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MUSCULAR CONTRACTION, BLOOD COAGULATION.

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CONTENTS.

Abbreviations used in this volume.

ATP = Adenylpyrophosphate or adenosinetriphosphate.
ADP = Adenylphosphate or adenosinediphosphate.
DRF = Double refraction of flow.

In this volume two expressions are used for the characterisation of actomyosin:

1. *% Actomyosin.* By % actomyosin is meant the quantity of actin in 100 parts of actomyosin. So for instance, 16,7% actomyosin contains 16,7 g actin in every 100 g of actomyosin.

2. *% Activity.* By % activity of actomyosin is meant the fall of viscosity of actomyosin, (dissolved in neutral 0,6 M KCl) on addition of ATP — relative to the fall of viscosity of a 16,7% actomyosin. If we say, for instance, that an actomyosin is 100% active, this means, it shows the same fall of viscosity as a 16,7% actomyosin. (16,7% is the activity of our myosin B, see vol. I. of these studies.)

The actin content of an actomyosin can be calculated from its % activity by means of the curves given by F. B. STRAUB in this volume.

The autocatalytic formation of thrombin and the clotting defect of hemophilic blood.*

by

K. LAKI

The coagulation of blood is the conversion of a soluble protein, the fibrinogen, into the insoluble fibrin. This conversion of fibrinogen is brought about by the action of thrombin, which is formed from prothrombin during the process of blood clotting. The formatin of thrombin increases during the process of clotting and follows the type of an autocatalytic reaction.

This autocatalytic type of the formation of thrombin has aroused considerable interest among students of blood coagulation and many theories have been put forward to

* This work was aided by a grant from the Duke of ESTERHÁZY.

explain it. As knowledge about blood coagulation has increased and more and more components taking part in it have been isolated and investigated, it has become evident that the classical theory of blood coagulation is inadequate as an explanation of this autocatalytic type of the formation of thrombin. The processes known at present to be taking part in coagulation are not autocatalytic. Purified thrombin is not able to catalyse the thrombin formation from purified prothrombin. The conversion of fibrinogen into fibrin[1] is not autocatalytic nor is the formation of thrombin from prothrombin by the action of tissue kinase.[2]

The possibility that some hitherto unknown substance or mechanism is responsible for the autocatalytic formation of thrombin was indicated by the experiments of FISCHER[3] and especially of ASTRUP.[2] ASTRUP demonstrated that in the so called Mellanby-fibrinogen solutions the autocatalytic formation of thrombin was broken down and that even a slight acidification of the normal plasma was enough to destroy the autocatalytic formation of thrombin.

In a series of investigations performed in recent years on the hemophilic patients of the Clinic of Internal Medicin of Szeged, in collaboration with L. SZÉCSÉNYI NAGY, we compared the blood coagulation of normal persons with that of hemophilic patients.* Following quantitatively the formation of thrombin during the whole process of coagulation we came to the conclusion that hemophilic blood lacked the autocatalytic formation of thrombin and that the solution of the problem involved the necessity of elucidating the mechanism responsible for the autocatalytic formation of thrombin.

According to ASTRUP's and our findings Mellanby-fibrinogen and hemophilic blood resemble each other, both lacking the autocatalytic formation of thrombin.

It was found in the course of the investigation on the clotting of hemophilic blood that small amounts of fibrinogen prepared according to the method of the author in vitro restored not only the time of coagulation to normal but also

* A brief account of this work was read before the meeting of the Hungarian Physiological Society in 1942, and the detailed account will appear elsewhere.

the formation of thrombin. 0,01 cc. of fibrinogen solution containing 17 γ of protein was enough to restore the clotting of 1 cc. of hemophilic blood to normal.

Experiments soon revealed that some contaminating substance present in fibrinogen solutions is responsible for the effect. I call this substance *„plasmakinin."*

The resemblance between hemophilic blood and Mellanby-fibrinogen became closer when it was found that the same plasmakinin solution which restored the clotting of hemophilic blood to normal also accelerated the clotting of Mellanby-fibrinogen solution. The Mellanby-fibrinogen thus seemed to be a suitable material for the investigation of the mode of action of plasmakinin.

In the present communication the preparation of a crude solution containing plasmakinin will be described together with experiments showing the action of plasmakinin on Mellanby-fibrinogen. It will be seen that the addition of plasmakinin to Mellanby-fibrinogen leads to the formation of kinase, and that this formation of kinase increases in time. With the increase of kinase also increases the quantity of thrombin. Thus in view of these findings plasmakinin shows itself as an important factor in the formation of thrombin and the lack of it explains the hemophilic clotting defect.

Experimental.

Preparation of plasmakinin.

Oxalated cattle blood (containing 0,2% Na-oxalate) collected in the slaughter house was centrifuged and the clear plasma was sucked off. To the clear plasma saturated ammoniumsulfate was added in small portions until 0,25 saturation was reached. The precipitate which consisted mainly of fibrinogen was centrifuged. After discarding the supernatant plasma, the precipitate was brought into a solution of 0,7% NaCl (containing 0,2% Na-oxalate). Starting with 300 cc. of plasma, the precipitate was brought into 80 cc. of the NaCl solution. The precipitate dissolved slowly and gave a turbid solution. To remove the ammoniumsulfate this solution was dialysed in a cellophane tube against ten volumes of NaCl

solution for about 6 hours in the cold. The solution was then thoroughly centrifuged and used as PK_1 (first plasmakinin solution) in the experiments. 0,01 cc. of this solution given to 1 cc. of hemophilic blood is usually enough to restore the clotting time to normal.

Further purification was achieved by coagulating the fibrinogen. For this purpose 4 cc. of purified thrombin* solution were added to 80 cc. of PK_1 solution. Within an hour a firm clot was formed. The fibrin clot, after standing for a few hours in the ice chest was broken into pieces by means of a spatel then put on a cloth and the liquid, enclosed in the clot, pressed out. The fibrin-free liquid was left to stand in the ice chest; some further formation of a small amount of fibrin occured. Removing the clot by centrifugation a clear solution was obtained, which was almost free of fibrinogen and contained practically no thrombin. (The thrombin was probably adsorbed by the fibrin and was removed with it.) This solution (PK_2) often has the same activity as PK_1 though usually the activity is somewhat less. The preliminary experiments about the nature of this substance show that boiling to 100° destroys the activity of plasmakinin solutions. Warming to 50° for 5 minutes in neutral or slightly alkaline solution does not influence the activity but warming to this temperature in acid solution (N/40 acetic acid) or precipitation with alcohol or with acetone completely destroys the activity.

Preparation of Mellanby-fibrinogen (MF).

Cattle oxalate plasma obtained as above was gradually acidified by adding *N* acetic acid. During the course of acidification the pH was controlled colorimetrically. In order to test the pH 1 cc. of plasma was pipetted into test tube and 1 drop of bromocresolpurple** was added to it. Acetic acid was added till the colour of the dye became greenish-yellow. The acidified plasma was then poured into ten volumes of distilled water: a voluminous precipitate appeared which was allowed to settle

* 0,1 cc. of the thrombin solution gave a clotting time of 1' 30" when added to 1 cc. of oxalated plasma.

** 0,1 g. of bromocresolpurple dissolved in 18,5 cc. of *N*/100 NaOH was diluted with water to 250 cc.

overnight in the cold. The fluid was then sucked off and 15 cc. of Ca-free Ringer* solution were added to the precipitate for every 100 cc. of plasma which was used for the preparation. This turbid solution, (— made up to 40 cc. with water —) was allowed to stand at room temperature for about 12 hours and then centrifuged. The precipitate was discarded and the solution placed into the ice chest overnight during which time some formation of fibrin clot occured. This clot can easily be removed and after centrifugation a clear slightly yellow solution is obtained. This solution contains 3—6 mg. of fibrinogen and yields, on the addition of tissue kinase and calcium, as much thrombin per cc. as 1 cc. of the original plasma. 0,03 cc. of this solution added to 1 cc. of hemophilic blood reduces the clotting time only slightly, which shows that the active substance responsible for the normal clotting is greatly reduced in MF solutions.

The Mellanby-fibrinogen can be kept for weeks in frozen state and gives a clear solution when thawed. Storing in an ice chest is not very convenient owing to the formation of fibrin clot which must be removed before using the solution. In order to serve as a test solution for the action of kinase or plasmakinin the MF was diluted five times with *M*/20 borate buffer. This clear solution clots only after the addition of Ca and it takes usually 25 minutes before the first fibrin clot is detectable. If the clotting time is much shorter, then the original undiluted MF solution should be left overnight in an ice chest. After removing the fibrin clot which has been formed the solution can be treated as above and a suitable test solution will be obtained.

Kinase and plasmakinin have a similar effect on MF: both reduce the clotting time.

The action of tissue kinase on MF.

The clotting time of MF depends on the quantity of kinase used as demonstrated by fig. 1. The curve was obtained by plotting the clotting time against the quantities of kinase. The experiment was performed in the following way: Into small test tubes different quantities of very diluted brain

* The Ringer solution contained 0,23% $NaHCO_3$.

kinase* were pipetted. Then 1 cc. of recalcified MF solution was added after being diluted with borate buffer of pH 7,8. (The concentration of $CaCl_2$ was 10^- *M.*) One tube without kinase served as control. By shaking the tubes gently from time to time the appearance of the first fibrin clot can be observed sharply and the time of its formation measured. It is necessery to use test tubes made of the same glass because the glass wall

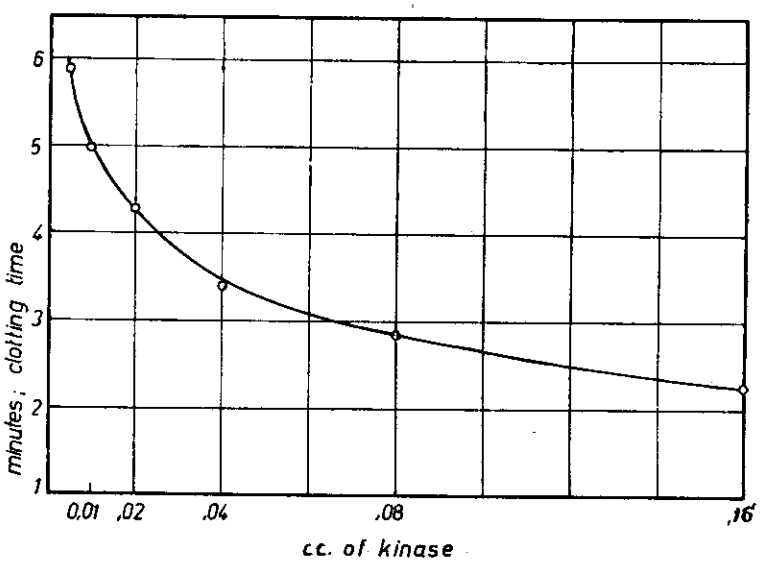

Fig. 1.

somewhat influences the clotting time of MF solutions. In all the experiments presented in this paper glass of Jena was used.

The concentration of Ca influences the clotting time to a great extent, the optimum is at 2×10^{-3} *M* $CaCl_2$. Fig. 2. gives the data of an experiment in which the concentration of Ca was varied and the kinase quantity kept constant. The experimental technic was the same as described above. The $CaCl_2$ solution and the kinase was pipetted into the tubes first, then 1 cc. of MF solution, which was diluted with borate buffer of pH 8,0 was added and the clotting time observed. In fig. 2. the clotting time is plotted against the $CaCl_2$ concentrations.

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* Rabbit's brain was extracted with an equal volume of 0,9% NaCl solution.

The action of plasmakinin on MF.

Like kinase, plasmakinin also catalyses the clotting of MF. Data of a similar experiment which was performed as above, using various quantities of PK_1 and PK_2 instead of kinase, are presented in table 1. It can be seen how the clotting time depends on the quantity of plasmakinin solutions. In the first column the dilution of plasmakinin is given and in the second the clotting time. PK_2 has somewhat longer clotting times indicating some loss in activity.

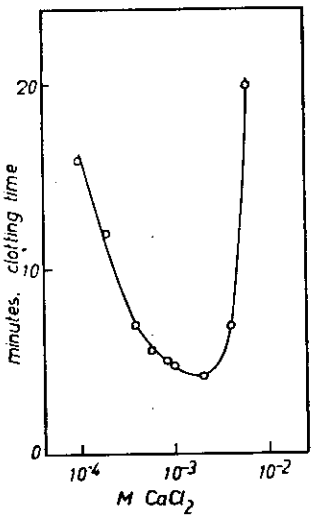

Fig. 2.

Table 1.

The clotting time of Mellanby-fibrinogen on the addition of various quantities of PK_1 and PK_2.

Quantity of PK_1 or PK_2 added to 1 cc. of MF.	Clotting time in the presence of PK_1 or PK_2	
0,05 cc. of . . . . . PK_1 or PK_2	10 min.	10 min.
„ 5 times diluted . . . . „	15 „	16 „
„ 25 „ . . . . „	25 „	29 „

Although the simplest explanation of the action of plasmakinin is that it acts as a kinase, it may be of interest to discuss briefly the possibility that other factor present in plasmakinin solutions might be responsible for the observed effect. The possibility that thrombin is responsible can easily be ruled out because PK_1 does not contain thrombin at all. The accelerating effect can hardly be attributed to prothrombin. MF contains much more prothrombin and the slight increase in prothrombin

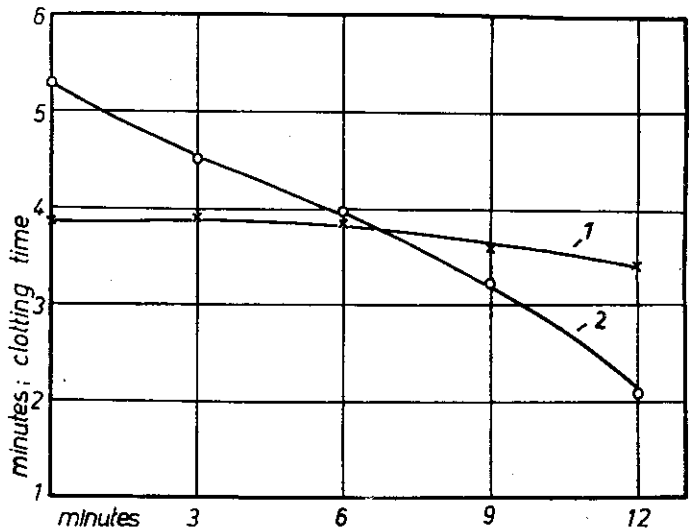

Fig. 3.

due to the addition of plasmakinin cannot be responsible for the effect. There was found no correlation between the fibrinogen quantities of plasmakinin solutions and their action on MF. The most plausible explanation remains that plasmakinin acts as kinase.

There is now increasing evidence that blood plasma contains some kind of soluble kinase. HOWELL,[4] using hemophilic plasma as a test, isolated a substance which, according to him, represents a kinase very similar in its action to the kinase in platelets or in tissues. According to FEISSLY[5] the kinase activity is connected with a viscous protein fraction of the serum. LENGGENHAGER[6] studying the clotting defect in hemophilic blood, postulated the existence of an inactive kinase present in normal plasma but lacking in hemophilic plasma. APITZ[7]

calls this plasma substance which the hemophilic plasma lacks, X-factor. WIDENBAUER and REICHEL[8] also came to the conclusion that blood plasma contained a kinaselike substance in an inactive prestadium, and whinh becomes active only when the clotting of blood starts.

It was found in our recent experiments that cattle plasma does not contain kinase whereas after clotting the serum does contain. Further experiments revealed that the kinase present in serum appears when the clotting starts and that this formation of kinase depends on the presence of Ca ions.

In view of these findings it seemed surprising that plasmakinin obtained by a simple ammoniumsulfate fractionation of plasma should act as kinase when tested with MF. The experiment in which the action of kinase and plasmakinin was compared showed that plasmakinin is not an active kinase but becomes kinase after it has been added to MF.

Comparison of the action of kinase with plasmakinin.

In the following experiment recalcified MF was allowed to stand at room temperature and at various times samples were taken out to test the activity of tissue kinase and of plasmakinin. The experimental procedure was the following: At various times samples of 1 cc. were taken out from the recalcinated MF and brought into small test tubes, one part of which contained kinase while the otthers plasmakinin solution. There were thus two series of test tubes. The tubes in one series contained 0,04 cc. of suitably diluted brain kinase and the tubes in the other 0,04 cc. of PK_1. Into the first tube of each series the MF solution was pipetted in the moment of recalcination. After 3, 6, 9, 12 minutes had passed MF was pipetted into the 2nd, 3rd, 4th and 5th tubes of each series. Thus there were tubes in which MF was added after it had stood for 0, 3, 6, 9, 12 minutes at room temperature. The appearance of the first fibrin clot in the tubes was observed and recorded as clotting time. The clotting time of MF without PK_1 or kinase was 25 minutes. The clotting times in the presence of PK_1 and kinase were much shorter as can be seen on fig. 3 where the clotting time is plotted against the time which elapsed after recalcination. Curve 1 represents the results obtained with kinase and curve 2 the results with PK_1.

In can be seen that it is almost immaterial for the action of tissue kinase how old the MF was. The clotting time in the first tube is nearly the same as in the last where MF stood for 12 minutes after recalcination. There was no substance formed to activate tissue kinase. In the case of PK_1 however the age of MF had marked effect. The older the MF solution is the more pronounced the effect. The first clotting time is much longer than the last. This shortening of the clotting time shows that in MF a substance was formed which acted on PK_1 in such

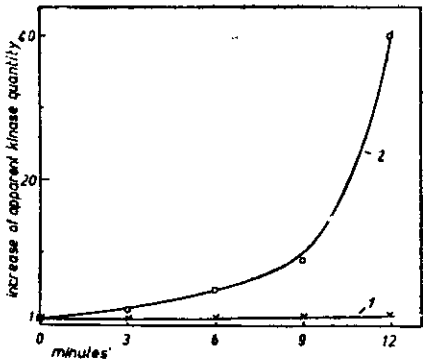

Fig. 4.

a way as if its kinase content had been increased. This apparent increase of kinase quantity is more pronounced when instead of the clotting time the apparent kinase quantity is represented. If the action of PK_1 on MF is equivalent to a certain quantity of kinase then every clotting time obtained with PK_1 corresponds to a certain quantity of kinase. This quantity of kinase can be read from the curve of fig. 1. Taking the kinase quantity corresponding to the first clotting time as a unit it can readily be calculated how many times the apparent kinase quintity has increased. If the increase of kinase quantity, calculated this way, is plotted against the time which passed after recalcination curve 2 of fig. 4 results. The data for curve 1 were calculated in a similar way: the first clotting time obtained with tissue kinase being taken as a unit, the increase in kinase quantity was calculated. As fig. 4 shows a certain quantity of kinase always represents the same quantity, independent of how old the recalcinated MF is. But the apparent kinase quantity of plasmakinin increases

according to the time which has passed after recalcination. In the light of this experiment plasmakinin seems to be an inactive substance which only gives rise to the formation of kinase upon the action of an other factor. This factor formed in MF is probably the thrombin itself, if not, it must be an as yet unknown substance, the formation of which strictly follows the formation of thrombin.

Summing up the results of the above experiment two facts emerge: a) During coagulation kinase is formed. b) For this formation of kinase the plasmakinin is responsible plus an other substance which is the thrombin or an unknown substance.

Discussion.

In view of the findings described in this paper the clotting of blood can be pictured in the following way: The reaction starts with the conversion of a small amount of prothrombin into thrombin by the action of platelets or tissue kinase. The thrombin converts fibrinogen into fibrin, parallel with this process kinase is formed from plasmakinin. This new kinase converts more prothrombin into thrombin, which is followed again by the formation of an other amount of kinase. Thus during the clotting process there is an increased formation of kinase and consequently an increased formation of thrombin too. This simultaneous formation of thrombin and kinase explains the autocatalytic type of the formation of thrombin.

The clotting system in hemophilic blood lacks this mechanism due to the lack of the plasmakinin. The addition of this substance restores the autocatalytic mechanism and with it the normal clotting.

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Adenosinetriphosphatase of muscle.

by

K. LAKI.

JACOBSEN,[1] BARRENSCHEN and LÁNG[2] demonstrated the existence of a phosphate splitting enzyme in animal tissues which exhibited great substrate specificity towards ATP. This enzyme, called ATP-ase liberates two inorganic phosphates from the ATP thus forming adenylic acid. Later T. SATOH[3] held the view that the dephosphorylation of ATP is accomplished by two enzymes, one is a pyrophosphatase producing ADP, the other is a phosphomonoesterase. One of them needs an activator while the other working at pH 9,0 needs Mg ions. LOHMANN[4] found that in crab's muscle only one phosphate is split off from the ATP. He found also that even in muscle tissues in which the ATP is broken down to adenylic acid, the washing of the muscle renders it incapable of splitting more than one phosphate group, and that the addition of Mg restores the full activity.

The ATP-ase attracted much attention when ENGELHARDT and LJUBIMOWA[5] concluded from their experiments, that myosin, the contractile element of the muscle, might be the ATP-ase itself. According to these authors the myosin splits one phosphate group from the ATP and the splitting of the second phosphate is brought about by a water soluble enzyme.

The experiments presented in this paper show that the enzymatic hydrolysis of two inorganic phosphate groups of the ATP molecule in the muscle tissue is not due to a single enzyme, but is a joint action of various factors present in the muscle. A muscle juice obtained by extracting the minced muscle with water is very active in splitting off two labile phosphate groups

from the ATP molecule. This turbid muscle juice can be separated into two parts by centrifugation. One of them is the precipitate, the other is the supernatant fluid. The phosphatase activity resides in the precipitate, the supernatant clear fluid. containing the water soluble part of the muscle, is inactive. It was found that the precipitate, when washed four or five times with water did not only lose some of its original activity but became altered in such a manner that it was able to hydrolyse only one phosphate group from the ATP molecule. It was also found that the full activity (the splitting of two phosphate groups) could be restored by bringing the precipitate and the inactive water soluble part of the muscle together.

It is clear that the insoluble muscle particles which consist mainly of actomyosin contain the enzyme responsible for the splitting of one phosphate from the ATP. The splitting of the second phosphate group is brought about by the joint action of the insoluble muscle particles and the water soluble part of the muscle. (Fig. 1.) This water soluble part of the muscle which I call aqueous extract exhibits phosphatase activity neither on ATP nor on ADP.

It can be seen from fig. 2 that the splitting of the first phosphate group has a pH-optimum at 7,4 (curve 1) and the splitting of the second phosphate at pH 8,6 (curve 3).

The analysis of the action of the aqueous extract showed that two factors were responsible for the reactivating effect of the extract: one of them is a protein like substance, the other is the Mg ion, which can be substituted by Co^{++} and Mn^{++}.

Pyrophosphate inhibits the splitting of the second phosphate group.

Experimental.

Preparation of the washed muscle suspension which splits one phosphate group from the ATP.: 50 gr. of pigeon breast muscle were minced in the Latapie mincer and mixed with 150 cc. of dist. water. The mixture was allowed to stand in the cold for two hours, then it was squeezed through a cloth. The turbid fluid obtained was centrifuged and the precipitate washed in the centrifuge four or five times, each time with about 150 cc. of dist. water. The pecipitate was finally suspended in 40 cc. of 0,6 *M* KCl solution.

It often happened that even after the fifth washing the muscle suspension retained some activity in regard to the splitting of the second phosphate group. In such cases it was enough to allow the muscle suspension to stand in the ice chest in order to obtain a suspension which splits only one phosphate group.

Preparation of the aqueous extract which activates the splitting of the second phosphate group: The muscle juice, from which the insoluble particles were separated by centrifugation, was dialysed overnight in the cold against dist. water. After centrifugation a clear fluid was obtained which showed no phosphatase activity but restored the full activity of the above muscle suspension. So this aqueous extract contains a factor which is necessary to activate the splitting of the second phosphate group.

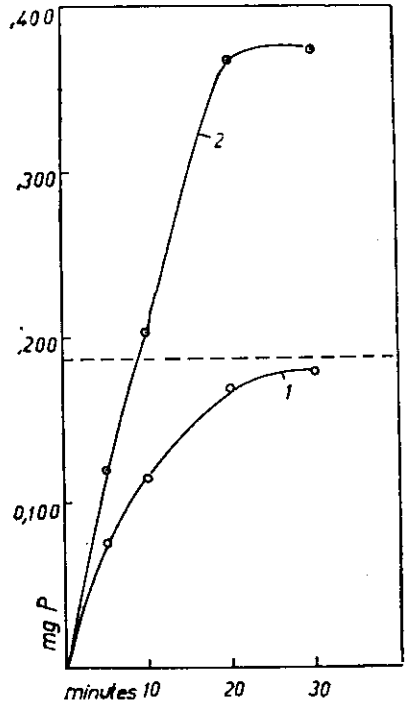

Fig. 1. Liberation of phosphate from ATP by the washed muscle suspension (curve 1), and washed muscle suspension + aqueous extract (curve 2). The dotted line represents the quantity of P corresponding to the splitting of one phosphate group from ATP.

Fig. 1. shows the result of a typical experiment demonstrating the role of the two factors. The quantities of the inorganic phosphate marked on the ordinate are plotted against the time on the abscissa. Curve 1 shows that the muscle suspension

splits one phosphate group. From curve 2 it can be seen that in the presence of the aqueous extract two phosphate groups are split off.

The experiment presented on fig. 1 was performed in the following way: The reaction mixture was pipetted into two Erlenmeyer flasks placed in the water bath (36°). The composition of the reaction mixture in the flasks was the folloving: into the first flask 5 cc. of M/20 borate buifer pH 8,5, 4 cc. of water, 2 cc. of muscle suspencion and 1.5 cc. of ATP (15 mg.) solution were pipetted. In the second flask in addition to the above components 1 cc. of aqueous extract was added replacing 1 cc. of water. The flasks were gently shaken during the experiment. The reaction started when the ATP solution was added to the reaction mixture. The reaction was followed by the estimation of the inorganic phosphate liberated. For this purpose from time to time 2,5 cc. were pipetted out of the reaction mixture into test tubes containing 1 cc. of 20% trichloroacetic acid. Then the solution in the test tubes was filtered and the inorganic phosphate was estimaed in the filtrate.

In all the experiments presented in this paper the inorganic phosphate was determined by the FISKE and SUBBAROW method with the modification of LOHMANN and JENDRASSIK.

Inhibition of the splitting of the second phosphate group. It was found that pyrophosphate had a strong inhibiting effect on the dephosphorylation of the ATP. Pyrophosphate in *M*/100 concentration prevented the liberation of the second phosphate group, but it had no effect on the splitting of the first phosphate group. The inhibiting effect of pyrophosphate can be paralysed by the addition of Mg.

Effect of pH on the dephosphorylation of the ATP. It can be seen from fig. 2 that the splitting of the first phosphate group accomplished by the muscle suspension has a pH optimum at 7,4 (curve 1). When two phosphate groups are hydrolysed the pH optimum is at pH 8,6 (curve 2). Subtracting curve 1 from curve 2, curve 3 results. This curve represents the activity —pH curve of the action of the aqueous extract.

The experiments which gave the results shown in curve 1 and 2 were performed in test tubes. Two sets of test tubes were used. The first set consisted of 8 tubes. Into each tube the felowing solutions were pipetted: 1 cc. of veronalacetate buffer with different pH-s, 0,3 cc. of the muscle suspension, 0,5 cc. of *M*/20 Na-pyrophosphate solution adjusted prior to the pH of the buifer solutions, and 0,4 cc. of water. The rôle of pyrophosphate in these experiments was to assure that no second phosphate splitting occured. In the second set of test tubes the reaction mixture was similar to that of

the first except that pyrophosphate was not added, but there was added 0,3 cc. of aqueous extract instead to achieve the splitting of two phosphate groups. The total volume of the solutions was brought up to 2,2 cc. by the addition of the necessary volume of water. The tubes were placed into the water bath (36°). After temperature equilibrium was reached to each tube 0,3 cc. of ATP (3,8 mg.) solution was pipetted. The reaction time was 16 minutes. Then the reaction was stopped by the addition of 1 cc. of

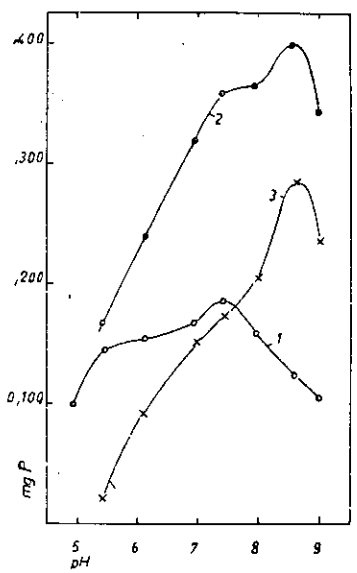

Fig. 2. The action of pH on the liberation of phosphate when only one phosphate group (curve 1), and when two phosphate groups are split off from ATP (curve 2). Curve 3 results by subtracting curve 1 from curve 2 and thus reprecents the activity pH curve of the oqueous extract.

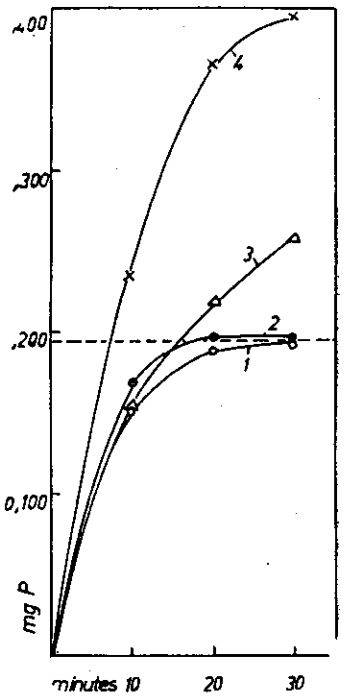

Fig. 3. The rate of liberation of phosphate from ATP by the muscle suspension (curve 1). Curve 2 represents the same in the presence of Mg, curve 3 in the presence of the aqueous extract and curve 4 in the presence of Mg and the aqueous extract together. The dotted line represents the quantity of P corresponding to the splitting of one phosphate group from ATP.

20% trichloroacetic acid. After filtration the inorganic phosphate was determined. In fig. 2 the curves are drawn by plotting the phosphate quantities against the pH-s at which the reaction proceeded.

Purification of the aqueous extract. 220 gr. of pigeon

breast muscle were minced in the Latapie mincer, then 440 cc. of dist. water was added and the mixture was allowed to stand in the cold. After four hours it was squeezed through a cloth. The 410 cc. of turbid fluid were centrifuged. To the supernatant fluid (400 cc.) 40 cc. of *N* acetic acid were added and the solution placed in a water bath (36°) for 15 minutes. 37,5 cc. of *N* KOH were then added and the great quantity of precipitate formed was discarded. To the clear reddish fluid ammoniumsulfat was added to reach a 0,6 saturation. The solution was filtered through fluted filters and the precipitate discarded. By the addition of more ammoniumsulfate to the solution the saturation was brought to 1,0. The precipitate, which contains the active substance, was collected on the filter paper and dissolved in 30 cc. of dist. water. The reddish solution was dialysed overnight against dist. water in the cold and then used for the experiments.

The activating effect of magnesium ions. The experiment, presented in fig. 3 shwos that the aqueous extract has almost lost its activity by this purification process (curve 3) and that it activates the muscle suspension only in the presence of Mg ions (curve 4), thus demonstrating that the activating effect of the original aqueous extract is due to two factors: one of them being a protein, the other the Mg ion. Curve 2 shows that Mg has no effect on the splitting of the first phosphate group. (The slight activation is very likely due to incomplete removal of the protein component of the extract). The Mg must be bound to the protein because it cannot be removed even by prolonged dialysis and the purification procedure was needed to remove it from the protein. Co, Mn ions can substitute the Mg ions in the same concentration, but Fe, Ni, Cu, Cn cannot: they even inhibit the reaction.

The experiment presented in fig. 3 was performed in Erlenmeyer flasks. Four flasks were used each containing the following solution: 5 cc. of borate buffer pH 8,5, 2 cc. of muscle suspension, 1,5 cc. of ATP (15,2 mg) solution which was added to the reaction mixture only when temperature equilibrium was reached. The addition of ATP marked the starting of the reaction. In addition to the above solutions 1,2 cc. of *M*/100 $MgCl_2$ solution were added to the second and fourth flask, 1,5 cc. of ten times diluted purified aqueous extract to the third and the fourth flask. With dist. water the total volume in each flask was adjusted to 12,5 cc.

At the 0, 10, 20 and 30th minute 2,5 cc. samples were removed from the flasks and the phosphate determined as above.

Summary.

The insoluble muscle particle in itself is only capable of splitting one phosphate group from the ATP molecule. The splitting of a second phosphate group is due to the joint action of the insoluble muscle particle, a soluble protein and Mg ion.

The splitting of the second phosphate group can be inhibited by the addition of pyrophosphate.

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Actin. II.*

by

F. B. STRAUB.

I. Introduction.

An improved method for the preparation of actin from rabbit's muscle is described in the present paper. During the course of these investigations it has been found that actin may exist in two different forms. In the absence of salts it is present in solution as *inactive actin,* in the presence of salts as *active actin.*

Both forms of actin would combine with myosin, but the properties of the resulting actomyosins are different. Active actin, when added to myosin, forms the actomyosin, the characteristics of which have already been described in earlier communications.[1] This is the actomyosin, which has a high viscosity and which reacts, on adding ATP, with a decrease of viscosity, the decrease being proportional to its actin contents. For the sake of clarity we shall now term this compound, *active actomyosin,* in contrast to the complex of *inactive actomyosin,* which is formed when inactive actin and myosin are brought together in salt solution. This latter complex has a viscosity practically equal to that of the myosin which is present in it. The viscosity of an inactive actomyosin is not influenced by the addition of ATP.

Inactive actin is easily transformed into active actin by the addition of small amounts of any salt which does not destroy the protein. Active actin partly reverts into inactive

* This work was aided by a grant from the Duke ESTERHÁZY.

actin on dialysis under certain conditions. Owing to the destruction of actin during dialysis, this tansformation has not yet been realized in a quantitative manner. But there is no dount that the reaction: inactive actin $\rightleftharpoons$ active actin is a reversible one.

Profound changes are observed in viscosity and in deuble refraction of flow during the transformation of inactive actin into active actin.

On addition of salt, the viscosity of an inactive actin solution rises rapidly and the readily flowing thin solution of inactive actin sets to a gel of active actin.

The viscosity of an inactive actin solution is very low compared with that of active actin. We found in one case the specific viscosity to be 0,032 for a 4 mg/ml solution of inactive actin, in an other case 0,03 for 3 mg/ml. In many other cases somewhat higher values were obtained. The variations must be ascribed to the presence of small amounts of active actin.

As the determination of active actin in small amounts can be made only with considerable error, we cannot state definitely what the viscosity of a completely inactive actin may be. It is either identical or lower than the values given above.

The viscosity of hemoglobin is somewhat lower, that of serumalbumin somewhat higher than the viscosity of inactive actin. Thus, by its viscosity, inactive actin is classed among the globular proteins.

Active actin, on the other hand, has a very high viscosity which equals the viscosity of polymer substances, such as nitrocellulose and rubber. It has therefore an extremely assymetrical molecule of considerable length.

The change of shape of the molecule during the activation of actin is quite unique. A trace of salt changes a globular protein into a highly assymetrical, fibrous one.

The great rise in viscosity of actin during its activation is partly due to the elastic anomaly of the viscosity of active actin. The relation between the viscosity and the pressure in the viscosimeter has been studied in the capillary viscosimeter previously specified.[1] A 12 l flask of air served to maintain uniform pressure during the measurement; the pressure was read on a water manometer. The results are shown in Fig. 1.

Even in dilute solution and at rather high pressures the orientation of the molecules is not yet complete. Contrary to earlier statements,[1] the results presented in Fig. 1. show that actin is not thixotropic; readings at different pressures were taken at random and yet no effect of previous treatment could be found.

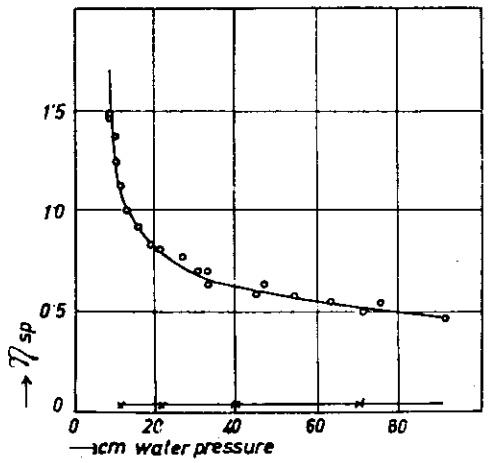

Fig. 1. Viscosity of inactive and active actin at different pressures. o——o 2 mg/ml active actin in 0,2 *M* KCl. ×——× 3 mg/ml inactive actin in dist. water at 0°.

That inactive actin is a globular protein whereas active actin consists of rod-shaped particles is born out by the study of the double refraction of flow of actin solutions.

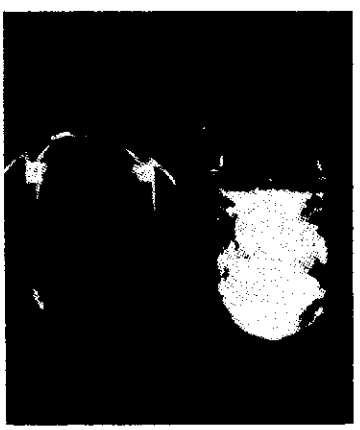

Fig. 2. Double refraction of actin solutions. Photographs taken through crossed nicols. Left: inactive actin. Right: the same solution after activation.

If a solution of inactive actin is sucked through a capillary and the rapidly moving fluid is observed between crossed nicols.

there is no double refraction of flow to be observed. Active actin has, on the other hand, a very strong double refraction of flow. Furthermore, a solution containing 3—5 mg active actin per ml remains doubly refracting more than half an hour after it has been gently stirred. (Fig. 2.)

It is obvious from these observations that the actin preparations described in the earlier communication[1] were solutions of active actin with occasional admixture of some inactive actin.

The transformation: inactive actin ——→ active actin, which we will call the *activation of actin,* may be brought about by any salt which does not destroy the protein. Non-ionizable substances have no effect on inactive actin. With the method to be described later in this paper, we have studied the velocity and the extent of the activation under different conditions. It was found:

1. that the activation proceeds to the same end point regardless of the nature of the salt concerned and of the temperature and pH, or — beyond a certain limiting concentration — of the concentration of the salt;

2. that the velocity of the transformation depends a/ on the purity of the actin, b/ on the concentration of the actin, c/ on the concentration of the salt, d/ on the temperature, e/ on the pH and f/ on the nature of salt used.

The conclusions which may be drawn from these results are the following. As non-dissociating substances have no effect on actin, the activation must be ascribed to *ions.* The concentration of salt needed for the same activation is inversely proportional to the *charge* of the positive ion.

About 10 times more KCl is needed than $CaCl_2$ to produce the same effect. Of the alkali chlorides the velocity of activation increases in the order: Rb < K < Na < Li, if all are used in the same concentration. Of the haloids, if all are used in the same concentration as Na salts, the velocity of activation increases in the order: J < Br < Cl < F. Thus NaF activates very fast, KJ very slowly. The effect of the charge of the anion is opposite to the effect of the charge of the cation, e. g. sulfates activate much slower than chlorides. Hydrogen ion is a more potent activator than any other ion, there is instantaneous activation when the pH is lower than pH 6.

II. Method of preparation.

The main features of the method previously described[1] have been retained. Slight alterations, however, were introduced so as to obtain the actin in the inactive form.

Rabbit's muscles from the back and from the legs are rapidly excised and cooled immediately by packing them into ice. They are minced first through a cold meat chopper (diameter of holes 2 mm) and then through a cold Latapie mincer. 300 ml of an alkaline KCl solution* are added to every 100g of the mince and stirred mechanically together at 0° for 20 minutes. The mixture is then centrifuged and the supernatant fluid, which contains the greater part of the myosin and some actin, is discarded. The residue is left to stand at 0° for 24 hours. At the end of this period it is weighed and mixed with 5 volumes of its weight of distilled water at room temperature. After standing for 1 hour at room temperature, the mixture is centrifuged. The washing of the residue with distilled water at room temperature is repeated, with the same amount of water as before, again standing for 1 hour. After this second washing the muscle residue is treated with 4 volumes of acetone at room temperature. After 20 minutes' standing at room temperature, the acetone is removed by pressing it out through a filter cloth. The residue is now mixed with a fresh lot of acetone (¼ of the former volume) and left to stand again at room temperature for 20 minutes. The acetone is again pressed out and the residue is spread over filter paper and left to dry.

The acetone dried muscle powder (after 10—15 hours of drying) is extracted with 20 volumes of CO_2-free distilled water at room temperature. Neither grinding of the muscle powder before the extraction, nor grinding nor stirring during the extraction is advantageous, as in either case the solution will become opalescent and, moreover, the actin will be partly or mostly activated. Therefore the muscle powder is mixed with the extracting water and then left to stand alone at room temperature for 10—15 minutes. At the end of this

* This is prepared by mixing 800 ml 0,1 *M* potassium borate with 200 ml 2 *M* KCl. — The potassium borate solution is made by dissolving 12,4 g boric acid in 100 ml *N* KOH, then making the solution up to 1 l with dist. water.

period the resulting pulp is poured in to a Buchner funnel and the solution is sucked off. It contains the actin in its inactive form. The protein content varies between 3—6 mg/ml. The purity of the actin in such a solution is mostly maximal (1,0)* but rarely below 0,7. If the purity of the preparation is not maximal, it cannot be purified any further. It has been found that the purification procedures described in the previous paper[1] do not lead to purification but to the activation of the actin, which was in an inactive form.

The yield in this new procedure is not very high. It might be increased by a more thorough extraction of the dry muscle. Yet in such cases the resulting solution will be opalescent, and the actin in it will become activated. Therefore higher yields have been sacrificed for the advantage of obtaining inactive actin in clear solution.

Solutions of the actin show varying degrees of stability. Inactive actin is less stable than active actin. The former would lose on the average 10—20 % of its potential activity in 24 hours at 0°.

Actin of the maximal purity has been analysed by DR M. KOVÁCS-OSKOLÁS. She found:

51,3 % C, 8,6 % H 15,09 % N

The ash content was 1,1 %. After wet ashing the P content was determined colorimetrically. Less than 0,07 % were found. These results indicate the actin to be a protein. The orcin test for pentoses is negative, the Millon and Pauly tests are positive.

III. Determination of active and inactive actin.

In the previous communication a method for the determination of actin has been described.[1] The method was based on the determination of the activity of the actomyosin, which results when the actin solution in question was mixed with a certain amount of myosin. The definition of the activity of actomyosin and its determination have been described in the same paper. The technique used there was followed in the experiments described in the present paper. Viscosity was

* The purity of actin is maximal (1.0) if 1 mg of it gives with 5 mg pure myosin a 100% active actomyosin.

always determined in a 0,6 *M* KCl solution which contains a veronal-acetate buffer of pH 7. Temperature: 0°.

When using a solution of crystallised myosin,[2] the formation of actomyosin was found to be the same as with the impure myosin solutions, used in earlier investigations. It was found, however, that of the purest actin preparations 1 mg is needed to activate 5 mg of myosin to 100 % activity. (With impure myosin we found a ratio of 1:6. The discrepancy is due to the fact that the myosin contents of impure myosin solutions were estimated wrongly.)

The method of determination of actin in solution, referred to above, is a determination of the active actin content only. The total actin content (active + inactive) can be determined only if the actin solution is first activated by salt solution. This is shown by the following observations.

If a solution of inactive actin is mixed with myosin, there is no rise in the viscosity of myosin (the viscosity of the added inactive actin being negligible compared with that of the myosin). If ATP is added, there is no decrease of viscosity. If, however, the inactive actin solution is first kept at room temperature (22°) in presence of at least 0,1 *M* KCl for 15 minutes and after that added to the myosin, the viscosity will be found to be high and ATP causes a drop of viscosity almost to the level of the viscosity of the myosin present in the solution. This is illustrated by the following experiment (Table I.):

Table I.

	Relative viscosity	
	alone	with ATP
6 mg myosin + 0,3 ml inactive actin, made up to 6 ml with 0,6 *M* KCl	1,30	1,30
6 mg myosin + 0,3 ml of the same actin after it has been standing at 22° in 0,1 *M* KCl for 5 minutes, made up to 6 ml with 0,6 *M* KCl	1,89	1,39

The result is somewhat surprising in view of the fact that during the determination of the viscosity there is 0,6 *M* KCl in

both solutions, which could at least partially activate the actin at 0°. It follows therefore that a combination must have taken place between inactive actin and myosin and that this combination prevents the inactive actin from becoming activated.

As an explanation of the effect of ATP on the viscosity of actomyosin it has been suggested that ATP splits the complex into its components: myosin and actin. For the case of the inactive actomyosin this is supported by the following observation.

18 mg myosin were mixed with a solution of actin, which contained the actin mostly in its inactive form. KCl was added to bring the KCl concentration to 0,6 *M*. The volume was then 4,5 ml. This mixture was divided into three parts. One part of it was immediately diluted with 0,6 *M* KCl to 6 ml and the activity of the actomyosin was determined. The other two samples were left to stand at room temperature for 1 hour, one of them with the addition of 0,05 mg ATP. During this time the small amount of ATP added was obviously completely split by the myosin, as addition of fresh ATP caused a marked decrease in viscosity. The determination of the viscosity was made in all cases at 0°.

Table II.

	Relative viscosity		% activity of actomyosin
	alone	with ATP	
6 mg myosin + 2 mg inactive actin determined immediately	1,325	1,25	30
Same after 1 hour at 22°	1,415	1,325	25
Same + 0,05 mg ATP after 1 hour at 22°	1,755	1,303	138

The activation of the inactive actin in the experiment, which contained ATP, is no doubt due to the fact that ATP has split the complex of inactive actomyosin, setting free the actin thus making it accessible to the activating effect of the salt. This effect is very likely instantaneous but it cannot be measured experimentally as one has to wait until the ATP is completely split, before one can measure the viscosity and the activity of the actomyosin.

As, according to the former experiment, there is no

activation of the inactive actin under the experimental conditions prevailing during the determination of actin, it follows that only the active actin content is determined by the method.* If we want to know the sum of active + inactive actin, we have first to activate all the actin. This was usually achieved by adding KCl in 0,1 *M* concentration to the actin solution and keeping it at 22° for 10—15 minutes. A determination of the actin after this treatment will give the total actin contents. An example of such a determination is shown in Table III.

Table III.

	Relative viscosity		% activity of actomyosin
	alone	with ATP	
5 mg myosin + 0,3 ml actin "258"	1,545	1,31	69
5 „ „ + 0,3 „ activated actin "258"	1,87	1,313	164
5 „ „ + 0,15 ml „ „ "258"	1,464	1,225	114

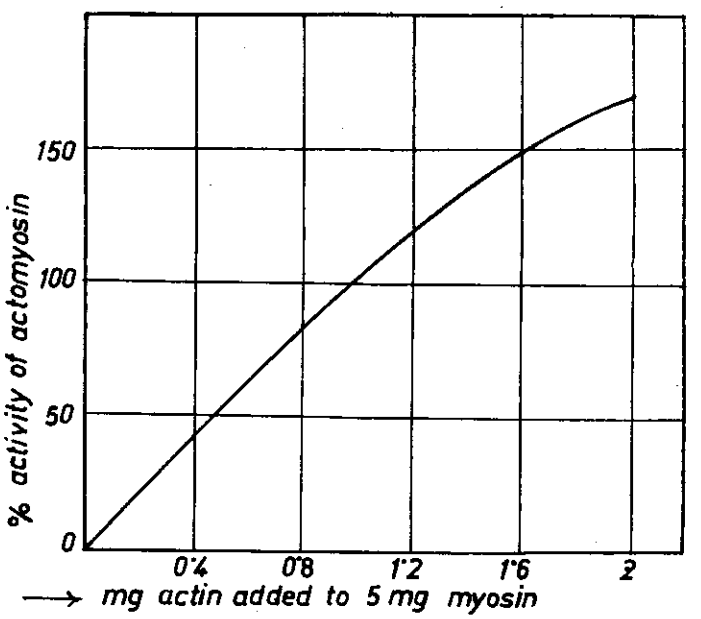

Fig. 3.

* Naturally, if an excess of inactive actin is added to the myosin, e. g. more than 3 mg to 5 mg myosin, it will become partly activated as the myosin will not be able to bind it completely.

To evaluate these data, we have to refer to the curve of Fig. 3. which shows the activity of the actomyosin as the function of the amount of active actin, added to 5 mg myosin. The curve was determined by using crystallised myosin and actin of the highest purity. It is seen from this curve that 0,67 mg actin are needed to produce a 69 % active actomyosin, and 1,15 mg to produce an 114 % active actomyosin from 5 mg myosin. It follows that the actin preparation „258" contains $\frac{0,67}{0,30} = 2,23$ mg/ml active actin and the total actin content is $\frac{1,15}{0,15} = 7,70$ mg/ml The active actin content of the preparation is therefore $\frac{2,23}{7,70} \times 100 = 29$ %, whereas the remaining 71 % is inactive actin. We say that in this preparation there is 29 % active actin.

It might be mentioned here that a given actin solution, which is partially active, gives varying activity according to the temperature at which it was kept before the determination. An example of this is found in Table IV.

Table IV.

	% active actin found
Actin "275" kept 1 hour at 23°	33
Same „ „ „ „ 35,5°	17

This is due to the fact that there is an equilibrium between the salts present and the fraction of salt bound to the actin. This equilibrum changes with the temperature and thus the active actin content changes also. If the actin solution is brought to another temperature, the new equilibrium will be reached only after considerable time. For this reason we made the activity determinations on partially active actin only after at least 2 hours standing ot 0°.

There is an even more pronounced change in the activity of a partially active actin solution after it has been frozen. Even a nearly completely inactive solution will appear to be completely active after it has been frozen and then left to thaw. If the solution is then kept at 0° this apparent activity

will be lost within one or two hours. The phenomenon may be repeatedly observed, that after freezing and thawing there is always a high activity to be found. During freezing there is a local increase in the concentrations of both actin and salts, which leads to activation. When the ice has melted, the thermal equilibrium is reached only after a considerable time.

IV. The factors influencing the activation of actin.

The endpoint of activation. That an actin preparation is activated to the same endpoint regardless of the nature of the salt used, is shown in the following table. A „100 % active actin" means the active actin content which was found when activated with KCl. Rather high concentrations were used to obtain, as far as possible, real endpoints of activation.

Table V.

2 mg/ml actin during activation, pH 7,0 Temperature: 22°

Salt	final concentration of salt, Mol/liter	incubation time, min.	% active actin found
KCl	0.2	15	(100)
„	0,2	120	100
LiCl	0,4	30	102
NaBr	0,4	30	100
NaJ	0.1	60	95
„	0,5	60	0
$CaCl_2$	0,02	15	92
Na_2SO_4	0,2	15	48
„	0,2	120	92
NaF	0,2	30	98

$CaCl_2$ gives a somewhat lower value in spite of fast activation, this is due to a visible denaturation. The reason why 0,5 *M* NaJ fails to activate at all, will be discussed later.

Time curve of activation. The velocity of activation depends upon the concentration of the salt. If a low concentration of salt is used, the time curve of activation can be studied. It is found that after a rapid rise the activation is considerably slowed down and the end point is reached very slowly. This fact points to the multimolecular nature of the reaction. (Fig. 4.)

Temperature effect. Table VI. shows the rapid rise in activation velocity by increasing temperature. 2 mg/ml actin in 0,05 *M* KCl were incubated at the temperature indicated and after 5 minutes 0,6 ml of this solution were added to 5 mg myosin and rapidly cooled to 0° at which temperature the determination of activity was done.

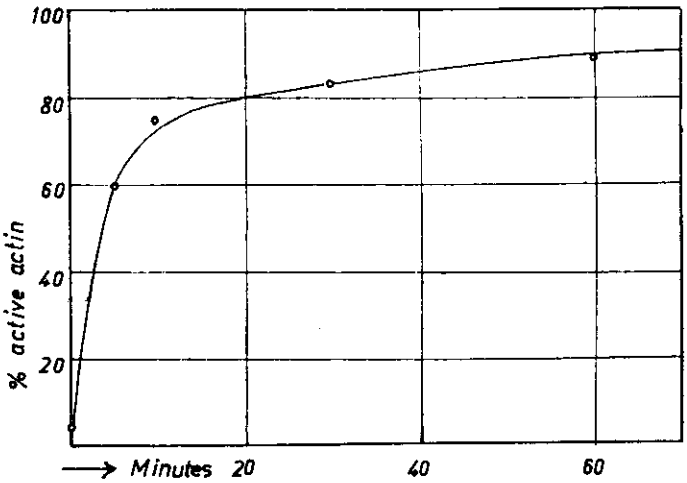

Fig. 4. Activation time curve in 0,02 *M* NaF at pH 6.5 and 35,5°.

Table VI.

Temperature	% active actin found
0	4
12	70
20	82
30	100
35,5	100

Effect of salt concentration. It is almost impossible to get a true picture of the effect of salt concentration because the endpoint and the velocity of activation both vary with the salt-concentration. It has already been shown that the endpoint is reached very slowly with smaller concentrations of salt. Therefore we believe that the curves presented in Fig. 5. do not express the true activation endpoints at smaller salt concentrations, the points indicated by the experiment being probably too low.

Activation by H ions. To 5 ml of an inactive actin solution various amounts of an 0,01 *N* HCl solution were added. The pH was determined colorimetrically and the % active actin content was determined with the usual procedure after the mixture had stood 15 minutes at room temperature. As the

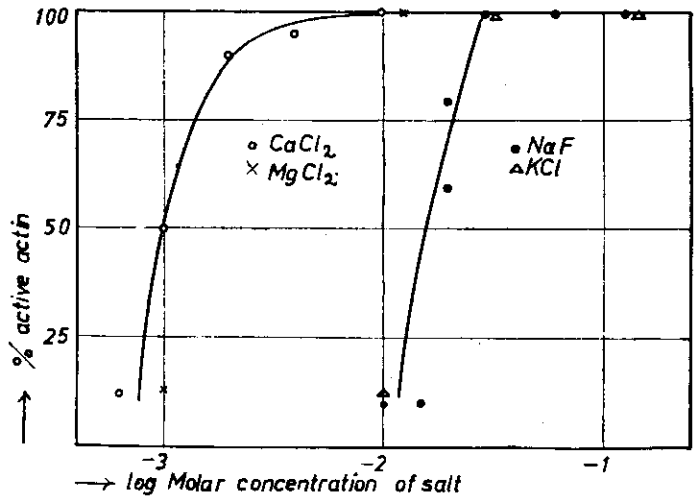

Fig. 5. Effect of concentration of salt on the activation of actin. 2,5 mg/ml actin incubated at 35.5° with salt of the concentration indicated on the abscissa. pH 7,4. The % active actin content was determined after 60 minutes incubation.

myosin solution used for the determination was buffered, the acidified actin solution was not neutralised before adding it to the myosin. During the determination of the activity of the resulting actomyosin the pH was 7.

Table VII.

Actin incubated for 15 min. at 22° at pH	% active actin found
7,0	6
6,0	20
5,7	75
5,3	100

The reversibility of the activation. If addition of salt causes activation, it could be expected that removal of the salt would effect inactivation of active actin. In spite of this, if

an actin solution, which contains just enough salt to keep its actin content in the activated form, is subjected to dialysis at 0°, no inactive actin will be formed. There will be a loss in the total actin content, but what remains is active actin. Inactive actin is however formed during dialysis if the actin solution is first made alkaline. An actin solution which contained 70 % of the actin in the active form, was mixed with $^1/_{10}$ of its volume of 0,1 *M* borate buffer of pH 10 and dialysed against CO_2 free distilled water for 24 hours. The pH of the dialysed solution was adjusted to pH 7. It contained only 2 % of the actin in its active form, the rest being inactive actin, which could be activated by the addition of salt. The total actin content decreased by 30 % during dialysis.

V. Secondary salt effects.

Any salt, if given in great excess over the concentration which brings about the activation of actin, will precipitate the protein from its solution. KCl will precipitate over 2 *M*, $CaCl_2$ and $MgCl_2$ already precipitate at 0,002 *M*. The precipitation usually does not set in immediately and it is always connected with partial denaturation.

If we add a KCl solution to a precipitate of actin, caused by the addition of small amounts of Ca ions, the precipitate may be redissolved. If the operations are not carried out rapidly enough, this redissolution will be incomplete and there will be a loss of activity.

Actin is precipitated from its solution at pH 5. This precipitate is redissolved by neutralizing the precipitate to pH 7. Minute amounts of salts, even KCl, if present during this precipitation, will cause denaturation of the actin.

Addition of dilute acid to the actin will first bring about its activation, the solution will become highly viscous, but transparent. Addition of more acid will cause precipitation, and of still more, dissolution of the precipitate. It is interesting to note that if the precipitate is only just dissolved by acid, it will not be viscous, neither does it show any double refraction of flow, comparable to that of the solutions which are obtained if the precipitate is dissolved by adding alkali. Actin dissolved

in acid is very sensitive towards anions: chlorides cause immediate denaturation, whereas phosphates do not precipitate.

At neutral pH the iodides like NaJ show an exceptional behaviour. At concentrations up to 0,1 *M* NaJ activates like any other salt. At 0,5 *M* it does not activate at all.(See Table V.) If an actin solution is activated by the addition of 0,1 *M* KCl and then NaJ is added to the solution to give a 0,5 *M* solution of NaJ, within half an hour, at room temperature, all activity of the actin is abolished. There is, however, no precipitation of protein. The viscosity and the double refraction of flow of the active actin disappear together with its ability to form active actomyosin. If an actin in 0,5 *M* NaJ was diluted 5 times or if the excess of salt was removed by dialysis, the inactivation was not reversed or if there was some reversal, this was only to a very small extent.

My thanks are due to Mr K. BALENOVIĆ for his helpful cooperation during part of this work.

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On the specificity of the ATP-effect.*

by

F. B. STRAUB.

Actomyosin, in 0,6 *M* KCl, is split into actin and myosin by the addition of small amounts of ATP. This is evidenced by the fall of the viscosity, the original high viscosity returning if the ATP is removed. We find that the same effect is also produced by inorganic pyrophosphate. This is shown in Table I. Adenylic acid, ortophosphate and metaphosphate were found to be without any effect. ADP did not show any effect if pure myosin and actin were used for the preparation of actomyosin, it was however active, if impure myosin and actin were used. This is most likely due to contamination of myosin in the latter case by the enzyme system, which brings about the transformation of ADP into some other compound with a pyrophosphate group.

Table I.

Added substance	Relative viscosity of actomyosin in 0,6 *M* KCl at 0°
None	1,70
0,0007 Mol/lit ATP	1,28
0,00002 „ „	1,28
0,0006 „ Na-pyrophosphate	1,28
0,00006 „ „	1,29
0,00001 „ „	1,375
0,000003 „ „	1,425

There is some difference between the effect of ATP and inorganic pyrophosphate. Whereas the effect of ATP is almost

* This work was aided by a grant from the Duke ESTERHÁZY.

instantaneous, the effect of pyrophosphate takes some time to develop, especially so at smaller pyrophosphate concentrations. As pyrophosphate is not split by the myosin, its effect is permanent, in contrast to the effect of ATP, which is soon abolished, due to the splitting of ATP by the myosin. If, however, the actomyosin, to which pyrophosphate was added, is precipitated and washed with dilute saline solution, pyrophosphate can be removed and the actomyosin shows again a high viscosity. Thus the effect of inorganic pyrophosphate is reversible.

The action of pyrophosphate depends on the temperature. At 0° and at 6,5° it acts like ATP in very dilute solutions. At 23° however, inorganic pyrophosphate has no effect at all, even in 3.10^{-3} *M* concentration. ATP is fully active not only at this temperature, but at 37,5° also.

If 6.10^{-4} *M* inorganic pyrophosphate is added to a solution of actomyosin at room temperature (22°), there will be no change in its viscosity. If this solution is cooled to 0° its viscosity will become low, just as if ATP would be present. Bringing the solution back to 22°, its viscosity will rise again and can be lowered by the addition of ATP.

From these data it appears that it is the pyrophosphate group of the ATP which is responsible for the viscosity effect. It might therefore be concluded that adenosinediphosphate, prepared through dephosphorylation of ATP by myosin, does not contain a pyrophosphate residue.

It may also be concluded that the splitting of ATP is not involved in its viscosity decreasing effect, since pyrophosphate has the same effect and is not split by myosin.

Dr T. Erdős has found in this laboratory (unpublished) that on addition of inorganic pyrophosphate actomyosin threads do not contract at 1,3°, whereas addition of ATP brings about their contraction.

Observations on myosin and actomyosin.

by

F. GUBA.

1. *Comparative Study.*

I compared the behaviour of the striated muscle of different animal species during extraction. I used the muscle of the rabbit, pigeon (breast muscle), frog (Rana esc.), fish (amiurus nebulosus), cattle, hen (leg and breast) and the heart of the rabbit. The muscles of the freshly killed animal were quickly cooled, minced on a cooled Latapie mincer, suspended in 0,6 *M* KCl, 3 ml being taken per g of muscle. The suspension was stirred at 0° C and then centrifuged. My problem was to see how much myosin is extracted and how active it is when the time of extraction is varied. The results are summed up in the two curves.

Fig. 1a. and 1b. show the relation between activity and the time of extraction. As can be seen the extract of rabbit muscle shows little activity during the first 15 min. and begins to rise after this time. This rise is continuous and after 3 hours the rabbit (as well as the pigeon) muscle has given a 120% active myosin. The activity of frog muscle extract rises steep in the beginning to remain unchanged afterwards. Very remarkable is the behaviour of the fish which gives in 20 min. a 120% active myosin. Other fishes (esox mucius) behave in like manner. The actin thus releases the stucture very easily. This might explain the easy digestibility of fish meat.

Fig. 2a and b. shows the relation between the time of extraction and myosin content of the extract. These fig. show

that the rabbit is an especially favorable object. The values obtained in 20 min. in the other animals seem to lie close to

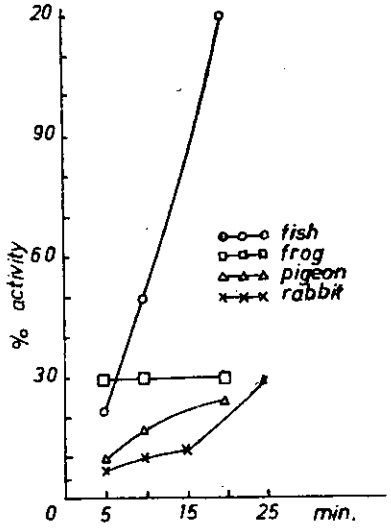

Fig. 1. a.

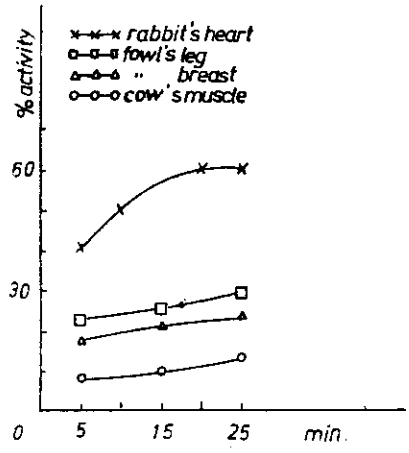

Fig. 1. b.

the limit, for, in the pigeon even after 3 hours, no more than 9 mg per ml was extracted.

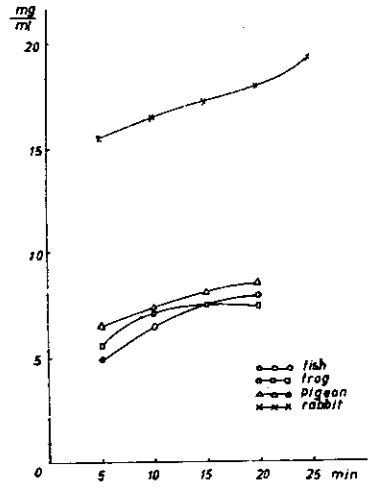

Fig. 2. a.

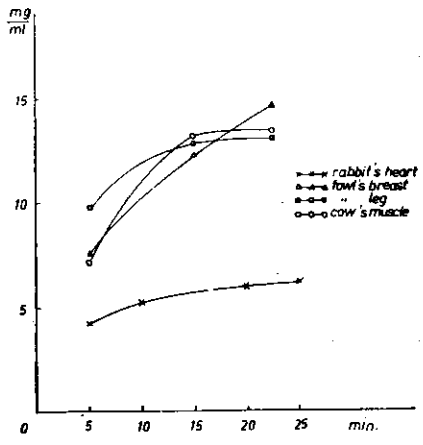

Fig. 2. b.

2. KCl curve and activity.

In these experiments I compared the viscosity of myosins of different activity — thus different actin content — at varied

KCl concentration with and without the addition of ATP. The 10% and 90% active myosin were prepared directly from muscle. The 50 and 150 % myosin were prepared by mixing actin and myosin. My solutions contained 1,6—1,8 mg myosin per ml. The pH was stabilised with veronal acetate buffer of pH 7 (see BALENOVIĆ and STRAUB[1]). The solution contained 0,001 *M* $MgCl_2$. To 4 ml of fluid 0,05 ml of a 1,2 % ATP solution

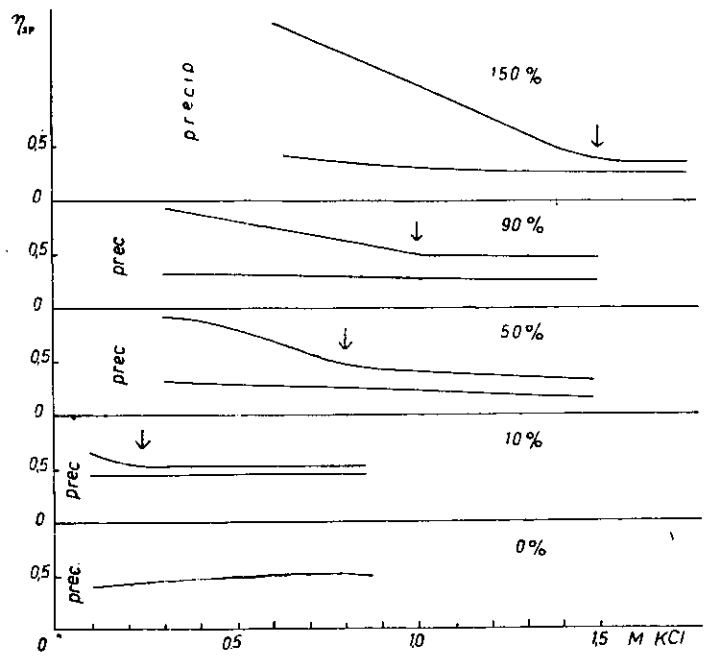

Fig. 3.

was added. Foaming was prevented by the addition of one drop of caprylic alcohol. The viscosity was determined in the capillary viscosimeter. The time of outflow of the salt solution varied between 70 and 120 sec. First the viscosity was determined without and then with ATP. Temp. 0°.

My results are summed up in Fig. 3. The blank zone on the left means precipitation. In every case the lower line is the ATP curve.

In the case of 0% active myosin ATP had no effect and the two curves were identical. F. B. STRAUB[2] found that ATP at a lower pH greatly affects the viscosity of myosin A. At that time, when those experiments were made, however, we

were not yet able to prepare entirely inactive myosin. Myosin A was not quite inactive; it had only a low actin content. Now, repeating these experiments with completely inactive myosin, ATP is found to have no effect at all.

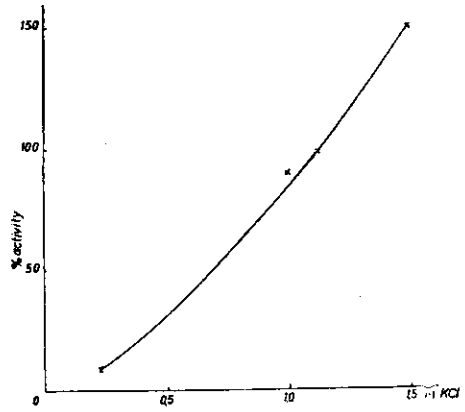

Fig. 4.

The other curves show that at a high KCl concentration the two curves lie close to each other and are parallel. A high

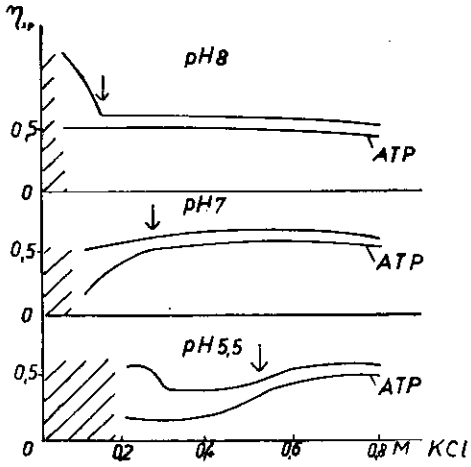

Fig. 5.

salt concentration has thus the same effect as ATP, i. e. it is capable of dissociating the actomyosin.

The curves show that according to the activity (actin content) of the actomyosin, the parting of the two curves lies at different KCl concentrations i. e. actomyosins of different

activity are dissociated by the salt alonce at different concentrations. This means that actomyosins of different % composition behave as individuals. The less actin they contain the less KCl is needed for their dissociation.

If the points, at which the two curves part, are plotted against the activity, Fig. 4. is obtained which is, in fact, the curve of complete hydration and dissociation of actomyosins of different actin content.

In the case of 10% active actomyosin I also studied the effect of pH (see curve Fig. 5.) I found that higher pH shifts the point of parting to the left, lower pH to the right. At pH 8 this point lies at 0,15 *M* KCl, at pH 5,5 at 0,6 *M* KCl. In both cases the pH was stabilised by a veronal acetate buffer.

As a practical consequence we may deduce that the measurement of activity of actomyosins of low actin content can be made with advantage at low KCl concentration and at lower pH, which, so to say, magnifies the effect of ATP on viscosity.

The theoretical consequence of these curves is still more interesting. We have seen that the 10% active myosin behaves, in absence of ATP, as an individual and not as myosin plus actomyosin. This 10% active actomyosin contains only 1,6% actin to 98.4% myosin. It can be concluded herefrom that actin is capable of coating itself with several layers of myosin. Myosin in itself has a tendency towards coaxial association and the actin seems to act as the nucleus of these associated particles. The force, by which actin holds this great mass of myosin can be but small because 0,25 *M* KCl suffices to cause dissociation. As has been shown elsewhere (F. B. STRAUB[3]) maximum activity is 170%. Also fermentatively this complex is the most active (BANGA[4]). It corresponds to the natural actomyosin of muscle and contains actin and myosin in the proportion of 1:3. Very probably this is the actomyosin in which the actin micels are coated with one layer of myosin. This one layer is held very firmly and a very high KCl concentration is needed for dissociation.

The smaller the % activity, the thicker this layer of myosin held by the actin, but the smaller the force by which myosin is held and, correspondingly, the smaller the KCl concentration which is capable of effecting dissociation.

If the salt concentration of the actomyosin solutions of different activity is gradually reduced at a certain point the solution precipitates. The more active the actomyosin, the more salt is needed to keep it in solution. This precipitation is reversible. If the salt concentration is increased again the precipitate dissolves. One very interesting fact, which emerges from these experiments of Fig. 3, is that if the precipitate is dissolved by increasing the salt concentration in presence of ATP the actomyosin not only dissolves but also dissociates at the same time into actin and myosin. Actomyosin, dissolved thus in presence of salt and ATP means dissociated actin and myosin.

If the KCl concentrations, at which the actomyosin dissociates without ATP, are plotted against the activity of the actomyosin, a straight line is obtained (Fig. 5).

References.

1. *K. Balenović* and *F. B. Straub.* These studies **1**, 43, 1941—42.
2. *F. B. Straub.* Ibid **2**, 6, 1942.
3. *F. B. Straub.* Ibid **1**, 43, 1941 42.
4. *I. Banga.* Oral comm.

Extraction of myosin.

by

F. GUBA AND F. B. STRAUB.

If rabbit's muscle is extracted with salt solution an actomyosin of varying activity is obtained. In order to prepare pure myosin it is essential to start from a possibly inactive material.

We have studied the amount and the activity of the extracted myosin under varying conditions. The mode of mincing, the pH, the salt concentration and the time of extraction were varied. Activity was determined by the method of STRAUB[1], myosin contents were calculated from the viscosity in presence of ATP.

We find that the extraction of coarsely minced rabbit's muscle for 10 minutes at 0° with 3 volumes of a phosphate-KCl mixture of pH 6,5 (which contains 0,15 *M* K-phosphate buffer of pH 6,5 and 0,3 *M* KCl) will give an actomyosin of low (3 %) activity.

1. The effect of mincing and time of extraction. In the first series the muscle tissue was minced through an ordinary meat chopper only* (= coarse mincing), in the second it was minced first through the meat chopper and then minced again through a Latapie mincer (= fine mincing). In both cases the mince was extracted at 0° with 3 volumes of a 0,6 *M* KCl solution under constant stirring for different lengths of time, as indicated in Table I.

* Diameter of holes: 2 mm

Table I.

Time of extraction minutes	Coarse mincing		Fine mincing	
	% activity	mg myosin/ml	% activity	mg myosin/ml
5	4,5	12	7	15,6
10	4,8	3,8	9	16,5
15	5,0	15,5	13	17,2
20	6,6	17,7	20	18
25	9,0	20,2	22	19
60	—	—	40	—
120	—	—	80	—

Table I. shows that there is a rise in the activity of the extracted myosin on prolonged extraction. We therefore extract the muscle tissue only for 10 minutes. Fine mincing results in somewhat more myosin being extracted, but the activity of the extracted myosin is in this case more than twice of that obtained from coarsely minced muscle. It is further seen from the table that even from the coarsely minced muscle KCl extracts a fairly active myosin. This extracting agent is not satisfactory also because the results are not strictly reproducible and show considerable variations.

2. *The effect of salt concentration.* The muscle was minced through a meat chopper and extracted for 10 minutes at 0° with 3 volumes of the KCl concentration indicated in Table II.

Table II.

Salt concentration of extracting fluid, *M*	% activity	mg myosin/ml
0,2	—	0,8
0,3	17	3,5
0,4	10	8,7
0,5	11	10
0,6	12	11,8

Table II. shows that a minimal KCl concentration of 0,4 *M* is needed to extract myosin. It is to be seen moreover that the activity of the extracted myosin has a minimum value at 0,4 *M*.

The effect of pH. The muscle tissue was extracted at 0° for 10 minutes with 3 volumes of a phosphate-KCl mixture. This contained 0,15 *M* phosphate (KH_2PO_4 and K_2HPO_4) of varying pH and 0,3 *M* KCl.

Table III.

pH	% activity	mg myosin/ml
5,9	12	4,3
6,3	7	10,5
6,5	3,5	12
7,0	4,5	10,7
7,5	5	10,3

The activity was found to be 15—20 % when the muscle tissue was extracted with WEBER's solution* under similar conditions and 1% activity if it was extracted similarly with a 0,6 *M* KCl buffered to pH 10 with borate buffer.

Phosphate ions seem to have a depressing effect on the activity of the extracted myosin as extracts made without phosphate at the same pH and ionic strength are always more active than those prepared with phosphate. That the low activity is due to the lower actin content and not to the phosphate present is shown by the fact that the myosin of the phosphate extract, if precipitated and redissolved in a phosphate free KCl solution, will show the same low activity.

4. Salt concentration of phosphate-KCl mixtures. The extraction was made at 0° for 10 minutes with 3 volumes of phosphate-KCl mixture. The latter always contained 0,15 *M* phosphate of pH 6,5 and varying concentrations of KCl.

Table IV.

M KCl	% activity	mg myosin/ml
0,1	19	2,8
0,2	3	7,1
0,3	3	11,9
0,4	4	12,8

The table shows that apart from the phosphate 0,3 *M* KCl is needed to give optimal results. In a great number of experiments identical results were obtained.

References.

1. *F. B. Straub,* These studies **2**, 3, (1942).

* This solution contains 0,6 *M* KCl, 0,01 *M* Na_2CO_3 and 0,04 *M* $NaHCO_3$.

Note on the viscosity of myosin.

by

F. GUBA AND F. B. STRAUB.

Several workers have, in the past, studied the viscosity of myosin and found it to be anomalous. As it is now clear that in all these cases impure myosin was used, these data must be revised. A small amount of actin influences the viscosity of myosin very strongly.

We have determined the viscosity of twice recrystallised

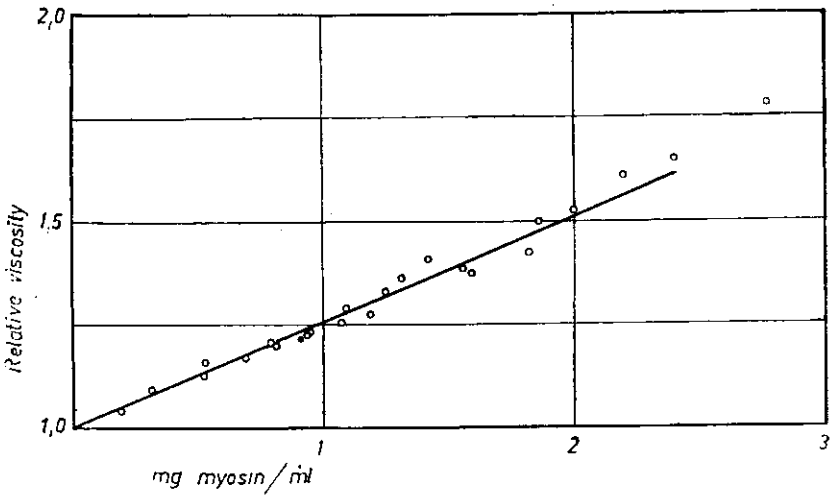

Fig. 1. Viscosity of recrystallised myosin in 0.6 *M* KCl at 0°.

myosin at 0° in 0,6 *M* KCl solution buffered with veronal acetate to pH 7. (For the composition of this solution see BALENOVIĆ and STRAUB[1].) The myosin content was determined in the following way: 1 ml of the myosin solution was diluted so as

to contain less than 0,2 *M* salt and then an equal volume of alcohol was added and the mixture was heated to 70° for 10 minutes. The precipitate was centrifuged off and washed with a 50 % alcohol, centrifuged and dried at 105°.

The viscosity of myosin solutions in relation to the concentration of myosin is shown in Fig. 1. No correction was taken for the change of specific weight on addition of myosin to the salt solution. The points were obtained with four different myosin preparations.

The dependence of the viscosity on the pressure was studied with the method described in this volume.[2] It was found that, between 5—100 cm water pressure, the viscosity is independent of the pressure within the limits of experimental errors. Thus it appears that the viscosity of myosin in 0,6 *M* KCl is not anomalous. The same conclusion can be drawn from Fig. 1. where it is to be seen that the viscosity of dilute solutions is proportional to the myosin content.

References.

1. *K. Balenović* and *F. B. Straub*, These studies **2**, 17 (1942).
2. *F. B. Straub*, ibid., **3**, (1943).

Rigor, contracture and ATP.

by

T. ERDŐS.

It has been shown in previous papers of this laboratory that the contractile substance of the muscle fibril is actomyosin, a complex composed of two proteins, actin and myosin. Actin is bound to the solid structure or rather forms the solid structure of the fibril itself. Myosin is attached to the actin in a loose, dissociable form. It has also been shown that ATP has a decisive influence on the physical state of actomyosin which, according to conditions, is contracted, relaxed or dissolved by ATP.

The object of this work was to see, whether in muscle ATP has any influence on the physical state of the actomyosin, especially, whether the relaxed state of the muscle is dependent on its presence.

Muscle contains the rather high concentration of 300—350 mg % of ATP. CASPERRSSON and THORRELL[1] have shown that a considerable part of the ATP is bound in the *I* band. One can thus expect no close parallelism between ATP concentration and the state of relaxation because the ATP is not evenly distributed. A really close parallel can be expected in rigor mortis only, where there is a post mortal disorganisation and sufficient time for the ATP to be distributed evenly within the muscle fiber. In other cases, the more the muscle is damaged and the more time given for even distribution of ATP the closer we may expect the parallelism to be. Apart from rigor mortis I also studied other forms of contracture, such as monoiodoacetic acid, caffeine, chloroform-contracture and the contracture of electrically stimulated muscle.

It has been found by several investigators (2, 3, 4) that the solubility of muscle proteins decreases during activity and in different forms of contracture. In the case of monoiodoacetic

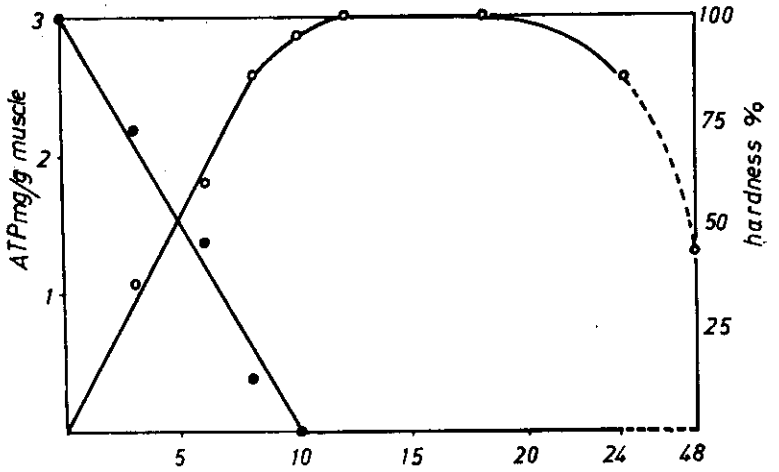

Fig. 1. a. ATP content during development of rigor mortis, in rabbit muscle.
● Hardness %/0%: fresh muscle. 100%: rigor mortis.
○ ATP mg/muscle

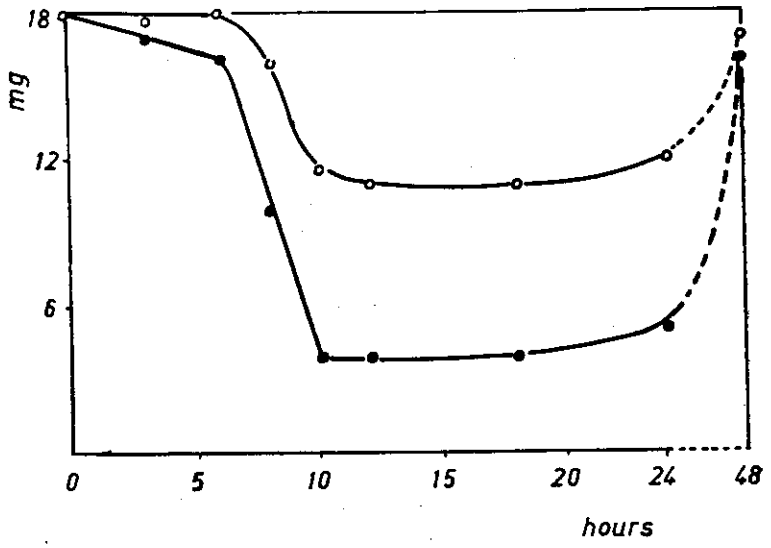

Fig. 1. b. Solubility of myosin during the development of rigor mortis. (Same experiment as in Fig. 1. a.)
● Myosin mg/ml. extracted with 3 vols. of. 0.6 *M* KCl.
○ Myosin mg/ml. extracted with 3 vols. of 0.6 *M* KCl. in presence of 100 mg% ATP.

acid, MIRSKY[5] found that the solubility of myosin is decreased. For this reason I also undertook to measure, parallel with the rigor and the ATP concentration, the solubility of myosin. My results were the following:

Rigor mortis: There is a close parallelism between hardness of the muscle and the ATP concentration. The rigor develops at the same rate as the ATP disappears. At the maximum of rigor there is no ATP at all. (Fig. 1.)

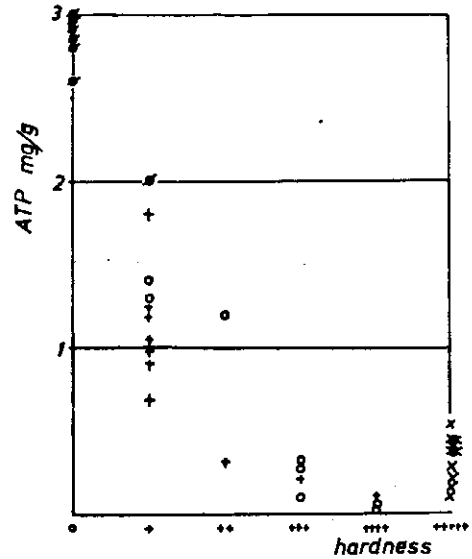

Fig. 2. ATP content and hardness of frog muscle, in different forms of contractures

0 is the hardness of fresh muscle.
+++++ is the hardness of muscle in maximal rigor.
⌀ Fresh muscle.
○ Muscle 24—48 hours after decapitation. (At 18—20°)
+ Electrically stimulated muscle.
× Isolated gastrocnemius exposed to chloroform vapours for 12 mins.
● Left leg
● Right leg, stimulated of frog poisoned with iodoacetate.

The hardness was measured *in situ*. The same muscles were used for ATP determination.

Parallel with the development of the rigor the myosin becomes more and more insoluble showing that both phenomena, rigor and insolubility are closely connected. By addition of ATP the solubility of myosin could be restored. This supports the conclusion that both the rigor and the decreased

solubility were, in fact, due to the reduction of the ATP concentration. In absence of ATP, myosin is practically insoluble.

Parallel to the relaxation of the rigor the myosin becomes soluble again and goes into solution even without addition of ATP. In this case, however, it is not myosin that goes into solution but actomyosin. It can be concluded that the relaxation of rigor mortis is thus due to desorganisation, i. e. to the release of actin from the structure.

In the other forms of rigor there is less close parallelism between contracture and ATP concentration than in the case of rigor mortis. (Fig. 2.)

Experimental part.

The hardness of the muscle was measured by MANGOLD'S[6] method. I worked throughout with 10 g weight.

Myosin was extracted with 3 ml 0,6 *M* KCl pro g muscle. The muscle was minced in the Latapie mincer, suspended in the KCl solution, stirred for ten minutes and centrifuged. In order to estimate the myosin content of this extract 1 ml. 0,5 *M*, pH 5,2 acetate buffer and 8 ml water were added to 1 ml of it. The precipitate was centrifuged off, washed twice, dried and weighed.

The ATP was estimated in extracts of muscle according to SZENT-GYÖRGYI (see page 93.).

Rigor mortis: The hardness was measured on one and the same place of the thigh of the decapitated and skinned rabbit. From the other thigh I excised, after certain periods, 10—10 g samples. 3 g were used to estimate the solubility of myosin without addition, 3 g with addition of ATP. (3 mg ATP was added pro g. muscle). In 3 g of the muscle the ATP was estimated. The results are summed up in Table 1. The data of the first series of experiments are shown also in Fig. 1, where the correlation between the different factors can be seen.

I was unable to pull threads from the myosin extracted from the muscle in rigor, which shows that there was very little actin present. The myosin extracted after relaxation can readily be pulled to threads. It was a 9% actomyosin and contracted on addition of K, Mg and ATP: 66%.

In the case of monoiodoacetic acid, and caffeine I injected the solutions into the lymphsac of the frog. (0.4 mg iodoacetate

Table I.

Hours	Hardness %	Soluble myosin mg/ml	Soluble myosin mg/ml in presence of 100 mg % ATP	ATP mg/g muscle
0	0	18	18	3
3	36	17	17.5	2.2
6	60	16	18	1.4
8	86	10	16	0.4
10	96	4	11.5	0
12	100	4	11	
18	100	4	11	
24	86	5	12	
48	43	16	17	
0	0	14	15	2.8
5	50	15	15	1.4
6	70	10	15	0.7
9	100	4	13	0
0	0	13	15	3
6	50	13	16	1.1
24	100	3	16	0
0	0	18	18	2.9
6	100	4	17	0
0	0	16	17	
10 days At 0°	40	14	14	
0	0	15	16	
8 days At 0°	45	14	15	

The hardness of fresh muscle: 0%.
Rigor mortis: 100%.

per g, 0,15 mg caffeine per g). After the rigor had developed I estimated the solubility of myosin with and without the addition of ATP. The results are summed up in Tab. 2. In one experiment the lower half of the spinal cord, innervating the lower limb, was destroyed. When rigor began to develop in the fore limb I stimulated one hind limb electrically for a minute and then used both limbs for estimation of rigor and ATP. The result of this experiment is summed up with other experiments in Fig. 2.

Table II.

Soluble myosin mg/ml extracted with :					
3 ml 0.6 *M* KCl/g muscle			3 ml 0.6 *M* KCl/g muscle in presence of 100 mg % ATP		
Fresh resting muscle	Muscle in iodoacetate rigor	Muscle in caffeine rigor	Fresh resting muscle	Muscle in iodoacetate rigor	Muscle in caffeine rigor
7	1.1	5	8	8.5	11
11	2.5	5.5	12	11	12
11.1	4		11	11	
9.5	3.6		10	9.2	
10	5		11	22	

The ATP content of muscles in rigor was 0.10—0.20 mg/g.

I am indebted to the CHINOIN Foundation for a grant.

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2. *H. G. Deuticke:* Pflügers Arch. **224,** 1, 1, (1930).
3. *G. Embden* u. *E. Metz:* Hoppe Seyler's Z. **192,** 4, 233, (1930).
4. *I. Banga* and *A. Szent-Györgyi:* These studies 1, 5, (1942).
5. *A. E. Mirsky:* Studies from the Rockefeller Institute for Medica, Research. **98,** 493, (1936). (Reprinted from The J. Gen. Physiology. **19,** 41 571, (1936)).
6. *E. Mangold:* Pflügers Arch. **196,** 200, (1922).

On the relation of the activity and contraction of actomyosin threads.

by

T. ERDŐS.

The object of my experiments was to determine the relation of the % activity of the actomyosin and the contraction of the threads. In order to obtain comparable results threads of equal diameter have to be used and the thread should have the same concentration of protein. It is not possible to use in the whole series of experiments threads of the same protein content. Threads of low protein concentration cannot be pulled from an actomyosin of low activity. Threads of highly active actomyosin contract rapidly only at a low protein concentration and become sluggish if the protein concentration is high. For this reason under 50 % activity I used threads of 1,6 %, above 50 % threads of 0,9 % protein content. The diameter was throughout 0.25 mm. The actomyosin was prepared from recrystallised myosin and actin. The actomyosin contained 0,5 *M* KCl and was squirted through a capillary into 0,05 *M* KCl containing 0,001 *M* $MgCl_2$. The contraction was measured by following the length of the thread in a 0,02 *M* pH 6,7 potassium phosphate solution containing 0,001 *M* $MgCl_2$ to which 0,16 mg ATP was added after the thread was soaked in the salt solution.

Two series of experiments were performed. In the first I compared threads made from actomyosin of 10, 20, 30, 40 and 50 % activity. In the second I compared threads made from actomyosin of 50, 100, 170 % activity.

The results were the following: up to 40 % activity the contractibility increased with the activity (see fig. 1.). Threads prepared from 40 and 50% active myosin give the same contraction. Threads of 50, 100 and 170 % active myosin, also give identical results.

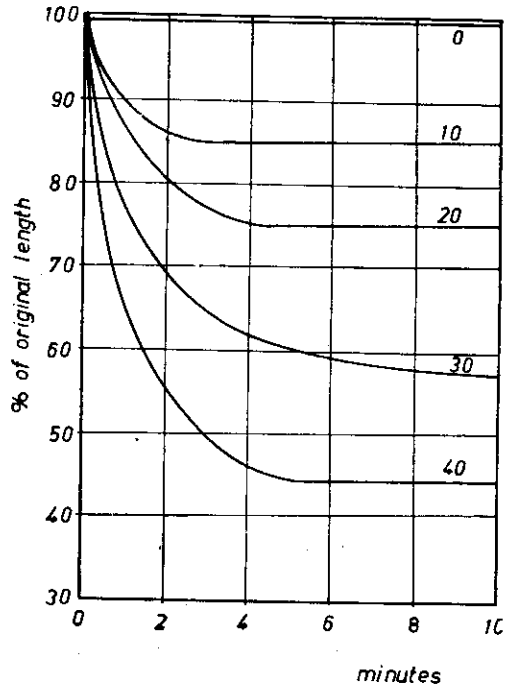

Fig. 1. Contraction of actomyosin threads of actomyosin of different activity. The numbers on the curves indicate the % activity of the actomyosin.

Summing up, thus, it can be said that the contractibility of actomyosin increases with increasing activity up to 40 % activity; 40 % actve myosin containing 6,5 parts of actin to 93,5 parts of myosin gives maximal contraction.

The ATP-creatine phosphopherase.

by

I. BANGA.

It has been shown by K. LOHMANN[1] that in muscle the easily hydrolysable phosphate of ATP is taken over by creatine. In dialysed muscle extract therefore the reaction taking place can be expressed by the equation:

ATP + 2 creatine = adenylic acid + 2 creatine-phosphoric acid

This reaction was investigated by H. LEHMANN[2] who demonstrated its reversibility, but the equilibrium constant = K calculated for a trimolecular reaction was not constant.

My investigations have proved that the above reaction takes place in two distinct steps and that the presence of two distinct proteins is necessary for this catalysis. The first reaction is:

ATP + creatine = adenosine-diphosphoric acid + creatine-phosphoric acid

The enzyme catalysing the reaction was called the ATP-creatine phosphopherase and was purified to a degree at which no secondary reactions occured. If the kinetics of this reaction were examined at varied concentrations of the reactants, the equilibrium constant calculated for a bimolecular reaction was found to be constant.

The second step of the reaction

Adenosine-diphosphoric acid + creatine = adenylic acid + creatine-phosphoric acid

is catalysed by another protein, which I called ADP-creatine phosphopherase, the isolation of which is in progress.

Experimental:

Test: Unit of the ATP-creatine phosphopherase is the quantity of enzyme which is capable of transferring 0,00213 moles P from ATP to creatine in a period of five minutes in the presence of 0.00475 moles of ATP and 0.0215 moles of creatine in a pH 8.55 veronal-acetate buffer. Half that quantity of enzyme transmits 0.00106 moles and a quarter of it 0.00053 moles of P during the same interval. The resulting creatine-phosphate was determined after FISKE and SUBBAROW.* In this method creatine-phosphate hydrolyses during the five minutes of incubation and appears as inorganic phosphate. There was no preformed inorganic phosphate in my experiments.

The activity of the enzyme is defined as: enzyme units divided by the quantity of the protein dry material, a $= \frac{\text{units}}{\text{mg protein}}$ The protein dry material was determined by placing a given quantity of enzyme in a weighed centrifuge tube and precipitating it with 5% of trichloro-acetic acid. The precipitate was washed twice in a tenfold volume of water, dried and weighed.

The process of isolation: The muscle of a freshly killed rabbit was minced on a Latapie mincer, suspended in 1.5 ml 0.1 *M* KCl per g and was then extracted by stirring it at 0° for 10 min. The muscle was then strained through a cloth and once more extracted in $^1/_3$ of the former volume of 0.1 *M* KCl for a period of ten min. The extracts were united, left alone for 1—2 hours and then centrifuged. The ATP-creatine phosphopherase activity of this extract could not be determined with accuracy because it also contained ADP-creatine phosphopherase. On the whole it was found that 1 g of fresh muscle of the rabbit contained 100—150, that of the pigeon 200—250 units of ATP-creatine phosphopherase.

*Preparation of ATP-creatine phosphopherase:***

First step: To every liter of the above fluid were added 80 ml of 0.5 *M* $NaHCO_3$. Then the fluid was half saturated with ammoniumsulfate and centrifuged once more. The precipitate was discarded. Activity of the fluid = 6.

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* as modified by LOHMANN and JENDRASSIK.

** All operations were carried out at 0°.

Second step: For every liter 30 ml of 0.5 *M* $NaHCO_3$ were added and as much ammoniumsulfate as was needed to bring it up to 0.7 saturation. The precipitate was filtered and dissolved in 250 ml buffer solution of 0.025 *M* borate of pH 8.55. Dry weight 30mg/ml. a=7. No loss of activity.

Third step: To 1 liter of enzyme solution 2.5 litres of alcohol of —20° were added. The precipitate was centrifuged off at 0° and dissolved in 250 ml of 0.02 *M* borate buffer of pH 8.55. The insoluble material was filtered off and the solution

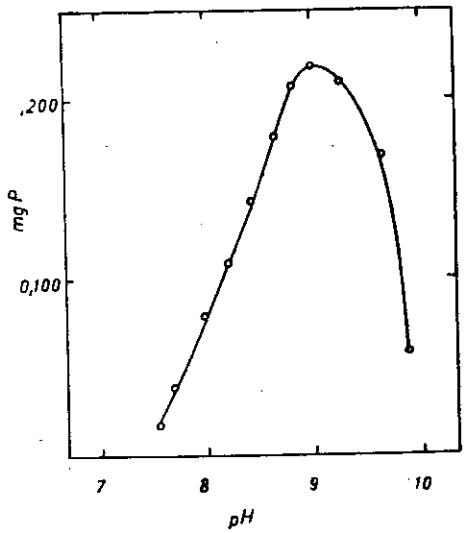

Fig. 1.

was dialysed for a period of five hours against thirty volumes of water. Dry material 7—10 mg/ml; a = 30. Loss 30 %.

Fourth step: Adsorption to γ $Al(OH)_3$. To every liter of dialysed fluid 300 ml of $Al(OH)_3$ suspension was added. This contained 20 mg of $Al(OH)_3$ per ml. After shaking for five minutes the solution was centrifuged. Elution with 250 ml of pH 8.55 borate buffer containing *M*/10 borate and 20 % ammoniumsulfate. Dry material 3—4 mg/ml; a = 95. Loss 45 %.

Fifth step: *M*/50 NH_3 and ammoniumsulfate was added till 0.5 saturation. The resulting precipitate was filtered and dissolved in *M*/10 borate buffer. a=100. Loss 10%.

1 γ of this purest enzyme preparation (a=100) transferred at pH 8.55 in one minute 2 γ P from ATP onto creatine. If the

molecular weight of the enzyme is taken to be 70,000, this would mean, that one molecule of enzyme did react upon 4.400 molecules of the substrate per min.

In the course of this isolation the ADP-creatine phosphopherase disappeared at step 3. together with ADP-isomerase (see page 66.).

With ATP-creatine phosphopherase, thus isolated. I determined how far the activity was dependent on the pH. As can be seen from Fig. 1., in presence of borate buffer the pH optimum was 9.05.

Determining the equilibrium constant at different pH, I found that the value of K depended to a great extent on the pH and reached its maximum at the pH optimum of the reaction. Table I. shows the equilibrium constant $= K$ at varied pH and with constant creatine and ATP concentrations. These investigations were complicated by the fact that the ATP itself acted as a buffer, and thus changed the pH of the original buffer solution. For this reason the pH of the ATP and creatine solutions were colorimetrically brought to the necessary pH and the buffers were used in high concentrations.

Table I.

Experimental technique: 1 ml of borate buffer solution of varying pH, 0.0195 *M* creatine, 0.00890 *M* ATP, 0.1 ml $= 60\,\gamma$ of ATP-phosphopherase. Volume 1.6 ml. Incubation at 38° until the state of equilibrium had been reached.

pH	Measured *M* creatine P	$K = \frac{Cr \,.\, P \,.\, ADP}{ATP \,.\, Cr.}$
7.50	0.00059	0.0023
7.60	0.00085	0.0049
7.80	0.00172	0.0239
8.20	0.00289	0.0925
8.50	0.00383	0.1910
9.05	0.00459	0.3651
9.50	0.00344	0.1382
9.70	0.00094	0.0061

In establishing the pH curve the following experimental technique was employed: 2 ml of buffer solution were mixed with 1 ml (0.01915 *M*) of creatine + 0.5 ml (0.00890 *M*) of ATP + 0.5 ml H_2O. Of this mixture 0.5 ml were removed for the pH determination. 1.5 ml were used to determine the rate of the reaction. 0.05 ml enzyme (30 γ) was added and the mixture was incubated for 5 min. at 38°. Another 1.5 ml in the presence

of enzyme were incubated for 30 min. at 38° in order to determine the equilibrium constant.

Table 2 shows the values of the equilibrium constant (K) at identical pH (veronal acetate buffer of pH 8.55) and with varied ATP and creatine concentrations. As can be seen, the equilibrium constant has the same value throughout. The value is lower in veronal acetate buffer than in borate buffer. This might be attributed to the complex formation of borate and ATP which complex might react faster with creatine than free ATP. To avoid such complications I employed for the estimation of the equilibrium constant a buffer solution of veronal acetate.

Table II.

Experimental technique: 1 ml of veronal-acetate buffer solution of pH 8.55 + 0.1 ml = 60 γ ATP-phosphopherase + varying quantities of ATP and of creatine. Volume: 1.6 ml. Incubation at 38°, until state of equilibrum had been reached.

M creatine added	*M* ATP added	*M* P found	$K = \frac{Cr \,.\, P \,.\, ADP}{ATP \,.\, Cr.}$
0.02390	0.00222	0.00104	0.040
0.02390	0.00444	0.00158	0.042
0.02390	0.00890	0.00232	0.038
0.02390	0.01331	0.00292	0.039
0.01030	0.00236	0.00078	0.040
0.01531	0.00236	0.00090	0.038
0.02040	0.00236	0.00104	0.040
0.02542	0.00236	0.00108	0.038

Further experiments with ATP-creatine phosphopherase showed that the pH stability curve did not coincide with the pH activity curve. After a storage for a longer period, the enzyme had changed its pH optimum.

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Enzyme Studies.

by

I. BANGA.

I. Enzymic activity of crystallised myosin.

SZENT-GYÖRGYI[1] described a method for the preparation of myosin free of actin. This myosin could be crystallised and purified by repeated recrystallisations. I have found that, in presence of KCl, this myosin, recrystallised twice, has the same enzymic activity towards ATP as earlier impure preparations. This can be seen from Fig. 1.

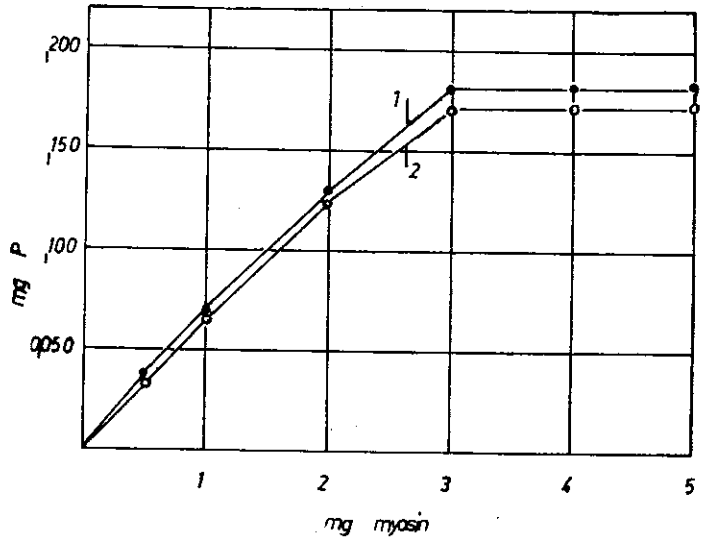

Fig. 1. The splitting of ATP by impure and recrystalliseed myosin. mg P split off at 38° in 5 min. from a mixture of 1 ml of 0.1 *M* veronal acetate buffer of pH 7.0. 0.05 *M* KCl. 3.6 mg ATP as neutral K salt and varying amounts of myosin. Total volum 3 ml.
Curve 1: purified myosin before crystallisation.
Curve 2: the same myosin recrystallised twice.

The reaction mixture was always deproteinised by 1/5 volume of 20% trichloroacetic acid and P was determined in the filtrate according to the method of FISKE and SUBBAROW, modified by JENDRASSIK and LOHMANN.

ATP has two phosphate groups which are readily hydrolysed in 15 minutes at 100° by *N* HCl. Only one of these is split off by crystallised myosin. This process of splitting off of phosphate is activated by KCl (see page 69.) and is inhibited by Mg. The Mg inhibition is shown in Fig. 2. The resulting ADP

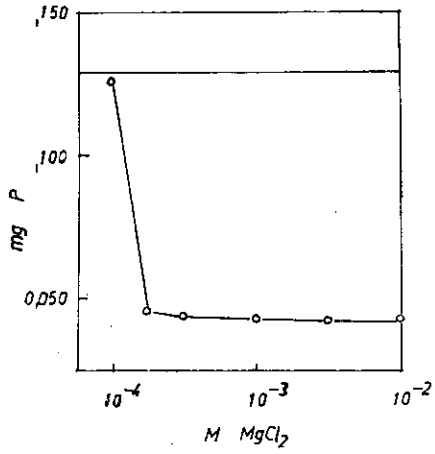

Fig. 2. Effect of $MgCl_2$ on splitting of ATP by crystallised myosin. mg P split off at 38° in 5 min. from a mixture of 1 mg myosin, 0.1 *M* KCl, 3.6 mg ATP and varying concentrations of $MgCl_2$. Total volume 3 ml.

was isolated from the reaction mixture. The Ba salt contained 3/2 Ba atom per molecule of ADP. From the two phosphate groups only one was readily hydrolysable.

The ADP was isolated by precipitating the inorganic phosphate by ammoniacal $MgCl_2$ and precipitating the ADP by $BaCl_2$. I proceeded as follows: 100 mg crystallised myosin and 1400 mg of ATP, in the form of neutral K salt, were dissolved in 300 ml 0.1*M* KCl. Incubation at 38° until samples taken showed that one P had already been split off. The protein was then removed by adding 30 ml of 20% trichloroacetic acid. Thereafter I added 3 g $MgCl_2$ and NH_4OH until the solution began to turn red in presence of phenolphtalein. The resulting mixture was stored overnight at 0° during which period the inorganic P had separated quantitatively in the form of $Mg(NH_4)PO_4$. In order to precipitate the neutral Ba salt of ADP. 3 g of $BaCl_2$ and 1/5 volume of alcohol were added to the liquid. The precipitate was washed first in 50% alcohol, then in

absolute alcohol and dried. The yield was 65 % of the theoretical value.

In order to obtain the ADP free of Mg, the Ba salt was dissolved in 5 % acetic acid and precipitated by Hg acetate. The salt was dissolved in 0.5 *N* HCl and the Hg removed as HgS. H_2S was removed by ventillation. By adding $BaCl_2$ and alcohol to the resulting liquid at neutral reaction the ADP was precipitated as a Ba salt.

Analysis of P: 100 mg Ba salt of ADP were dissolved in presence of HCl in 8 ml of H_2O and K_2SO_4 was added (one molecule of K_2SO_4 to every molecule of Ba salt). Thereafter I neutralised with KOH and brought the volume up to 10 ml with water and centrifuged. 0.1 ml of this solution contained 0.002 mg of inorganic P, 0,046 mg readily hydrolysable P and a total P of 0.093 mg. The proportion of the readily hydrolysable P to the stable one, was found to be 1 : 1.

II. ADP-isomerase.

When adenosine triphosphate was added to impure actomyosin (myosin B)[2] then both of the readily hydrolysable phosphate groups were split off. When all the soluble protein had been removed from actomyosin by four consecutive precipitations, it was found that only one of the two readily hydrolysable phosphates was split off. The other phosphate would split off upon addition of a muscle extract from which all substances, insoluble in water, had previously been removed. K. LAKI[3] showed that in addition to an enzyme system, insoluble in water and liberating only one phosphate group from ATP, a soluble protein and Mg were necessary to split off a second phosphate. My investigations confirmed LAKI's findings. The soluble factor must have been a protein since it was thermolabile and could be precipitated by trichloroacetic acid. In presence of this protein actomyosin readily attacked ADP. This protein was named ADP-isomerase for reasons to be shown hereafter.

By the following method the ADP-isomerase can be obtained 50 times purer than the original muscle juice. Freshly minced muscle of rabbit was suspended in 0.1 *M* KCl, 1.5 ml being taken per g of the muscle. Two hours later the insoluble

parts were removed by centrifugation. Ammonium sulfate was added to 0.5 saturation and the precipitate discarded. The liquid was neutralised and ammonium sulfate added to 0.7 saturation. The resulting precipitate contained the ADP-isomerase. It was dissolved in 0.1 *M* borate buffer of pH 8.5, dialysed for 24 hours and the insoluble parts removed by centrifugation. The isomerase was adsorbed to γ $Al(OH)_3$. From the latter it was eluted with 0.1 M borate buffer of pH 8.5. From this solution the isomerase was purified by precipitating it between 0.5—0.6 saturation of ammoniumsulfate. 50 γ of this protein in 3 ml. activated the splitting of ADP by actomyosin.

The question might be raised whether this new protein is not identical with the myokinase described by KALCKAR,[4] which dismutates ADP into adenosinetriphosphate and adenylic acid. My experiments showed that this is not the case since the new protein when incubated with ADP, did neither produce ATP nor adenylic acid. The product of incubation was not split by crystallised myosin, thus it was not ATP, nor was it reacted upon by deaminase, therefore the product could not have been adenylic acid either. When ADP had been incubated with isomerase, the product was split by actomyosin which suggested that isomerase had changed the molecular structure of ADP. It was for this reason that the name isomerase was given to this new protein since its effect consisted not in dismutation but in isomerisation. There was no appreciable change observed in the relation between the labile and the stable phosphate of ADP if incubated with isomerase.

Actomyosin is able to split off phosphate from the compound formed by the action of isomerase on ADP, whereas it is unable to attack ADP itself. If therefore ADP is incubated first with isomerase and the reaction product is added afterwards to actomyosin, inorganic phosphate will be formed to the extent to which the isomerisation reaction has proceeded. By this method I was able to study the isomerisation reaction itself.

Isomerisation of ADP: 3 mg K-ADP were incubated at 38° for 10 minutes at pH 8.5 in the presence of 50 γ isomerase in 1 ml. I deproteinised with 2 ml of 20% trichloroacetic acid, centrifuged and neutralised it by addition of *M* KOH.

Splitting of the isomerised product: The reaction mixture contained 2 mg of 25% actomyosin produced from cryst. myosin and actin, 1 ml 0.1 *M* veronal acetate buffer. To this mixture I added the substances noted in Col. I. Total volume 3 ml. Incubation for 10 min. at 38°. The results are shown in Table I.

Table I.

Splitting of the isomerised product of ADP by actomyosin

	mg P split off	Percentage ADP converted
Actomyosin + 3 mg ADP	0.00	
" + 3 mg ADP + 50 γ isomerase	0.104	(100)
" + 3 mg ADP previously incubated with isomerase	0.022	20
" + 3 mg ADP previously incubated with isomerase + Mg	0.064	60
" + 50 γ isomerase + 3 mg ADP previously incubated with isomerase + Mg	0.107	

Upon addition of Mg, the effect of the isomerase could be increased by 200 per cent. Thus while in a certain interval isomerase alone turned only 20% of ADP into a product which was split directly by actomyosin, 60 % were transformed in the presence of 0.001 *M* $MgCl_2$.

As to the question what molecular changes ADP had undergone upon the effect of isomerase, we must remember on one hand that isomerisation did not affect the relation of the labile and stable P of ADP. It is seen on the other hand from Table II. that only one half of the readily hydrolysable phosphate of ADP is split off by actomyosin + isomerase, i. e. only one quarter of the total P.

Table II.

10 mg of 25% actomyosin, 0.5 mg isomerase, 10 ml 0.1 M veronal-acetate buffer of pH 8.5, 30 mg ADP containing 2.25 mg readily hydrolysable P and 4.50 mg total P. The mixture was made up with water to 30 ml. Incubated at 38°. At definite time intervals samples of 3 ml were taken out and deproteinised with 1 ml of trichloroacetic acid. The experiment was finished when no more P was liberated. This usually was reached at about the end of 20 min. No more P was liberated even if the experiment was protracted to an hour.

	Inorg. P	Readily hydr. P	Total P
At the outset of experiment in 3 ml	0.00	0.225	0.450
At the end of incubation in 3 ml	0.115	0.110	0.450

III. Adenosinediphosphatase.

When ADP was added to crystallised myosin no reaction whatever took place. ADP was not split by crystallised myosin, not even when isomerase had been added. But if a third protein, — extracted in a soluble form from a washed, acetone

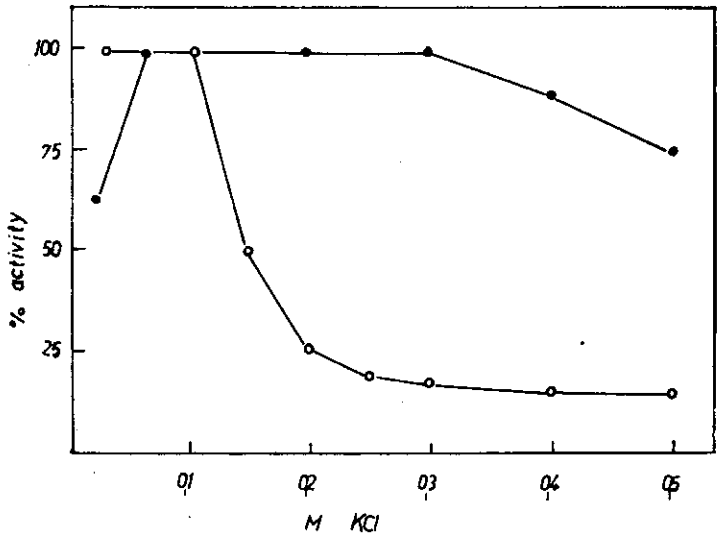

Fig. 3. Effect of KCl concentration on splitting of ATP and ADP. 100% is the maximal amount of P split off under optimal conditions.
○ — ○ — P liberated from ADP
● — ● — P liberated from ATP
2 mg of myosin, 3 mg of K ATP or ADP, KCl of varying concentrations. Total volume 3 ml. Incubated for 5 min. at 38°. In the case of ADP, isomerase and third protein were added, too.

dried muscle — was added to a mixture of crystallised myosin + isomerase, then one half of the readily hydrolysable phosphate of ADP was liberated. The optimum pH of the ADP splitting was at 8.5. The reaction was very sensitive to the KCl concentration. While the ATP-phosphatase activity of crystallised myosin was constant along a wide range of KCl concentration, the ADP splitting of the above three-enzyme system was optimal only within a narrow range of KCl concentration. (Fig. 3, curves 1, 2.)

The third protein could be replaced by actin prepared according to F. B. STRAUB's[5] method, therefore it seemed likely that the two proteins were identical. The experiments, numbered 1—6 below, seem to indicate, that the protein which splits

ADP is actomyosin itself. This, however, could not be proved conclusively.

1. No solution of the third protein could be extracted from a fresh muscle in water or in weak salt solution, since it was always the insoluble part of the fresh muscle which contained the third protein. But, similarly to actin, this protein became soluble in water when the muscle had previously been washed, treated with acetone and dried. When this washed and acetone dried muscle had been extracted in a tenfold volume of water for about 10—20 minutes, a 0.1% solution of protein was obtained of which 0.1 mg manifested the third-protein-activity. In case of small amounts the activation was

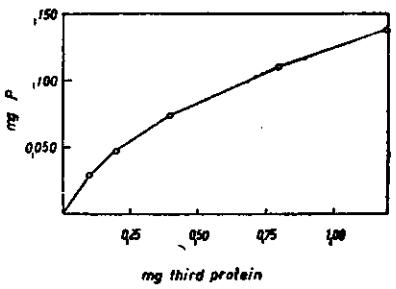

Fig. 4. Effect of varying amounts of the third protein on the splitting of ADP by myosin in presence of isomerase. 1 mg of crystallised myosin, 50 γ isomerase, 1 ml veronal acetate buffer of pH 8.5, 3 mg ADP as K salt and varying amounts of third protein. Total volume 3 ml. Incubated for 5 min. at 38°.

proportional to the quantity of the protein, whereas with greater amounts the curve for the activity flattened as shown by Fig. 4.

Actin prepared by the method of F. B. STRAUB shows a third-protein-activity proportional to its actin content.

2. If 0.01 *M* $CaCl_2$ had been added to a solution of the third protein, the Ca precipitated the third protein quantitatively just as it precipitated actin.

3. When I added the third protein to crystallised myosin in a 0.5 *M* KCl, actomyosin was formed which could be precipitated upon a 5—10 fold dilution. This actomyosin could again be dissolved in a 0.5 *M* of KCl and once more precipitated by dilution. Actomyosin, thus produced, splits ADP in the presence of isomerase and shows the same activity as myosin + actin, or myosin + third protein. Since the third protein as well as the myosin were soluble in water, it was evident that a combination had taken place between myosin and the third protein, making the former insoluble in water.

It is clear from all this that the third protein produced the same effect as actin.

4. Similarly to actin the third protein could be precipitated from its solution by addition of weak acids (pH of about 4.8).

5. The KCl sensitivity (Fig. 3) of the ADP splitting, also corroborated the surmise that it was the actomyosin, which played a part in the reaction since actomyosin, in a 0.2 *M* KCl solution, dissociates into myosin and actin, as proved by the experiments of F. GUBA.[6]

6. The various impure myosin preparations split ADP in the presence of isomerase proportional to their actin content.

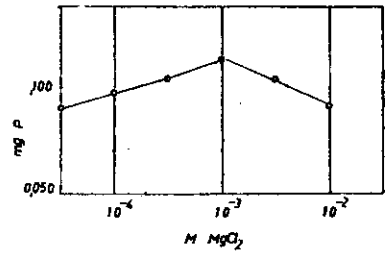

Fig. 5. Effect of varying amounts of $MgCl_2$ on the splitting of ADP by the three-enzyme-system. 1 mg of crystallised myosin, 50 γ isomerase, 0.5 mg third protein 1 ml 0.1 *M* veronal acetate buffer of pH 8.5 and varying concentrations of Mg Cl_2. Total volume 3 ml. Incubated for 5 min. at 38.°

The ADP splitting of the three-enzyme system was activated by $MgCl_2$ to about 30% (see Fig. 5) whereas isomerisation itself was activated to a considerable greater degree (200%) upon addition of Mg. The reason of this is probably that the whole reaction is limited by the relatively slow splitting off of phosphate, which is not activated by Mg.

The ADP splitting in presence of actomyosin and isomerase was inhibited by 0.01 *M* pyrophosphate to an extent of 50—75%, depending on the length of incubation with the inhibitor. Initial velocities were inhibited by 75%, whereas in case of longer incubations the inhibition fell to 50%.

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4. *H. M. Kalckar,* J. Biol. Chem. **143**, 299, (1942).
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The influence of salts on the phosphatase action of myosin.

by

I. BANGA and A. SZENT-GYÖRGYI

One of us has studied before the influence of salts on the phosphatase activity of myosin.[1] It seemed desirable to repeat part of this work with crystallised myosin.

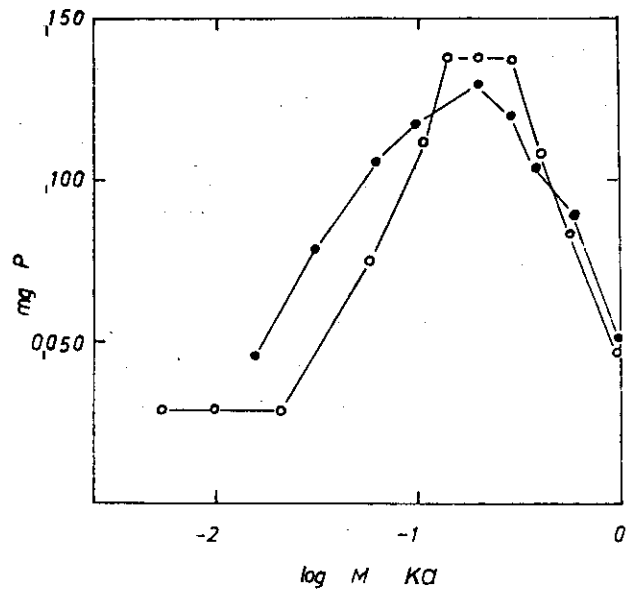

Fig. 1. Effect of KCl on the phosphatase activity of myosin and actomyosin. Formation of inorganic P determined by the method of *Fiske* and *Subbarow*. 1 mg myosin or 1 mg myosin plus 0.3 mg actin in 3 ml of water, 3,6 mg ATP as neutral K salt. Incubation for 5 min. at 38°.
Points = myosin, circles = actomyosin.

The influence of the KCl concentration on the phosphatase activity of myosin and actomyosin is given in Fig. 1.

It can be seen that there is no great difference between the KCl curve of myosin and acto-myosin. Both have a distinct KCl optimum at 0,2 *M* KCl.

The influence of $MgCl_2$ concentration at two different KCl concentrations is given in Fig. 2. (0,01 *M* KCl) and Fig. 3 (0.1 *M* KCl).

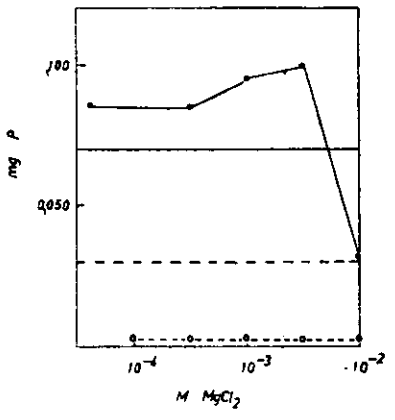

Fig. 2. Effect of $MgCl_2$ on the phosphatase activity of myosin in the presence of 0.01 *M* KCl. Broken line = myosin, full line = actomyosin. The straight lines indicate the phosphatase activity without $MgCl_2$.

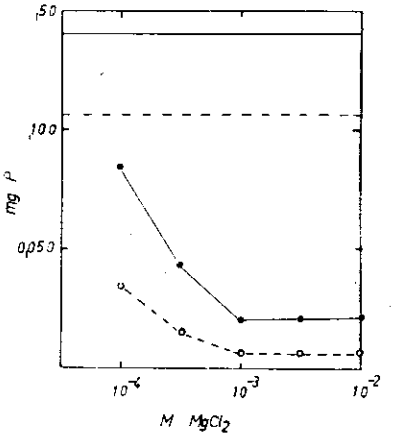

Fig. 3. Effect of $MgCl_2$ on the phosphatase activity of myosin in the presence of 0.1 *M* KCl. Broken line = myosin, full line = actomyosin. The straight lines indicate the phosphatase activity without $MgCl_2$.

It can be seen that the phosphatase activity of myosin is completely inhibited even by the smallest $MgCl_2$ concentrations regardless of the KCl concentration. The influence of $MgCl_2$ on the activity of actomyosin depends on the KCl concentration. In the presence of very small KCl concentrations $MgCl_2$ enhances the phosphatase activity with a maximum between 10^{-3} and 5.10^{-3} *M* $MgCl_2$. Higher concentrations inhibit. In presence of 0,1 *M* KCl the activity of actomyosin is inhibited by all Mg concentrations, similar to the action of $MgCl_2$ on myosin. (The intermediary KCl concentrations of 0,025 and 0,045 had an intermediary effect: Up to 5.10^{-3} *M* Mg had no effect at all, higher concentrations inhibited.)

The question arises how this action of KCl should be

explained? The results of this laboratory obtained by viscosimetric methods[2] allow of a simple explanation. It has been shown that in the presence of KCl and ATP the actomyosin dissociates into actin and myosin. This dissociation depends on both the KCl and ATP concentrations. In our experiment this dissociation took place at 0,1 *M* KCl, hence Mg had at this KCl concentration on actomyosin the same inhibiting effect as on myosin.

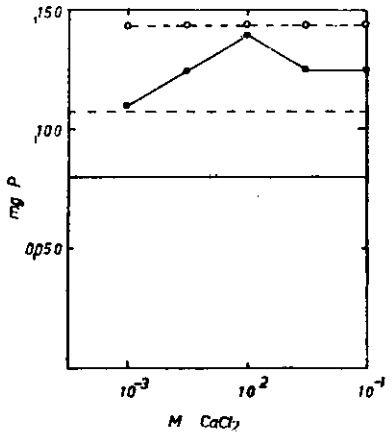

Fig. 4. Effect of $CaCl_2$ on the phosphatase activity of myosin in the presence of 0.01 *M* KCl. Broken line = myosin, full line = actomyosin. The straight lines indicate the phosphatase activity without $CaCl_2$

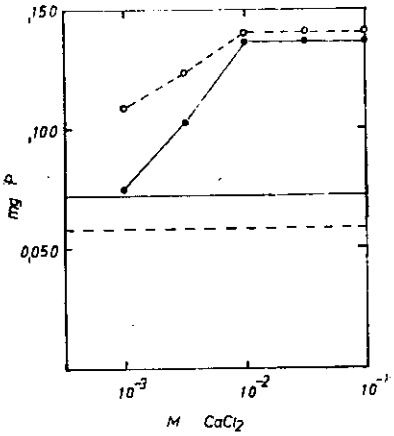

Fig. 5. Effect of $CaCl_2$ on the phosphatase activity of myosin in the presence of 0.1 *M* KCl. Broken line = myosin, full line = actomyosin. The straight lines indicate the phosphatase activity without $CaCl_2$.

Fig. 4 and 5 show the action of varied $CaCl_2$ concentrations in presence of 0,01 and 0,1 *M* KCl respectively. The experimental conditions are the same as in the experiments of Fig. 2. It will be seen from this curve that $CaCl_2$ has a strong enhancing influence at all concentrations between 10^- and 10^{-1} *M*. The effect is identical at both KCl concentrations. Since myosin and actomyosin are equally activated by $CaCl_2$ dissociation will have no effect and identical curves will be obtained with myosin and actomyosin at both KCl concentrations.

In Fig. 6 is given the effect of varying concentrations of $MgCl_2$ in presence of 0,01 *M* $CaCl_2$ and 0,01 *M* KCl. It will be seen that the phosphatase activity which has been increased

by $CaCl_2$ is depressed by all concentrations of $MgCl_2$. The experiment gave in presence of 0,1 *M* KCl the same results. Mg and Ca, though in themselves both capable of increasing the phosphatase activity of actomyosin are, if given together, not synergetic but antagonistic.

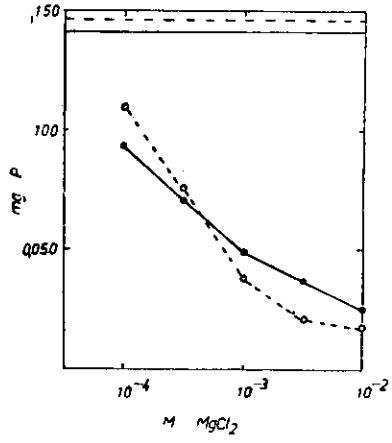

Fig. 6. Effect of $MgCl_2$ on the phosphatase activity of myosin and actomyosin in presence of 0.01 *M* $CaCl_2$ and 0.01 *M* KCl. Broken line = myosin, full line = actomyosin. The lines on the top give the phosphatase activity in absence of $MgCl_2$.

We want to use this occasion to correct an earlier statement. One of us (B.) has found before[1] that quinine and nicotine strongly inhibited the phosphatase action of myosin. This was a rather important statement because these poisons do not inhibit the contraction of actomyosin. It was found since that quinine and nicotine do not inhibit the enzymic action of myosin. They only inhibit the detection of the phosphate hydrolysed. We could not corroborate either the earlier finding that oxalate inhibits the enzymic action of myosin. The question, thus, whether splitting of ATP is necessary for contraction, is still open.

1. *I. Banga.* These studies 1, 27, 1941–42.
2. *F. Guba.* This vol. p. 40.

The crystallisation of myosin and some of its properties and reactions.

by

A. SZENT-GYÖRGYI.

It has been shown in the previous papers of this laboratory (1. 2.) that the contractile substance of striated muscle is „actomyosin", a compound of two proteins: actin and myosin.

The methods, hitherto employed by different authors for the preparation of „myosin" always yield a myosin more or less heavily (1—3%) contaminated with actin. All the data of literature relate to such undefined mixtures. Since even small traces of actin greatly modify the properties and reactions of myosin it seemed desirable to obtain myosin free of actin.

In this paper I will describe the preparation of actin-free myosin, its crystallisation, will describe some of its properties and reactions.

The preparation of myosin.

In all our experiments only chemicals of high purity were employed. The distilled water was redistilled from glass vessels. As material the striated muscle of the rabbit was used.

The animal was killed by decapitation, quickly skinned, eviscerated and dipped into ice-water. After a few minutes the body-muscles were cut out and packed between ice. Then they were minced on a cooled meat mincer with a sieve plate of holes of 2 mm diameter.

Principle. The separation of myosin from actin is based

on the observation that ATP will precipitate, in presence of different salt concentrations, actomyosins of different composition. The higher the salt concentration the less myosin is taken down by a given amount of actin but at the same time the less complete the precipitation. We have to work at the salt concentration at which the precipitation is fairly complete and not too much myosin is taken down by the actin. So for instance, in presence of 0,05 *M* KCl and at pH 6,5 a 0,5 % actomyosin will precipitate as such, that will say that one part of actin will take down with it 200 parts of myosin. In presence of 0,1 *M* KCl a 1,5 % actomyosin will be precipitated from the same solution and one part of actin will take down only 66 parts of myosin. Above 0,2 *M* KCl the precipitation becomes incomplete. The separation of actin involves always the loss of a relatively great quantity of myosin. It is thus essential to start with myosin of possibly little activity.

This precipitation with ATP will not yield a completely actin-free preparation (0.1 % actomyosin). The muscle extract seems to contain also some inactive actomyosin which is not precipitated by ATP. This actomyosin can be precipitated by diluting the KCl to 0.04 *M* at a slightly alkaline reaction. Myosin is soluble, actomyosin insoluble in this solvent. The relative loss in myosin in still bigger than in the case of the ATP precipitation and still more myosin is taken down by the same amount of actin. This method can thus be used only with extracts which have been rendered very poor in actin by the preceding ATP-precipitation.

Myosin cannot be freed from actin by crystallisation since actomyosin behaves as an individual substance. As shown by F. GUBA (oral comm.) even a 2,5% actomyosin can be brought to crystallisation as such.

First step. The muscle is suspended in the ice-cold KCl-Phosphate mixture of GUBA and STRAUB which contains 0,3 *M* KCl and 0,15 *M* K-phosphate of pH 6,5. The muscle is extracted for ten minutes under constant stirring and then centrifuged at 0°. The precipitate is discarded, the fluid diluted with four volumes of water of room temperature (22°) and stirred gently. After one or two hours suddenly a flocculent precipitate is formed. What happened was that the ATP present was used up to such an extent that it caused no

more a dissolution but a precipitation of the actomyosin. (If this precipitation takes place without stirring a very fine colloidal precipitate is formed which cannot be separated on the centrifuge.)

Myosin is rather sensitive to heat and even short (10 min.) incubation at 37° causes a rise of its viscosity and partial loss of its enzymic activity. Therefore it is important to work at low temperature. Unfortunately this is not possible throughout, for in two of the steps the precipitaion would be incomplete at 0°. Fortunately myosin is stabilized by ATP, as observed by ENGELHARDT and LJUBIMOWA (3) and corroborated by myself. In the first steps of our preparation the myosin is protected by the ATP present. The fluid contains even at the moment of the precipitation some ATP.

The precipitate thus formed is separated by rapid centrifugation at room temperature and the opalescent fluid is diluted with 1,5 vols. of ice-cold water. This water is run in slowly, in about 10 minutes under constant energetic stirring. The myosin separates in the form of fine, needle-shaped crystals. If the water is added suddenly and without stirring an amorphous precipitate is obtained.

The fluid is allowed to stand for an hour or two at 0°, decanted and the myosin separated on the centrifuge.

If necessary this crystalline precipitate can be washed by suspending it in 0,04 *M* KCl and separating the myosin again on the centrifuge.

Example: 357 g minced muscle. Extract 750 ml. Contains 7 g 0,35% actomyosin. The first precipitate contains *2* g 1,5% actomyosin. The final myosin precipitate contains 3,4 g myosin. Thus only about ½ of the myosin present in the extract is isolated. The other half is lost as actomyosin or is left behind in the fluid.

Second step. The crystalline myosin precipitate of step 1 is dissolved in a 0,02 *M* K_2CO_3 containing 10 ml of 1% alcoholic phenolphthalein in every liter. We add the carbonate solution in small quantities and homogenise carefully with strong stirring. Carbonate is added till the fluid retains a faint rose colour (pH 8,3) and add for every g of myosin present 4 ml of *2 M* KCl. Then we dilute with water adding 50 ml for every ml of KCl solution. This water is of room tempera-

ture (22°) and contains 0,001% phenolphtalein and sufficient K_2CO_3 to give it a faint rose colour. The water is added under strong stirring. A voluminous, loose precipitate is formed which is separated on the centrifuge. The faint rose coloured opalescent fluid is poured off and cooled. The precipitate is treated once more in the above way, *i. e.* if its colour has faded out we restore it by adding K_2CO_3, then we add KCl and finally water and centrifuge, the only difference being that this second time we add only half as much KCl and water as the first time. The precipitate is discarded and the fluids united. From this point on the preparation is continued at 0°.

The fluid is stirred energetically and 1% acetic acid is run in very slowly till the fluid is neutralised. The myosin separates in the form of somewhat irregular needles and is centrifuged.

Recrystallisation.

The precipitate is dissolved by adding 2 *M* KCl in small kuantitites. The fluid is carefully homogenised after each addition. KCl is added till the concentration of the KCl reaches 0.6 *M*. Then we dilute further with 0,6 *M* KCl till the fluid loses its very high viscosity and contains about 3 % myosin.

The myosin solution is stirred very energetically and water is run in very slowly till the KCl concentration drops to 0,04 *M*. The addition of this amount of water should take about one hour. The myosin separates in the form of beautiful needleshaped crystals. About half of the myosin isolated in the first step will be obtained in this form. This myosin contains no actin or only negligible traces of it (0—0,02 %).

Some properties and reactions of myosin.

Myosin crystallises in the form of very fine needles (Fig. 1.) with a strong tendency of lateral association. Needles, observed with high power, will often be found to be a bundle of finer needles. Sometimes the needles associate into fine, long threads, fibrils (Fig. 2.).

Analysis. Recrystallised myosin was precipitated with alcohol and extracted with boiling abs. alcohol for two hours.

The alcohol was evaporated, the residue extracted with chloroform. The chloroform was evaporated and the residue

Fig. 1. Myosin crystals. Magn. 1:90.

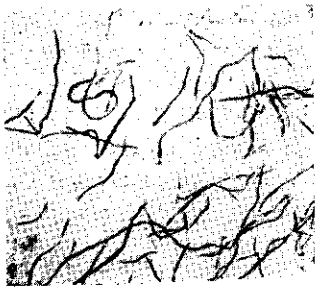

Fig. 2. Myosin threads. Magn. 1:40.

extracted with ether. After evaporation of the ether the residue was weighed. The lipoid matter was partly insoluble in acetone and constituted 3% of the dry weight of myosin.

The lipoid-free myosin was dried *in vacuo* at 120° C. The elementary analysis was kindly made by DR. M. KOVÁTS OSKOLÁS. It showed the following result:

C	50,04%
H	7,70%
N	16.15%
S	1.14%
Ash	1.23%

According to this analysis myosin is a protein. It contains 6 atoms of S for every 17000 g.

Stability. Myosin, if dissolved in 0,5 *M* KCl and neutralised, can be kept at 0° C without loss of enzymic activity for a fortnight. Finer colloidal properties, however, seem to be impaired after a few days storage, as indicated by the sluggishness of the fall of viscosity of an actomyosin, prepared from it, on addition of ATP (oral comm. of F. B. STRAUB).

Solubility. If the crystalline myosin is suspended in water and is rendered salt-free by dialysis it swells up to a glassy mass which shows only a slight opalescence. On dilution it dissolves in water giving a clear and very viscous solution from which no myosin can be separated by centrifugation. According to the water-solubility in entire absence of salts myosin is not a globulin. On the other hand, on adding ammoniumsulfate, the myosin precipitates as we pass half saturation. In this respect myosin conforms with globulins.

Myosin is precipitated by small concentrations of neutral salts and dissolves in presence of higher salt concentrations. If dissolved in water or in 0,1--0,2 *M* KCl it shows a very strong DRF. This DRF, however, is not due to the single myosin particles but to their coaxial association, for the DRF becomes weaker at 0.3 *M* KCl and disappears entirely at 0.4 M KCl. It also disappears if the solution is rendered alkaline. Evidently the higher salt concentration or pH prevents association. At the low velocity gradients of our experiments myosin shows, in 0,6 *M* KCl, no DRF.

Hand in hand with the association the limpid myosin solution becomes more and more opalescent.

If a sufficiently strong 0.5 *M* KCl-solution of myosin is diluted with water and is stirred, the turbidity assumes a silky appearance as the concentration of the KCl falls below 0,4 *M*. Between crossed Nicolls the fluid shows a strong double refraction. This indicates that the myosin particles aggregate

coaxilly to anisodimensional particles. As we dilute the solution more and more the aggregation becomes stronger and as we reach the limit of the solubility of myosin about 0,05 *M* KCl the particles become visible in the form of crystals. Strong mixing is essential if we want to obtain well formed crystals, for this mixing provides the coaxial orientation of particles which is necessary for crystallisation.

Reaction with salts. The most striking property of myosin is that it is readily precipitated by small concentrations of alkali salts. It is almost quantitatively precipitated by 0,05—0,01 *M* KCl; even 0,0015 *M* KCl causes precipitation.

In table 1. the precipitating action of different salts is compared.

Table I.

	0,2	0,1	0,05	0,025	0,0125	0,006	0,003	0,0015	0,0008	0,0004
KCl	0	0	+	++	++	++	+	+	0	0
KF	+	+	+	++	++	++	++	++	+	0
KJ	0	0	+	++	++	+	0	0	0	0
LiCl	0	0	+	+	++	++	++	+	+	0
NaCl	0	+	+	++	++	++	+	+	0	0
$MgCl_2$	0	++	++	++	++	++	++	++	++	++
$CaCl_2$	0	++	++	++	++	++	++	++	++	++

0,5 ml. of the salt solution was added to 2 ml of a 0,1% salt free myosin solution. Upper line: the final molar concentration of the salt. O means no change, + means turbidity or precipitation.

The table shows that the action of the different alkali halogens is rather similar. Bivalent cations give a rather voluminous precipitate through the whole range without a well defined maximum. This makes it evident that the precipitation is rather the action of the cation than that of the anion. The valency of the anion has less influence and even the trivalent phosphate as K salt has about the same action as KCl.

It was interesting to know whether the precipitation of myosin by salts is connected with a loss of charge. On my request K. LAKI has kindly undertaken to investigate this question. His report reads as follows:

„The migration of charcoal particles coated with myosin was followed in the microscopic cataphoretic apparatus described by J. H. NORTHROP und M. KUNITZ (J. gen. Physiol. 7. 729, 1925). It was found that the myosin, dissolved in dist. water, is negatively charged and readily migrates under the influence of an electric field. In KCl solution of 0,015 *M* the migration is slowed down. At 0,025 *M* the migration is still detectable but at 0,05 *M* the particles ceased to move. Tests were made also at higher KCl concentrations up to 0,2 *M* but no migration was detectable.

From these preliminary experiments the conclusion can be drawn that the charge of the myosin particle is diminished or lost at KCl concentrations where the myosin precipitates. The results obtained are presented in the following table:

M KCl	Conc. of the myosin	
0,00	6,0 mg/cc	Moves to the pos. pole.
0,012	0,6 „	Moves slowly „ „
0,025	3,0 „	Moves slowly „ „
0,05	0,6 „	No migration
0,05	0,3 „	„
0,08	2,0 „	„
0,10	6,0 „	„
0,10	3,0 „	„
0,20	2,0 „	„

Casein, treated in the same way, migrates in 0,2 *M* KCl to the positive pole".

The effect of pH on precipitation is shown in Tab. II. The buffer acted as salt.

The table shows that at a higher pH the precipitation is limited to a smaller range and is weaker. If we raise the pH further there will be no precipitation at all. At low pH (the three lowest lines), where we approach the isoelectric point, the myosin separates in form of a gelatinous mass instead of giving a precipitate. The maximum precipitation is observed at pH 6,4 and 6,7.

The asterisks mean that the precipitate, on mincing, has a silky appearance and shows a strong DRF, is thus crystalline.

Table II.

$\frac{Na_2HPO_4}{KH_2PO_4}$	0,1	0,05	0,025	0,0125	0,006	0,003	0,0015	0,0008	pH
1/0	0	0	0	0	+	+	+	0	
16/1	0	0	0	++	++	+	+	0	8
8/1	0	0	0	++	++	+	+	0	7.7
4/1	0	0	+	++	++	+	+	0	7.3
2/1	0	0	+*	++	++	+	+	0	7
1/1	0	0	++**	+++	+++	+	+	0	6.7
1/2	0	+**	++**	++*	++	+	0	0	6.4
1/4	+	+	++	++	++	+	0	0	6.1
1/8	+	+	+	+	+	+	0	0	5.8
1/16	+	+	+	+	+	+	+	0	5.5
0/1	+	+	+	+	+	+	+	0	

Isomolar Na_2HPO_4 and KH_2PO_4 were mixed in different proportions (Col 1). The final molar concentration of PO_4 is given in the upper line.

This shows that crystallisation is limited to a narrow pH range and has its maximum about pH 6,5.

This pH of 6,5, at which the myosin precipitates and crystallises most readily, at which the most inactive myosin can be extracted (see GUBA and STRAUB), seems to correspond to the pH of thoroughly washed myosin, as determined by SLATER (5), the flocculation maximum as found by COLLIP (6) and the minimum of acid-base binding capacity as given by EDSALL (4).

It seemed to be interesting to know whether myosin binds any K at those KCl concentrations at which it is precipitated. This question was answered in the following way: the crystalline myosin, after its recrystallisation, was centrifuged, K was estimated in the supernatant fluid. The crystalline precipitate was weighed, dried, weighed again, combusted and its K estimated. I am indebted to Prof. E. ERNST for the K estimations.

The precipitate contained 4,5---7% crystalline myosin and a KCl solution the concentration of which was identical with that of the supernatant fluid and varied between 0,3—0,1 *M*. Knowing the quantity of the water in the precipitate and the

KCl concentration in the supernatant fluid, it can be calculated how much of the total K of the precipitate falls to the water and how much to the myosin. If the K of the water is subtracted from the total K the difference is the amount held by the myosin These data show that one g atom of K is held in the different experiments by the following quantities of myosin: 10000, 12000, 12000, 12000, 14000. On average 12000 g myosin bind one atom of K. This K can be removed by dialysis.

Reaction with ATP. If ATP is added to the watery solution of myosin the viscosity will decrease and the DRF will become somewhat weaker. This indicates that ATP disaggregates to some extent the associated myosin.

ATP will show a solvatising action also in presence of salts. If ATP is added along with the salts the precipitate formation will be weaker, or no precipitate will be formed at all. So, for instance, 0,05 mg ATP per ml will be sufficient to prevent any precipitate-formation by neutral potassium-phosphate.

If the salt precipitates the myosin in amorphous form the DRF disappears and can be brought back by the addition of ATP which dissolves the precipitate.

These experiments indicate that the myosin particles are present in their solution in a straight, distended form. They do not change their shape not even if discharged or precipitated or if ATP is added. This conclusion is also supported by our earlier observation that myosin threads do not contract.

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2. Ibid. **2**, 1942.
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Observations on Actomyosin.

by

A. SZENT-GYÖRGYI.

If a 0,5 *M* KCl-solution of myosin and a solution of actin are mixed actomyosin is formed. If the solution is sufficiently strong it can be pulled into threads.* If it is diluted with water it precipitates and can be washed salt-free giving a fine and fairly stable suspension.

Actomyosin is much less soluble than myosin or actin is. While myosin dissolves in water actomyosin only swells up. While 0,2 *M* KCl dissolves myosin 0,6 *M* KCl will be needed to dissolve actomyosin. But on the whole the collodial reactions of actomyosin are a reflection of the reactions of myosin.

Free actin is not precipitated by alkali salts, myosin is. Actomyosin will be precipitated similarly to myosin. In Tab. I. the precipitation of myosin and actomyosin by KCl are compared and it will be seen that the precipitation of both, at small salt concentrations, go parallel.

ATP has a solvatising action on myosin and will, in absence of salts, dissolve actomyosin also.

While myosin and actomyosin react thus to salts and ATP in a similar way; they will respond in a different way to the joint action of salt and ATP. While in the case of myosin the sole effect of ATP was to weaken the precipitating action of salts, in the case of actomyosin small amounts of ATP will greatly intensify the action of the salt. If the salt, in itself, had a precipitating action, ATP will make this precipitation much

* Technic of threads see vol. 1 of these studies.

Table I.

M . KCl	0,2	0,1	0,05	0,025	0,0125	0,006	0,003	0,0015	0,0008	0
Acto-myosin	++	++	+++	+++	++	++	+	+	0	0
	15	14	11	12	16	20	28	44	65	65
Myosin	0	0	+	++	++	++	+	+	0	0

Upper line: molar concentration of KC . Middle line: volume of the actomyosin precipitate in an arbitrary unit. Crosses mean precipitation. 0,1% solution of myosin and 0,1% suspension of a 25% actomyosin.

Table II.

The action of varied KCl and ATP concentration on an actomyosin suspension

M KCl	mg ATP per ml						
	0,4	0,2	0,1	0,05	0,025	0,0125	0
0,4	!!!	!!!	!!!	!!!	!!!	!!!	!!
0,3	!!!	!!!	!!	!!	!	0	!
0,2	!!	!	xx	xx	xx	x	++
0,05	xxx	xxx	xxx	xxx	xx	xx	+++
0,0125	!	!	0	0	x	x	+
0,003	!!	!!	!	!	!	x	+
0,0008	!!!	!!!	!!!	!!	!	0	0

0, 1 % suspension of 25% actomyosin.

+ = precipitation, x = superprecipitation, ! means clearing up of the suspension. !!! means complete dissolution. O means no change. In this experiment first the KCl was added to the actomyosin suspension, the resul noted and then the ATP introduced.

stronger. If the salt (owing to its higher concentration) solvatised actomyosin in, ATP will make the dissolution complete. (Tab. II.).

Not only will the precipitation, caused by KCl in presence of ATP, be much intenser. The whole character of the precipitate will be changed: it will become granular and settle quickly to a small volume. This change is due not only to the intenser precipitation but at the same time to the strong shrinking of the particles. I will call this effect „super-precipitation" to distinguish it from the simple precipitation given by salts alone.

The shrinking of actomyosin can better be observed on actomyosin threads which give under the same conditions a violent contraction which is, in fact, but an extreme degree of shrinking. Superprecipitation and contraction are identical phenomena caused by the shortening of the actomyosin micels, as proved by the anisodimensional contraction of oriented myosin threads (M. GERENDÁS[1]). We can thus sum up our results by saying that if salts act on an actomyosin particle, they will simply discharge and precipitate it. If they act on the ATP complex of actomyosin they will cause not only discharging and precipitation of the particles but also their shortening. This is what has been called superprecipitation and what is observed in threads as contraction.

We must distinguish between the action of small and high concentrations of ATP. Small concentrations will have the effect described. Big doses will have a solvatising action only. Comparing the solvatising action of this excess of ATP at different salt concentrations (Tab. II.) we will find that the stronger the KCl precipitation and thus the stronger the KCl—ATP superprecipitation, the more ATP will be needed to cause dissolution.

Similarly to precipitation also superprecipitation is reversible. Any agent that brings about a dissolution and dissociation of actomyosin reverts it to its uncontracted form. It has been shown earlier (3) that contracted actomyosin threads can be brought to relaxation by alkaline salt solutions or by the combined action of salts and ATP (4). These relaxed threads are capable of contracting again.

Superprecipitated or contracted myosin can be dissolved by 0,05 % ATP in absence of salts. It is also dissolved by

higher salt concentrations or salt plus ATP. Substances that will dissolve it will also restore to it its DRF, restore thus also the original shape and dimensions of its particles.

ADP.* Threads prepared form synthetic actomyosin contract energetically in presence of salts and ATP. They are somewhat more labile than threads prepared from the impure natural actomyosin (our earlier myosin B). It was repeatedly observed that a freshly pulled thread gave only a sluggish contraction and became very active spontaneously in a few hours. The initial sluggish contraction could be speeded up by soaking the thread in an aqueous muscle extract.

There is one very sharp difference between the reaction of threads prepared from pure actomyosin and from myosin B. If the myosin B thread is suspended in a 0,05 *M* KCl containing 0,001 *M* $MgCl_2$, and 0,1% ADP is added, the thread will contract in the same way as if ATP had been added. If the pure actomyosin thread is treated likewise no contraction occures. If, however, in addition to ADP also $^1/_{10}$ parts of an aqueous muscle extract** are added, the thread will contract.

The aqueous extract can be inactivated by 20 minutes boiling. The contraction of the thread will be faster if the extract is mixed with the ADP solution before this latter is added to the thread. If the extract is allowed to stand with the ADP for a few minutes and the mixture then boiled for twenty minutes, it will cause rapid contraction if added to the thread. This makes it evident that the aqueous muscle extract contains a thermolabile substance which alters the ADP in such a way that it is capable of causing contraction. Evidently this catalyst is identical with K. LAKI's watersoluble factor and I. BANGA's isomerase. In all probability it restores to the nucleotide the active pyrophosphate configuration.

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* The ADP was prepared by I. BANGA from ATP by having one phosphate split off by myosin.

** The aqueous extract was prepared in the following way: The muscle was minced, suspended in water, 3 ml being taken for every g of muscle. The suspension was stored over night at 0° during which time the ATP present was split. The clear filtrate contains no ATP and added to threads, gives, in itself, no contraction.

This experiment was repeated with a purified isomerase solution of I. BANGA. It was found that the protein solution reactivated the ADP only in presence of Mg (0,001 *M*). If isomerase and ADP were allowed to stand in absence of Mg, boiled and then added to the muscle, no contraction ensued.

We can thus say that the difference between myosin B and pure actomyosin is due the presence of isomerase in myosin B As shown by I. BANGA, myosin B can be freed from isomerase by repeated washing. Threads, prepared from such myosin B. behave in respect to ADP as the synthetic actomyosin. As shown by I. BANGA, such an isomerase-free actomyosin does not split ADP either. There is thus, at this point, a close analogy between splitting and contraction.

Salts. It has been shown before that there is a close analogy between the precipitation of actomyosin by KCl and the superprecipitation elicited by KCl and ATP. This analogy between salt-precipitation and superprecipitation breaks down in the case of Ca and Mg. Both ions have an equally strong precipitating action on actomyosin and could both be expected to act as K and give contraction in presence of ATP and enhance the K-ATP contraction. But contrary to this expectation Ca will give with ATP no contraction at all and Mg will do so only in presence of higher ATP concentrations which makes it probable that the contraction, in this case, is also due to the joint action of the Mg and K, the latter being present as cation of the ATP. Ca inhibits the K-Mg-ATP contraction also. There is thus an antagonism between K and Ca on the one side and Ca and Mg on the other side which makes it probable that Ca and Mg exert their influence by some other mechanism than K does. The antagonism of K and Ca in living muscle has been known for a long time. The predominance of K causes tetany, Ca suppresses it. Also the antagonism of Ca and Mg has been known from pharmacology (*Melzer* narcosis).

Example: actomyosin thread prepared from 12,5% actomyosin. The thread was soaked for a few minutes in the salt solution and then 0,125 % ATP was added in the form of its neutral K salt. The numbers give the % linear contraction reached in one minute, as measured by the length of the thread.

KCl 0,05*M*	38
KCl 0,05*M* and $MgCl_2$ 0,001 *M*.	52

KCl 0,05*M* and $CaCl_2$ 0,01 *M* 2
KCl 0,05*M*, $MgCl_2$ 0,001 *M* and $CaCl_2$ 0,01 *M* 18

The bending of actomyosin threads as a model of contraction. It has been shown that, under action of salts and ATP, actomyosin threads shrink rapidly i.e. become shorter and thinner: they contract. If, however, there is an assymmetry in the system which makes that the two long sides of the thread do not contract equally, then the thread, before

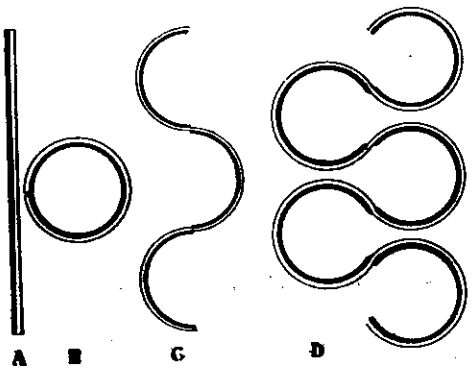

Fig. 1.

contracting, will bend or curl up. This is shown in fig. 1A and B where the more reactive side is marked with black. Such an assymmetry in the reaction can be produced by introducing the ATP on the one side of the thread. An assymmetry may also be found in the substance of the thread. Threads, if not pulled under special precautions, have some such assymmetry in their composition and tend to bend before they contract. One very often observes the curling up as shown in fig. 1B. It is immaterial what the source of the assymmetry is, the result will be the same: the bending of the thread. This is not a new phenomenon, it is an undesirable experience of every-day life: if two boards are stuck together and shrink or swell unequally then humidity will bend them.

Such an assymmetrically built myosin thread may serve as a model of the actomyosin micel. If a myosin and actin micel stick together side by side an assymmetrical structure will be obtained, as symbolised in fig. 1A. But myosin and actin

are both hydrophil colloids and rather similar in their constitution and reactions. A high degree of assymmetry will be introduced by ATP which reacts only with the myosin moiety of actomyosin forming with it an ATP-myosin* complex which is most sensitive to ions. The one sided shrinking of the actomyosin micel will have to cause its bending. This bending will make the micel effectively shorter and so the whole mass of the actomyosin gel will shrink and the thread, made herefrom, shorten. The maximum contraction will be reached when the micels have curled up as in fig 1B. In this case the thread has to contract to $^1/_3$ of its original length (more exactly $l = \frac{l_0}{\pi} + d$ d being the diameter of the thread). The maximum contraction of actomyosin threads was found to be 66%.

It is also evident that in threads in which the actomyosin micels have been arranged coaxially to the thread the curling of the micels must give anisodimensional contraction: the thread must become shorter and thicker and at the same time its double refraction must be lost.

Figure 1B makes it evident that, if a contracted actomyosin is brought to dissociation, we obtain the original uncontracted actin and myosin which, if put together, will give uncontracted actomyosin, as is actually the case in the experiment.

The geometrical limitations given above do not hold if the actin forms a continuous structure, as is the case in muscle. Combination with myosin and ATP will give shortening also here but in this case the limit will not be $1/\pi$. The contraction will theoretically have no limits. In fig. 1C is pictured a shortening of $^1/_3$, in fig. 1D a shortening of $^3/_4$.

References.

1. *M. Gerendás.* These studies, **1**, 47, 1941—42.
2. *A. Szent-Györgyi.* Ibid. p. 67.
3. „ „ „ „ „ 22.
4. „ „ „ „ **2**, 25, 1942.

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* Should it be found that myosin is phosphorylated then the only change will be in regard of this model that we will have to write P-myosin in stead of ATP-myosin.

Observations on muscle extracts.

by

A. SZENT-GYÖRGYI.

If the minced muscle is extracted for ten minutes with 0,6 *M* KCl and centrifuged a fluid is obtained which contains about 1—1,2% of actomyosin with a 0,5—1% actin content. If the extract is stored over night at 0° the ATP is split. If the extract is diluted now with 4 vol. of water containing 0,001 *M* $MgCl_2$, a slightly turbid fluid is obtained which contains about 0,1 *M* KCl and the actomyosin in the form of a stable suspension.* If ATP is added now, according to its concentration, a turbidity, or a clearing up will be seen. It seems to be logical to observe this before a black back-ground with side illumination. This mode of observation, however, gives in this case erroneous result for flocculation often goes hand in hand with a decrease of luminosity.

Good results are obtained if the flocculation is observed before a black background with light falling in from behind the test tube at a very small angle. To make this into a simple method the test tubes were immersed into a waterbath with glass walls. At the back wall a paper-screen with black lines was fixed (Fig. 1). The source of light was placed behind this screen. Now the light falls in from between the black lines at a very small angle and the black lines form the black background. Precipitation will cause the black strips to appear hazy and grey. Clearing up will have the opposite effect.

The ATP was always introduced with a small spoon

* The water should be added suddenly. If it is run in slowly the actomyosin flocculates.

(Fig. 1) in a volume of 0,1 ml. If the spoon is pulled once or twice through the fluid within the fractions of a second complete mixing can be obtained. Stirring was continued throughout the observation.

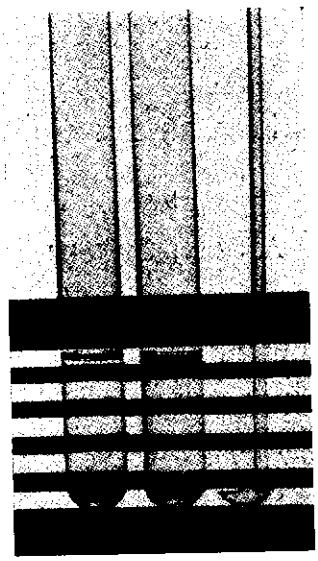

Fig. 1.

What will happen on addition of ATP depends on the first place on the quantity of the ATP added. If a very small quantity is added a precipitate will be formed at once. If more ATP is added the fluid will clear up. After the excess of ATP has been split the fluid will suddenly become turbid and precipitate will be formed.

If we plot the quantity of ATP against the time required for the formation of the precipitate we obtain the time curve of the splitting of ATP. Such an experiment is reproduced in the fig. 2. (0,1 *M* KCl). The splitting proceeds at a low ATP concentration at a fairly high rate to slow down suddenly at a somewhat higher ATP concentration. At a high ATP concentration the splitting becomes faster again. An increase of the KCl concentration from 0,1 to 0,15 and 0,2 *M* caused a flattening of the curve while the decrease of the KCl concentration from 0,1 *M* to 0,075 *M* made the curve much steeper. At 0,05 *M* KCl

the curve is still steeper. All the curves were S shaped. (On the curves of 0,15 and 0,2 *M* KCl the right half of the S falls outside the figure.)

The explanation of this change of slope is given by the experiments of I. BANGA who is showing in her paper that Mg very greatly inhibits the splitting of ATP by myosin and enhances the splitting of ATP by actomyosin. The increasing KCl concentration, as shown by F. GUBA makes the actomyosin dissociate into actin and myosin; the phosphatase action of the

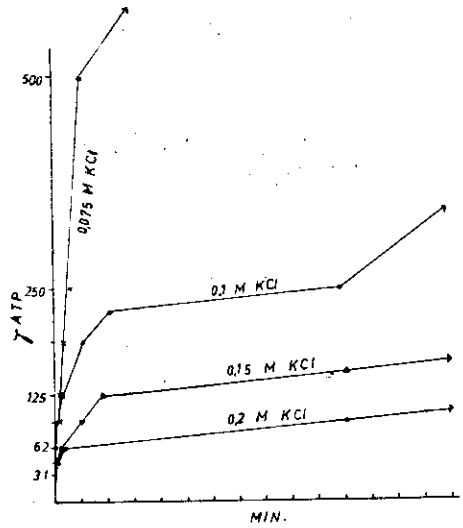

Fig. 2.

latter is then inhibited by Mg. The flattening of the curve indicates thus the dissociation of actomyosin.

This explains also the paradoxical flattening of the curve caused by the increase of ATP concentration. Evidently the excess of ATP also promotes dissociation.

A number of minor problems can be approached by the method described.

1. *ATP estimation.* If an unknown ATP solution is added to the 0,1 *M* KCl extract and its effect is compared with the effect of a known ATP solution, conclusion can be drawn on the ATP content of the unknown solution, provided it did not materially change the salt concentration.

2. *Maximum rate of splitting of ATP by myosin.* If we

measure phosphatase action by estimating the P liberated we have to take greater amounts of ATP and longer periods. Higher concentrations of ATP, as shown, inhibit the enzymic action. The described experiment allows to work with small ATP concentrations. In our experiment, working with small ATP concentrations at 0,05 *M* KCl, 100,000 g of myosin split 10 g molecules of ATP per second.

3. *Reversibility*. A small quantity of ATP causes precipitation, a bigger dissolution (0,1 *M* KCl). If we add a small quantity of ATP and, as soon as the precipitate is formed, we add a bigger dose, the precipitate will dissolve. If, however, we wait for a minute or so before adding the second ATP there will be no dissolution or the dissolution will be incomplete. This shows that very quickly unspecific cohesive links are formed in an actomyosin precipitate.

Note on plasmakinin.

by

K. LAKI.

Since the preceding papers have been closed down the experiments on plasmakinin showed that all the different globuline fractions of oxalated plasma obtained by ammoniumsulfate fractionation were active. If, however, the tests were made quantitatively it was found that the bulk of the active substance was brought down at 0,25—0,30 ammoniumsulfate saturation at which the fibrinogen precipitates. At the other saturations only 1 or 2 percent of this quantity came down.

If the fibrinogen, precipitated by the ammoniumsulfate is redissolved and then treated with alcohol or acetone, the precipitate is found to be inactive even if the precipitation was performed at low temperature where the proteins were not denatured.

Further experiments showed that an alcohol-soluble lipoid could be obtained from plasmakinin which showed almost the original activity when tested on Mellanby-fibrinogen.

The lipoid can be obtained in alcoholic solution in the following way: Fibrinogen is precipitated from the oxalated cattle plasma by 0,25 ammoniumsulfate saturation. The precipitate is centrifuged down and thoroughly washed with acetone and extracted with alcohol at room temperature. The alcohol is evaporated in a vacuum and the residue emulsified with water and tested on Mellanby-fibrinogen.

The active substance can also be extracted from the acetone-treated powder with petroleum ether. If the petroleum ether solution is concentrated in a vacuum, the addition of absolute alcohol produces a small quantity of an inactive precipitate, while the active substance remains in the alcoholic solution. This lipoid thus behaves similarly to the analogous alcohol-soluble lipoid fraction of brain. When this lipoid is exposed to air it looses the activity, probably owing to oxidation.

Note on actomyosin.

by

A. SZENT-GYÖRGYI.

Since the preceding papers have been closed down a new method has been worked out for the study of contraction. This method shows that actomyosin contracts also under the sole influence of alkali salts. This contraction is much weaker than the contraction caused by the joint action of salt and ATP. Its maximum lies at the same salt concentration as the maximum of the contraction caused by salt plus ATP.

It has also been found that, during contraction, active actomyosin goes over into inactive actomyosin. The change: inactive actin $\rightleftharpoons$ active actin seems thus to be involved in contraction. The breaking up of the fibrous active actin micels into the globular inactive actin can be explained by the model described in my second paper. If the actin forms the outer circle it will be stretched. If it could not break up it would resist bending.