The phosphatase activity of myosin.

by

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

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

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

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

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

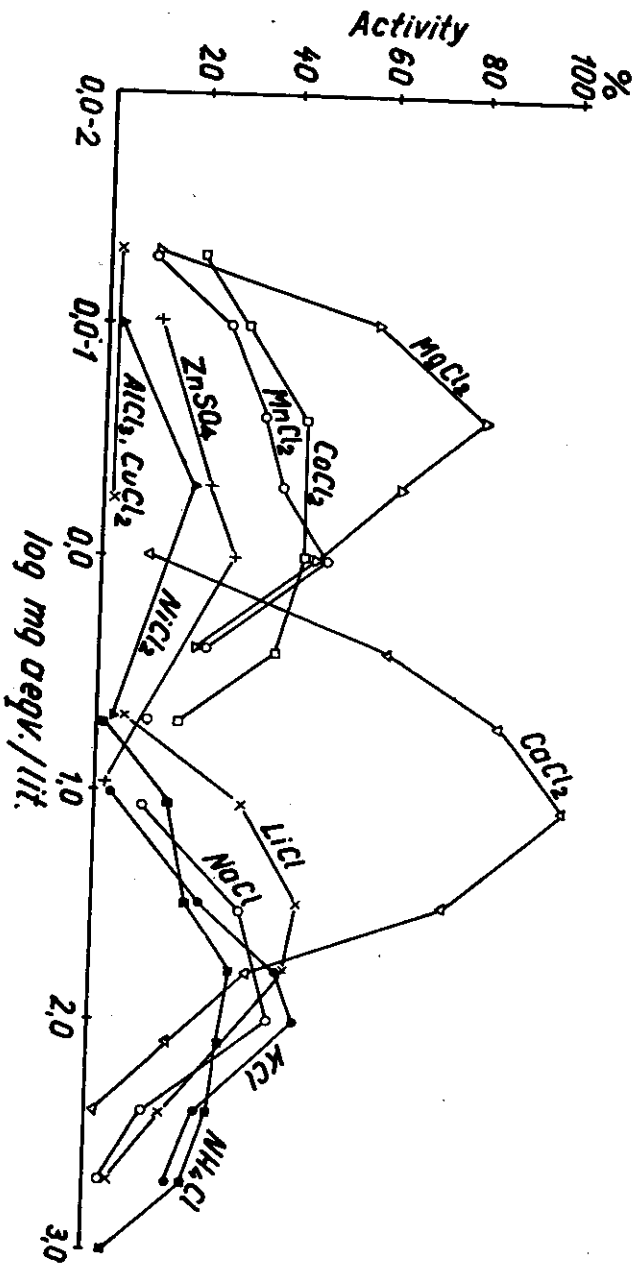
Activity
20
40
60
80
100
%
0·0-2
0·0-1
0·0
0·1
0·2
0·3
log mg equiv./lit.
MgCl2
ZnSO4
MnCl2
CoCl2
AlCl3, CuCl2
NiCl2
CaCl2
LiCl
NaCl
KCl
NH4Cl

Fig. 1.

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

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

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

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

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

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

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

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

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

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

Tab. I.

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

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

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

Tab. II.

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

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

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

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

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

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

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

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

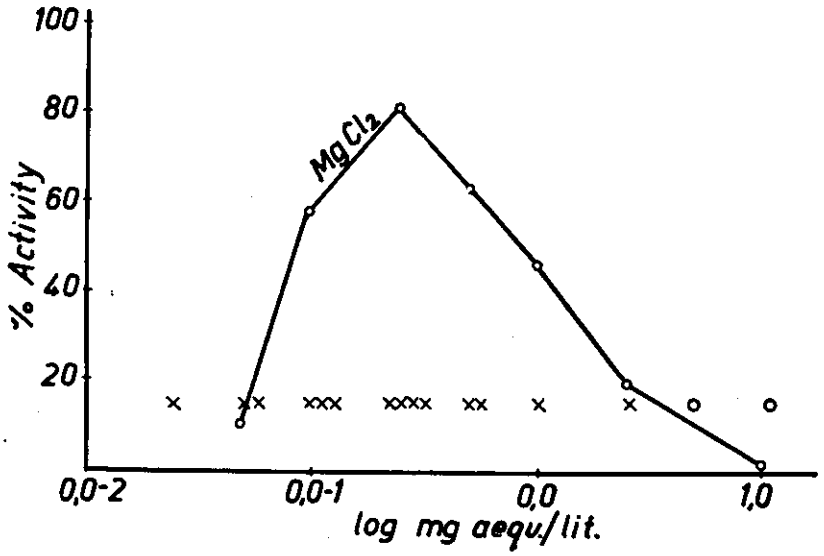

Fig. 2.

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

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

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

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

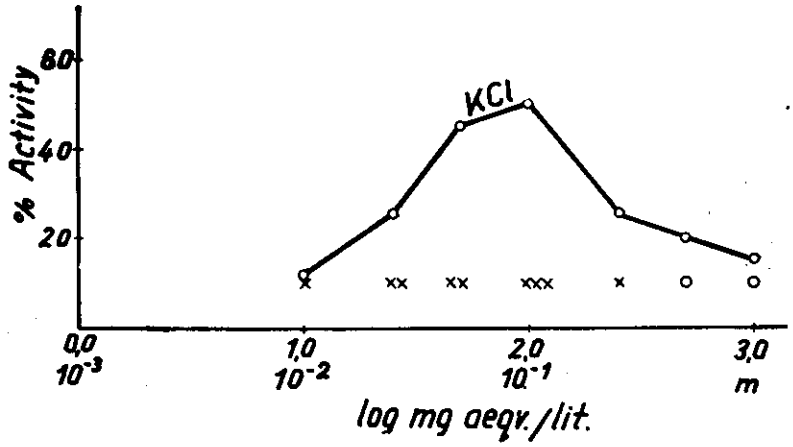

Fig. 3.

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

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

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

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

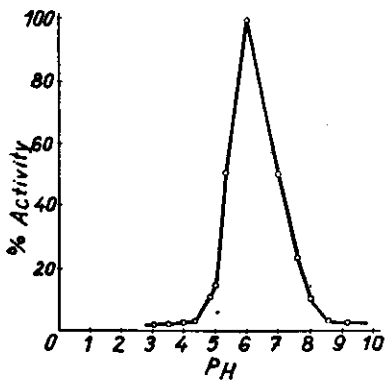

Fig. 4.

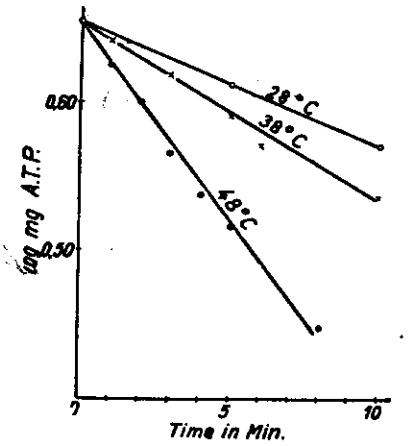

Fig. 5.

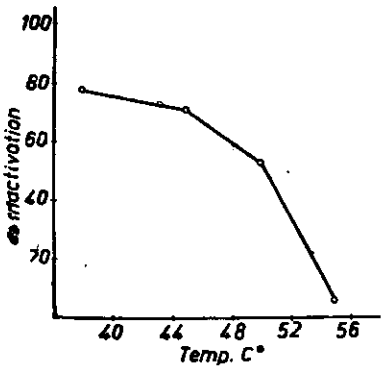

Fig. 6.

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

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

Reversibility. If our myosin contains no other en-

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

Tab. III.

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

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

Tab. IV.

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

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

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

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

Summary.

ENGELHARDT and LJUBIMOWA are corroborated.

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

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

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

Literature.

1. *U. A. Engehardt* and *M. N. Ljubimowa:* Nature *144,* 668. 1939.
2. *M. N. Ljubimowa* and *U. A. Engelhardt:* Biokhimija *4,* 716, 1939.
3. *K. Lohmann:* Biochem. Z. *271,* 264, 1934.