aus 0,5—5% Myosin gezogen werden. Zur Untersuchung der Kontraktion eignen sich Fäden vom 1—2% am bessten. Die aus konzentrierteren Myosinlösungen hergestellten Fäden ziehen sich minder gut zusammen, verdünntere Lösungen geben zu lockere Fäden. Myosin B Lösungen über 5% sind zu viskös um überhaupt verarbeitet zu werden.

Material.

Fäden können unmittelbar aus dem 24 stündigen Extract (s. BANGA and SZENT-GYÖRGYI) gezogen werden, wenn der Extrakt mit Salzlösung im Verhältniss von 100:80 werdünnt und zentrifugiert wurde. Eine konzentrierte Myosinlösung lässt sich erhalten, wenn man den Extrakt mit einer geringen Menge von ATP (0,014%) verflüssigt, rasch zentrifugiert, die Flüssigkeit abgiesst und wartet bis diese wieder gelatinisiert d. h. das zugesetzte ATP gespallten ist.

Der 20 Min. Extrakt, wenn frisch zubereitet, gibt überhaupt keine Fäden obwohl es etwa 2% Myosin enthält. Das Myosin fällt flockig aus, oder zerfliesst am Boden des Gefässes. Der Grund hierfür liegt in der Anwesenheit von ATP das in Ge-

genwart der hohen Salzkonzentration verflüssigend wirkt. Um Fäden aus dem 20 Min Extrakt zu erhalten muss man diesen erst über Nacht im Eisschrank stehen lassen, in welcher Zeit das ATP gespalten wird. Aber selbst dann geben die Auszüge meistens keine Fäden, da das Myosin A, aus dem das Myosin dieser Auszüge vorwiegend besteht, viel schwächere Kohäsionskräfte hat, wie das Myosin B des 24 Stündigen Extraktes. Um aus dem 20 Min. Extrakt Fäden zu erhalten muss der Extrakt neutralisiert werden. Ich gebrauchte zur Neutralisierung 5% Essigsäure. Die berechnete Menge der Säure wurde zugesetzt und die Lösung sehr energisch umgerührt. Die Neutralisierung hat keinen Einfluss auf die Kontraktionsfähigkeit der Fäden. Ohne Neutralisierung lassen sich nur sehr dünne Fäden u. zw. bei sehr kleiner Auslaufsgeschwindigkeit herstellen.

Die Myosinextrakte sowie die Fäden können bei O° mehrere Tage bis über eine Woche ohne Verlust an Aktivität bewahrt werden.

Sollten Fäden aus gereinigtem Myosin hergestellt werden, so wurden die Extrakte anlehnend an J. T. EDSALL, mit 5-fachem Vol. Wasser verdünnt und neutralisiert, das Prezipitat abzentrifugiert, mit Wasser gewaschen, dann wieder in Salzlösung gelöst. Das Salz wurde in Form eines feinen Pulvers oder in Form einer konzertrierten Lösung zugesetzt. Zur Homogenisierung liess ich die Lösungen vor Gebrauch einige Stunden stehen und entfernte die Luftbläschen durch Zentrifugieren. Alle Manipulationen wurden bei O° ausgeführt. Das Myosin kann unter diesen Umständen wiederholt umgefällt werden, ohne seine Aktivität zu verlieren.

Das an der Zentrifuge abgeschiedene Myosin ist zu locker um eine genügend konzentrierte (1—2%) Myosinlösung zu geben. Um eine solche zu erhalten wurde das Prezipitat bei -15° befroren. Nach auftauen lässt sich das Myosin leicht auf ein geringes Volum bringen. Oft genügt es schon das aufgetaute Prezipitat mit einem Glasstabe zu zerschlagen, wonach man das Wasser einfach ablaufen lassen kann. Das Myosin soll aber nicht zulange in gefrorenem Zustande gehalten werden da es sonst körnig wird.

Die Fäden, die aus derart gereinigtem Myosin hergestellt wurden, kontrahieren ebenso als di aus dem primären Extrakt hergestellten. Werden aber die Fäden aus derart gereinigtem

Myosin in destilliertem Wasser hergestellt, so schwellen diese nach einiger Zeit enorm an. Dieses Schwellen kann durch geringe Mengen von Neutralsalzen hintangehalten werden. 0,002 mol KCl oder 0,0001 mol CsCl ist deutlich aktiv. Aus diesen Gründen haben wir Fäden aus prezipitiertem Myosin stäts in 0,1 mol KCl und nicht in Wasser hergestellt. Wird die Myosinlösung vor dem Fadenziehen neutralisiert, so wird kein Schwellen beobachtet und der Faden kann besser in Wasser gezogen werden.

Untersuchung der Fäden.

Zur Beobachtung und Messung der Kontraktionerscheinungen bringt man am bessten kurze, etwa 3 mm lange Stückchen des Fadens in der betreffenden Lösungen auf ein ausgehöhltes Objektglas. Der Faden kann dann unter dem Mikroskop beobachtet und mit dem Okularmikrometer gemessen werden. Wir gebrauchten besonders dicke, tief ausgehölte Objektgläser.

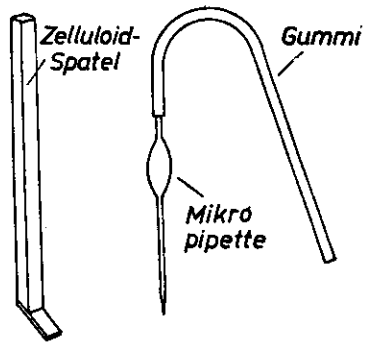

Fig. 2.

Soll die Länge des Stückes gemessen werden, so ist es wichtig dass die Schnittfläche an den Enden scharf sei. Zur Herstellung und Übertragung derartiger Faderstücke eignet sich ein aus Celluloid hergestellter Spatel besonders gut. (Fig. 2. a.) Das Myosin klebt zum Celuloid nicht. Der etwa 3 mm breite Spatel wird unter den Faden gelegt, etwas aufgehoben, dann streift man mit einem gebogenen, dünnen Glasstäbchen über die Ränder, wobei man den Faden abschneidet. Das abgeschnittene kleine Stückchen liegt am Spatel und kann mit diesem leicht aus der Flüssigkeit gehoben und auf das Objektglas übertagen werden.

Sollte die Kontraktion auf Zugabe von ATP in verschiedenen Salzen verfolg werden, so wurde die abgemessene Menge der Salzlösung (0,35 ml.) auf das Objektglas gebracht und das ATP (0,05 ml) aus einer kleinen, zur Kapillare ausgezogenen Pipette zugesetzt, (Fig. 2 b.) u. zw. derart, dass die Lösung

in einem dünnen Strahl energisch eingeblasen wurde. Wird dann noch Luft auf die Oberfläche der Flüssigkeit geblasen so wird die Lösung hierdurch energisch durchgerührt, was sehr wichtig ist. Es ist auch wichtig darauf zu achten, dass der Faden sicht nicht an das Glas anhafte und nicht am Rande, sondern frei in der Flüssigkeit liege, also von allen Seiten der Diffusion frei zugänglich sei.

In den Arbeiten vorliegenden Bandes wurde die Kontraktion stäts mit % der Verkürzung ausgedrückt. Diese Ausdruckweise ist bequem aber irreführend. Die richtige, aber mehr komplizierte Weise wäre die Kontraktion durch Messen des Volumes des Fadens auszudrücken.

Dies sei mit folgendem Beispiel erleuchtet: nehmen wir an dass wir die Kontraktion zwier Fäden vergleichen. Der eine Faden verkürzt sich (isodiametrisch) um 25, der ander um 66%. Man wäre also geneigt zu denken dass letzterer sich bloss 2,5-mal mehr kontrahierte als ersterer. Wird aber das Volum berechnet, so findet man, dass während der erste Faden sich nur um etwa 40% zusammenzog, letzterer eine Kontraktion von 97% zeigte. Handelte es sich um Fäden, die ursprünglich 1,5% Myosin enthielten so verlor der erste Faden 40% letzterer 98,5% seines Wassers. Während der Myosingehalt im ersteren Faden von 1,5 auf 2,5 stieg, stieg der Myosingehalt im letzteren auf 50%. Dieser letztere Faden besteht nun also praktisch genommen aus festem, benetztem Myosin.

Diese Berechnung erklärt auch warum in dem Vessuchen über die in diesem Bande berichtet wird, die maximale Kontraktion nie 66—70% überschritt. 66% Kontraktion wird sehr oft gefunden, aber kaum überschritten. Zugleich verstehen wir warum der kontrachierte Faden undurchsichtig wird.

Doppelbrechung und Kontraktion.

Die Myosinfäden, die in der beschriebenen Weise hergestellt wurden ziehen sich in der entsprechenden Salzlösung auf Zugabe von ATP in allen Richtungen gleichmässig zusammen, d. h. die Kontraktion ist isodiametrisch. Fig. 3. gibt die % Kontraktion eines Myosinfadens in 0,1 M KCl. Wie ersichtlich bleibt Kontraktion in der Querrichtung kaum hinter der Kontraktion der Längsrichtung zurück. (Abszisse: Zeit in Min.)

Dieses von der Muskelfaser abweichende Verhalen der Myosinfäden ist dem Umstande zuzuschreiben, dass die Myosinmicellen im Faden, im Gegensatzt zum Muskel, nicht, oder nur ganz schwach geordnet sind. Selbst wenn sich die einzelnen Micellen anisodiametrisch zusammenziehen muss die Kontraktion des Fadens isodiametrisch werden wenn die Micellen nicht geordnet sind. Meistens hat aber der Faden eine sehr geringe Doppelbrechung, also eine ganz schwache Orientierung der Micellen und darum bleibt auch die Kontraction im Querdurchmesser hinter der Verkürzung meistens etwas zurück (Fig 3).

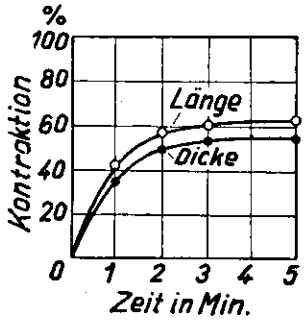

Fig. 3.

Um die Micellen im Faden zu orientieren und somit die Verhältnisse des Muskels, so gut wie mőglich, nachzuahmen, muss man den Faden spannen. Dann ordnen sich die Micellen koaxial mit dem Faden. Diese Orientierung kann durch Beobachtung der Doppelbrechung messend verfolgt werden.

Leider aber können unsere, in Wasser gezogenen Fäden nicht oder nur sehr unwesentlich (10—15%) gesteckt werden, da sie zerreissen. Die in gewőnhlichem destillierten Wasser oder $CuCl_2$ hergestellten Fäden sind dehnbar sind aber inaktiv und geben keine Kontraktion mit ATP (s. Tab. 1.). Bis zu einem gewissen Grade können die in KCl gezogenen Myosin Fäden derart orientirt werden, dass man sie auf ein Objektglas bringt, mit einem Deckglas bedeckt und durch Bewegen des letzteren die Fäden hin und herrollt. Der Faden zerreist hierbei, aber die entstandenen fadenförmigen Fragmente zeigen eine ausgesprochene Doppelbrechung. Auf Einwirkung von ATP werden diese Fadenstücke kürzer und gleichzeitig um etwa 15—20% dicker. (S. Tab. I.)

Ich bestrebte mich Verhältnisse zu finden unter denen dehnbare und doch aktive Fäden erhalten werden können. Ich versuchte Fäden an Stelle von Wasser, in verschiedenen Salzlősungen oder organischen Lősungsmitteln herzustellen. Nachdem die frisch gezogenen Fäden 5 Min. lang in der betreffenden Lősung verweilten untersuchte ich ihre Dehnbarkeit, dann über-

Tabelle I.

Lösung	Dehnung	Doppelbr. vor der Dehnung	Doppelbr. nach der Dehnung	ATP — Wirkung	
				Länge %	Dicke %
0,1 KCl	10—15%	—	In Spuren	— 62	— 53
0,001 $CuCl_2$	200%	$0{,}33.10^{-4}$	$18{,}5.10^{-4}$	—	—
0,1 KCl gewalzt	—	—	$7{,}5.10^{-4}$	— 7	+ 15
25% glycerin	200%	—	$15{,}6.10^{-4}$	— 16	+ 20
0,001 $ZnSO_4$	200%	In Spuren	$11{,}6.10^{-4}$	— 30	+ 55

trug ich sie in 0,1 mol KCl und untersuchte nach weiteren fünf Minuten unter ATP Zugabe (0,18%) ihre Kontraktonsfähigkeit. Zur Untersuchung der Dehnbarkeit gebrauchte ich den in Fig.

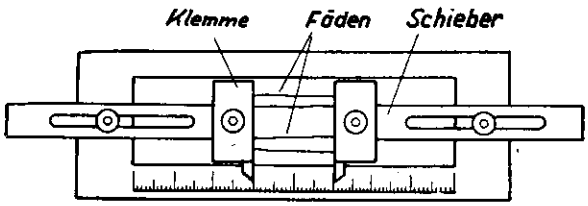

Fig. 4.

4 abgebildeten Apparat. Die Fäden wurden an beiden Ende zwischen zwei Plättchen eingeklemmt. Dann wurden die Schieber auseinander gezogen. An der Skala konnte die Dehnung abgelesen werden, bei der die Fäden rissen.

In Glycerin gezogen, werden die Fäden dehnbar ohne ihre Kontraktilität zu verlieren. Am bessten eignet sich 25% Glycerin, die Kontraktilität geht aber auch in reinem Glycerin nicht verloren. In reinem Glycerin steigt aber die Dobbelbrechung auf Dehnen nicht.

Die untersuchten Kationen können in drei Gruppen geteilt werden (s. Tab. II.):

1. Die die Aktivität nicht vermindern aber auch die Dehnbarkeit nicht steigern (Li, Na, K, Mg, Mn.)

2. Die die Dehnbarkeit steigern aber die Aktivität vernichten, also das Myosin denaturieren (Cu.)

3. Die die Dehnbarkeit bei gewissen Konzertrationen stei-

Tabelle II.

Lösung	Norm. Konz.	Dehnbarkeit	ATP — Kontraktion % (Länge)
LiCl	0,1	20	52
	0,01	20	60
	0,001	30	58
NaCl	0,1	—	55
	0,01	—	51
	0,001	20	58
KCl	0,1	10	62
	0,01	10	62
	0,001	10	62
$CaCl_2$	0,1	10	59
	0,01	20	55
	0,001	20	55
$MgCl_2$	0,1	Zerfliesst	—
	0,01	15	58
	0,001	20	55
$MnSO_4$	0,1	—	43
	0,01	—	50
	0,001	—	00
$CuCl_2$	0,1	203	—
	0,01	230	—
	0,001	80	6
$ZnSO_4$	0,1	130	10
	0,01	220	38
	0,001	75	40
$Al_2(SO_4)_3$	0,1	100	14
	0,01	100	21
	0,001	10	47
$FeCl_3$	0,1	40	17
	0,01	95	23
	0,001	30	40
$Co(NO_3)_2$	0,1	30	44
	0,01	30	54
	0,001	20	50
$NiSO_4$	0,1	70	42
	0,01	60	44
	0,001	50	40

gern aber die Aktivität nur in geringerem Masse vermindern (Zn, Al, Fe, Co, Ni). Natürlich ist der Unterschied kein absoluter und bei höherer Konzentration wirken auch diese Katione denaturierend.

Es ist scheinbar unmöglich Ione zu finden, die den Faden dehnbar machen und die Kontraktionsfähigkeit ganz unbeeinflusst lassen. Die Dehnbarkeit scheint bereits ein Ausdruck der Denaturierung zu sein.

Am günstigen fand ich die Verhältnisse bei 0,001 N $ZnSO_4$,

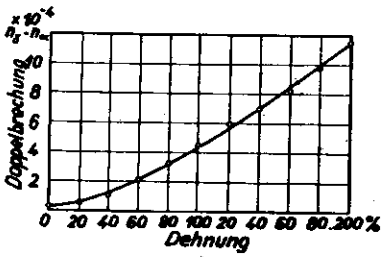

Fig. 5.

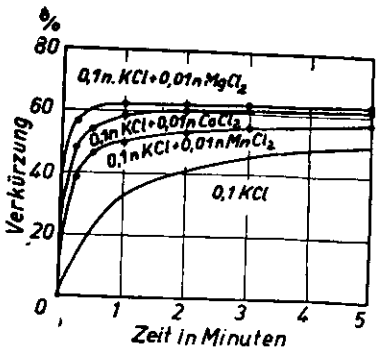

Fig. 6.

In der Fig. 5. wird der Zusammenhang zwischen Dehnung und Doppelbrechung gegeben.

Werden die gestreckten Fäden losgelassen, so ziehen sie sich wieder zusammen, die Dehnung ist also elastisch. Werden aber die Fäden 10 minuten lang in gedehntem Zustande gehalten, dann losgelassen, so behalten sie ihre länge. Offenbar entwickeln sich zwischen den benachbarten Micellen neue Verbände. Werden nun die also „gesetzen" Fäden in 0,1 KCl übertragen, hier 5 Min. lang belassen, dann ATP zugesetzt, so verkürzen sie sich, so wie der Muskel, unter gleichzeitiger Zuhnahme ihres Querdurchmessers (s. Tab. 1.) Gleichzeitig verschwindet ihre Doppelbrechung.

Anlässlich dieser Versuche untersuchte ich ob die Wirkung von Mg auf die Kontraktion spezifisch sie. Wie durch Szent-Györgyi beschrieben, kann die aktivierende Wirkung von KCl durch $MgCl_2$ befördert werden. Ich fand, dass Mg in diesen Versuchen durch Mn and Co ersetzt werden kann. Das Ergebniss meiner Versuche ist in beistehender Fig. 6 zusammenge-

fasst. Ohne KCl geben $CoCl_2$ and $MnCl_2$, so wie das $MgCl_2$, mit ATP nur eine schwache und langsame kontraction.

Zusammenfassung.

Technik der Herstellung und Untersuchung von Myosinfäden wird beschrieben.

Es wird gezeigt, dass Myosinfäden, in üblicher Weise hergestellt, sich isodiametral zusammenziehen. Gesteckte Fäden, in denen die Myosinmicellen koaxial zur Fadenaxe orientiert sind, ziehen sich anisodiamatral zusammen: werden gleichzeitig kürzer und dicker.

Ähnlich wie im Muskel verschwindet die Doppelbrechung während der Kontraktion.

Die Wirkung verschiedener Ione auf Dehnbarkeit und Kontraktilität wird untersucht.

Es wird gezeigt dass Mn und Co bei der Kontraktion das Mg ersätzen können.

Litteratur.

1. *H. H. Weber:* Arch. ges. Physiol. *235*, 205, 1934.
2. *M. Gerendás* and *A. Szent-Györgyi:* Enzymologia, 9, 117, 1940.
3. *M. Gerendás:* Ibid. p. 123.

The influence of K and Mg on the contraction of myosin.

by

T. Erdős

(Chinoin research student).

It has been shown by SZENT-GYÖRGYI that myosin B threads are capable of giving a contraction in the presence of adenyltriphosphate („ATP") and KCl. The rate of contraction was greatly increased by $MgCl_2$. At higher salt concentrations and in the presence of ATP, dissolution was obtained instead of contraction. Myosin A was found to be relatively inactive.

The object of this paper is to obtain more detailed information about the influence of salt concentration. For the sake of convenience I also will call the threads prepared from the 20 min. extract (see BANGA and Sz.) myosin A threads and those prepared from the 24 h. extract, myosin B threads, although the 20 min. extract always contains small quantities of myosin B and the 24 h. extract may contain some myosin A.

For the preparation and measurement of threads I used the technique of M. GERENDÁS.

Effect of varied KCl concentrations.

Table 1 gives the result of an experiment with varied KCl concentrations. The experiment was made on two different threads which I will call *a* and *b*. *a* was prepared from muscle extract which had been stored for two days, *b* from an extract which had been stored for ten days at O°C. It will be seen that storage makes no difference.

The freshly prepared threads were allowed to stand for 30 min. at room temperature and were then transeferred into the corresponding salt solution and measured. After 5 min. ATP (0,09%) was added and readings made 5 min. after that.

Table I.

Mol KCl	Myosin B				Myosin A			
	I.		II. + 0,001 mol $MgCl_2$		III.		IV. ATP + KCl simult	
	a	b	a	b	a	b	a	b
0.10	66	66	66	66	23	20	14	23
0.17	70	58	50	66	19	9	17	16
0.18	59	62	64*	56*	16	14	13	15
0.19	69	66	59*	66*	—	—	—	—
0.20	60	59	?	?	—	—	—	—
0.21	45	66	—	—	—	—	—	—
0.22	66	60	—	—	—	—	—	—
0.23	58	62	—	—	—	—	—	—
0.24	67	62	x	—	—	—	—	—
0.25	66	66	x	x	—	—	—	—
0.26	61	63	x	x	—	—	—	—
0.27	69	57	x	x	—	—	—	—
0.28	58	61	x	x	—	—	—	—
0.29	66*	70*	x	x	—	—	—	—
0.30	52*	59*	x	x	—	—	—	—
0.31	30*	30*	x	x	—	—	—	—
0.32	?	?	x	x	x!	x!	—	—
0.33	?	x	x	x	x!	x!	—	—
0.34	x	x	x	x	x!	x!	—	—
0.40	x	x	x	x	x!	x!	—	—
0.41	x	x	x	x	x!	x!	x!	x!

The whole experiment was repeated also in the following way: the thread was put directly into the salt-ATP mixture instead of putting it into the salt solution first and adding ATP afterwards. This made no appreciable difference with myosin B but changed the behaviour of the myosin A threads considerably. For this reason I am giving the effect of ATP and varied KCl concentrations on myosin A with the simultaneous addition of both substances.

The result of this experiment agrees with the result of other similar experiments except for a small variation of the KCl concentration at which the changes from inactivity to contraction or to dissolution occure. The experiment was also repeated with purified myosin which gave the same results except for the slighlty greater solubility of myosin A which dissolved in KCl at 0,28 mol. instead of 0,32. The experiment was also repeated with threads which had been strored for 12 hours at room temperature (25° C). This made no difference to myosin B. Myosin A threads lost their contractility.

Columm I and II give the data for myosin B, col. I. with pure KCl, col. II in the presence of 0,001 mol. $MgCl_2$. Col. III and IV relate to myosin A in pure KCl. In col. III the thread was put into KCl and ATP added afterwards. In col. IV KCl was added simultaneously with ATP.

The numbers give the % of shortening. The asterisks mean that the threads were very fragile and prone to fall into pieces if agitated. *x* means dissolution. *x!* means that the thread dissolved in the salt solution before the addition of ATP. *?* means a doubtful result: partial contraction and partial dissolution. — means inactivity, i. e. that there was no contraction or dissolution.

If we go over col. I we will notice a variation in the numbers, in spite of the fact that the contraction is evidently equally strong from 0,1 to 0,29 mol. KCl. This variation is due partly to errors of measurement and to a greater extent, to the different reaction of different threads; also to local conditions surrounding the thread which inhibited diffusion (too close proximity of the glass). So no great value should be attached to single measurements, only the general trend is of importance.

Now if we go over col. I we will find equally strong contractions from 0,1 mol. KCl up to 0,30. At 0,31 mol. the contraction is weaker and at 0,32 part of the thread dissolves, part of it contracts. At 0,34 mol. KCl there is dissolution only. (Whithout ATP the thread is not dissolved, not even by mol. KCl).

It will be seen from this that the change from maximal contraction to complete dissolution is not any too sharp and takes place within a range of 0,04 mol. (0,3—0,34). The KCl concentration, at which the change takes place, is fairly high.

Now if we compare this with col. II it will be seen that the 0,001 mol $MgCl_2$ present decreased the KCl concentration necessary for the dissolution of the thread, greatly. An entirely new phenomenon appears also: between contraction and dissolution there is a zone in which the thread neither contracts, nor dissolves, but is just inactive. The myosin has thus three different states: the contracted, the inactive and the dissolved state. The transition from one state into the

other is sharp and takes place within a concentration difference of 0,01—0,02 mol. The experiment gave the same result in the presence of 0,01 mol. $MgCl_2$ as with 0,001.

Col. III shows that myosin A behaves quite differently. With smaller KCl concentrations it gives the usual weak contraction. Then follows a wide zone of inactivity even without $MgCl_2$. At 0,32 mol KCl we find dissolution without the addition of ATP. From 0,19 mol KCl upwards ATP has thus no effect at all. The KCl concentration at which, in the presence of Mg, myosin B still gives a maximum contraction (0,19 mol.) myosin A is entirely inactive. Addition of 0,001 Mg made no difference.

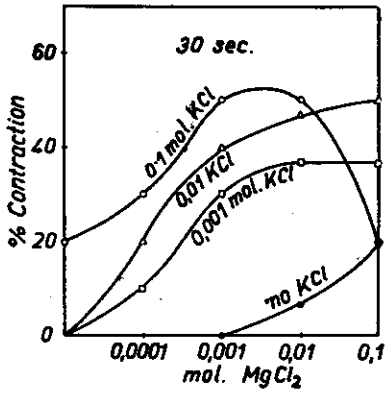

Fig. 1.

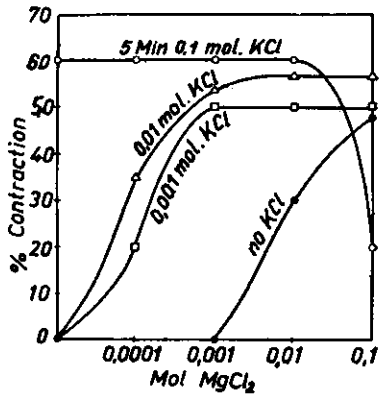

Fig. 2.

Col. IV shows that if ATP is added simultaneously with KCl, the zone of inactivity is very much extended and much higher KCl concentrations are necessary for the dissolution of the thread.

If the ATP is added first, and allowed to act for 5 minutes and the KCl then added, the effect of sequence is stronger still and the thread dissolves only in 0,47 mol. KCl while it is still inactive in 0,46.

Varied KCl and $MgCl_2$ concentrations.

In fig. 1. and 2. I am giving the effect of varied KCl and $MgCl_2$ concentrations on a myosin B thread. The thread was allowed to stand for five minutes in the salt solution and the ATP then added (0,09%). Readings were made 30 sec. (fig. 1) and 5 min. (fig. 2) later.

It will be seen that KCl, without $MgCl_2$ is inactive in a 0,01 or a 0,001 mol. concentration. 0,1 mol. KCl gives a relatively slow but strong contraction. On the other hand 0,001 mol. $MgCl_2$, without KCl is inactive, while 0,01 mol. $MgCl_2$ shows a slow, weak action and 0,1 gives a slow, but fairly strong contraction.

In the presence of 0,001 mol. $MgCl_2$ even 0,001 mol. KCl becomes strongly active while in the presence of 0,01 mol KCl even 0,0001 mol. $MgCl_2$ gives a strong contraction.

Effect of varied ATP concentrations.

I am giving the effect of varied KCl and ATP concentrations on a myosin B thread prepared from precipitated myosin in Tab. II. Owing to the higher myosin content the thread was somewhat less contractile than the thread of Tab. I.

Table II.

% ATP	0,4 KCl	0,3 KCl	0,2 KCl	0,1 KCl	0
0,7	x	x	x	48	50
0,35	x	x	41	47	35
0,18	x	x	46	47	8
0,09	x	x	42	44	0
0,045	x	x	32	21	0
0,022	x	x	22	15	0
0,011	x	2	12	6	0
0,006	4	0	8	0	0
0,003	0	1			

The thread was allowed to stand for 5 minutes in the salt solution, then the ATP was added and readings made 5 min. later. It will be seen that at a certain KCl concentration we obtain the same effect at all ATP concentrations, *i. e.*, contraction or dissolution. (The dissolution in 0,7% ATP at 0,2 mol. KCl, ist due to the additional KCl introduced with the ATP.) The nature of the action is thus independent ot the ATP concentration. Either we obtain contraction or dissolution or no effect at all.

In the absence of KCl, ATP is inactive in concentrations in which it still gives a maximal effect in the presence of KCl. This justifies the conclusion that the contraction observed in

the highest concentrations of ATP is due to the K present partly as the kation of the ATP and partly as a KCl impurity and that ATP in itself, without K, is inactive.

Varied concentrations of the muscle extract.

The fresh, minced muscle was suspended in water, 1 ml. of water being taken per g of muscle. The suspension was stored over night at 0° and filtered next day, first through a cloth and then through paper. The neutral juice was diluted with water; the dilution is given on the abscissa of Fig. 3. The myosin B threads were placed into the solution for 5 minutes and then 0,09% of ATP was added. Readings were made after 30 seconds. As will be seen the juice gives, even in 1/4 dilution, a maximal contraction. With further dilution the effect becomes wearker and eventually disappears. It may be concluded thus that the juice obtained in the described way contains the ions necessary for maximal contraction in excess.

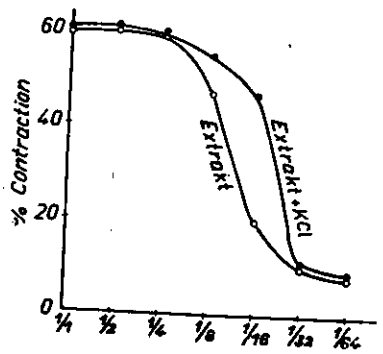

Fig. 3.

In a second experiment 0,1 mol KCl was added to the same juice. It will be seen (Fig. 3) that even at a dilution of 1/4 the effect of the extract is not increased by this addition of KCl thus there was K present in sufficient concentration to give maximal contraction. At a higher dilution of the extract the contraction is increased showing that there is still a sufficient concentration of Mg to enhance the action of KCl. At a dilution of 1/32 the Mg becomes insufficient too.

Variation of pH.

Some time ago I studied the action of salts on the isoelectric point of casein. The isoelectric point of casein is shifted by neutral salts and a precipitation or a dissolution can be obtained by their addition, at certain pH-s. It seemed

possible that the precipitating and dissolving action of salts and ATP in the case of myosin could be explained in a similar way. For this reason I studied the influence of pH on the physical state of myosin in the presence of different concentrations of KCl, with and without ATP, Mg and muscle extract. Contrary to my expectation I found that KCl did not shift the range of insolubility at lower concentrations to either side of the pH scale, nor did the ATP or Mg or muscle extract. The contraction, precipitation and dissolution of myosin can thus not be explained by a shift of the insolubility range on the pH scale.

I want to give the chief result of this very extensive work in a few words only. Threads prepared from precipitated, washed and redissolved myosin A and B were placed into the corresponding solutions, the pH of which was adjusted by the addition of HCl or KOH and controlled colorimetrically. Solutions of pH 2-pH 11 were prepared. After 5 min. the effect was observed.

Myosin B threads begin to dissolve above pH 8,5 and below pH 4. At pH 9,5 and 2,5 dissolution is complete. The range of insolubility lies thus between pH 4 and 8,5. This range is not shifted to either side by KCl not even by KCl in a 0,2 mol. concentration. At 0,4 mol. there is a slight shift towards the smaller pH: the thread begins to dissolve above pH 8 and is insoluble even at pH 2. ATP alone or in the presence of 0,25 mol KCl makes the thread insoluble on the acid side but has no effect on the alkaline side. At higher KCl concentrations ATP makes the thread somewhat more insoluble on the alkaline side too. The thread dissolves only at a somewhat (0,5—1) higher pH than without ATP. At 0,4 mol KCl the ATP dissolves the thread at all pH-s except on the extreme acid side, pH 2—3. Mg or muscle extracts increase the effect of ATP without shifting the insolubility range to either side of the pH scale.

Myosin A is insoluble between pH 4—7.5 in the absence of KCl and ATP; its insolubility range is somewhat narrower than that of myosin B. Up to 0,3 KCl the range remains unchanged. At 0,4 mol KCl the thread is insoluble between pH 2—5,5 and soluble at higher pH's. Here too the ATP makes the thread insoluble on the acid side but has otherwise

little effect. Mg or extract is inactive, they only broaden the insolubility-range to some extent.

Miscellaneous observations.

A number of experiments were made with $CaCl_2$. In 0,01 mol concentration this salt inhibits the contraction given by ATP in the presence of KCl or KCl + Mg. This inhibition may be complete but could not be reproduced regularly. Several of our myosin pereparations were not inhibited or inhibited only if precipitated myosin was used for the preparation of the myosin threads.

The Ca-inhibition is reversible. If the Ca is washed out again the thread behaves normally.

The action of 0,1% nicotine, quinine and K-oxalate were also studied. None of these reagents had any inhibitory action on contraction. This is important because BANGA found that the splitting of ATP by myosin is completely inhibited by these reagents. Myosin can thus contract without splitting ATP.

Summary.

The contraction of myosin A and B threads is studied at varied KCl, $MgCl_2$, ATP and H^+ concentrations. Differences in the behaviour of A and B myosin are pointed out. It is shown that in the presence of ATP and Mg very slight changes in KCl concentration are sufficient to change the physical state of the myosin B thread and make the inactive thread contract or the contracted thread become inactive or dissolve.

Contraction is not inhibited by the complete inhibition of the phosphatase activity of myosin.

Discussion.

by

A. Szent-Györgyi.

It has been shown in the previous papers that myosin can be extracted from muscle in two forms: as the relatively inactive myosin A and the very reactive myosin B. The myosin of previous investigators, prepared by EDSALL's method, corresponds to our myosin A with a small admixture of myosin B as an impurity. We have shown that myosin A is transformed into B if it stands in prolonged contact with muscle particles.

If we want to correlate our data with muscle physiology, our first question must be: what is the relation of the two substances that we called myosin A and B and what is the nature of the A→B transformation.

Experiments of F. B. STRAUB, now in progress, difinitely show that myosin B is a stoichiometric compound of myosin A and another substance. We will call this other substance „actin" and the myosin-actin complex will be called „acto-myosin". Actin, in itself, is insoluble in salt solution. It is, together with cytochromoxidase and succinodehydrogenase, part of the insoluble muscle residue.

There in thus, according to these results of F. B. STRAUB which will be presented later, but one myosin, and the substance that we called myosin B is acto-myosin.

As has been shown by MOMMAERTS and STRAUB myosin B, just as myosin A, forms a well defined complex with ATP also. Myosin can therefore exist in four different forms: 1. as free myosin (myosin A), 2. as ATP myosin, 3. as acto-myosin (myosin B), 4. as ATP-acto-myosin. If we want to discuss the influence of salts on myosin we do better if we talk about the influence of salts on these four different forms.

In table I. I am giving the table of T. ERDÖS somewhat

Table I.

Mol. KCl + 0,001 mol. $MgCl_2$	Myosin	ATP-Myosin	Acto-Myosin	ATP-Acto-Myosin
0	—	—	—	—
0,01	—	+	—	+++
0,1	—	+	—	+++
0,17	—	+	—	+++
0,18	—	+	—	+++
0,19	—	+	—	+++
0,20	—	—	—	?
0,21	—	—	—	—
0,22	—	—	—	—
0,23	—	—	—	—
0,24	—	—	—	x
0,25	—	—	—	x
0,30	—	—	—	x
0,31	—	—	—	x
0,32	x	—	—	x
0,33	x	—	—	x
0,45	x	—	—	x
0,46	x	—	—	x
0,47	x	x	—	x
0,48	x	x	—	x
0,60	x	x	—	x

completed and translated into these new terms. The signs mean the same as in ERDŐS's paper: — means inactivity *i. e.* no contraction and no dissolution, × means dissolution; ? means partial contraction, partial dissolution. The % of contraction has been marked with crosses, +++ meaning maximal, + weak contraction. There was 0,001 mol $MgCl_2$ present everywhere.

It will be seen that free myosin is fairly soluble in KCl and gives no contraction. ATP-myosin is less soluble in salt and gives a weak contraction at a low KCl concentration. Acto-myosin is insoluble in salt and gives no contraction. By forming the ATP compound this complex becomes most sensitive to salts: according to the concentration of the salt present the ATP-acto-myosin may be inactive, maximally contracted, inactive again or dissolved, and all this at a KCl concentration below 0,25 mol.

If we try to apply this experience to muscle, the first question is whether in muscle the myosin is in sufficiently intimate contact with the actin to form a complex. It has been shown by BANGA and myself that in the absence of ATP the myosin of the muscle cannot be extracted with EDSALL's fluid, which means that it is insoluble in salt solutions and behaves thus like acto-myosin. I have shown that the myosin in the frozen and extracted muscle reacts as myosin B. It can therefore be stated that myosin is in sufficiently intimate touch with the actin in muscle to form acto-myosin.

In living muscle, under normal conditions, there is always a fairly high concentration of ATP which is kept constant throughout life. The living as well as the freshly minced muscle contains thus the highly sensitive ATP-acto-myosin.

Our viscosity measurements indicate that, at the high salt concentration of EDSALL's salt solution (0,6 mol KCl), the complex dissociates into actin and ATP-myosin. This helps us to understand what happens in the 24 h. extraction. If we suspend the muscle in EDSALL's fluid, the ATP-acto-myosin dissociates into actin and ATP-myosin. The former is insoluble in salt solution or is linked to the insoluble residue and will therefore remain undissolved while the ATP-myosin goes into solution. For this reason we always obtain myosin A from fresh muscle containing ATP whether the myosin, present in muscle, was bound to the actin and was thus present as myosin B or not. On storage the ATP is split and the dissolved ATP-myosin goes over into free myosin which forms acto-myosin with actin. This complex being stable even in the presence of 0,6 mol. KCl, the myosin which is already dissolved will, by the formation of this complex, bring the actin into solution. (Possibly the connections of the actin with the residue are loosened up meanwhile by the alkaline reaction). If we start with muscle free from ATP, the myosin will be present in the form of the stable and insoluble acto-myosin which is insensitive to salts, and so will not be extracted by the salt solution.

Evidence indicates thus that myosin is present in muscle as ATP-acto-myosin. As shown by the last column of the table, this complex can exist in different states which depend on the KCl concentration: if there is no salt present the

complex will neither contract, nor dissolve, i. e. it will be inactive; on addition of very small amounts of KCl (0,01 mol) we will get (in the presence of Mg) maximal contraction. The change from 0 to 0,01 mol. concentration will be sufficient to cause the inactive myosin to contract maximally. A similar jump in the opposite direction will be observed between 0,18 and 0,20 mol, the change of 0,02 mol in the KCl concentration being sufficient to cause a maximal effect. Any of these two changes might be analogous to what happens in muscle when the wave of excitation arrives and the muscle goes over from relaxation to contraction. It is not impossible either that relaxed muscle corresponds to our dissolved myosin which is formed at 0,24 mol. KCl.

Naturally I do not mean to say that there is KCl in muscle and that the change of the KCl concentration is the cause of contraction. This would be in contradiction with primitive facts. What I mean to say is that our experiments show that slight changes in ionic concentration are capable of inducing changes in the structure of the protein and that analogous changes in the protein might occur in muscular contraction.

As contraction is brought about in our threads by a disturbance of the ionic equilibrium, so, in its turn, contraction might act on the ionic balance. It does not seem impossible that in muscle the contraction of one ATP-acto-myosin unit might cause the disturbance responsible for the contraction of the next unit and so forth. In this case the sharp distinction between the wave of excitation and contraction would be unjustified.

No attempt has been made in this series of papers to explain the inner molecular mechanism of the contraction of myosin. In our experiments the changes in the physical state of the ATP-acto-myosin-complex have been brought about by salts and are thus, to some extent, analogous to other colloidal reactions. As has been shown by BANGA and ERDŐS the splitting of ATP is not involved in the developement of the contraction. The myosin becomes fermentatively active only when contracted. Translated into terms of muscle physiology this would mean that the energy of the splitting of ATP is used for relaxation; relaxed muscle again is inactive. This causes

adequate quantities of ATP to be split and only adequate quantities of energy to be liberated, since it is the function itself (contraction) which liberates energy. Naturally I do not mean to say that contraction, as such, activates phosphorilysis. The contraction as well as the enzymic activity are consequences and expressions of the same change in the finer structure of the molecule.

I want to close with a rather subjective remark. I was always led in research by my conviction that the primitive, basic functions of living matter are brought about by ions, ions being the only powerful tools which life found in the sea water where it originated. Contraction is one of the basic primitive functions and the results reported in this volume corroborate me in my conviction.

PS. After this volume was sent to press reprints have been received from *U. D'Ancona* (Protoplasma *17*,388,1932) who emphasises the dehydration of the muscle fibril during contraction. This is in agreement with our observations on myosin.

A reprint has also been received from *Fr. Verzár* (Schweiz. Med. Wochenschr. *72*, 661,1942). He stresses the importance of K in contraction and brings the known facts about K, carbohydrates and adrenals into one ingenious theory. He also quotes *J. Needham* and collaborators (Nature 147,766,1941) who found that the double refraction of flow of myosin disappears in presence of small concentrations of KCl and ATP. Very unfortunately this paper is not accessible here at present.

CONTENTS

Page

1. *I. Banga* and *A. Szent-Györgyi:* Preparation and properties of myosin A and B — — — — — — — — — — — — 5
2. *A. Szent-Györgyi:* The contraction of myosin threads — — — 17
3. *I. Banga:* The phosphatase activity of myosin — — — — — — 27
4. *W. F. H. M. Mommaerts:* Quantitative studies on some effects of adenyltriphosphate on myosin B — — — — — — — — 37
5. *F. B. Straub:* Reaction of myosin A with Adenyltriphosphate — — 43
6. *M. Gerendás:* Technisches über Myosinfäden nebst einigen Beobachtungen über ihre Kontraktion — — — — — — — — 47
7. *T. Erdös:* The influence of K and Mg on the contraction of myosin 59
8. *A. Szent-Györgyi:* Discussion — — — — — — — — — — 67