Adenosinetriphosphatase of muscle.

by

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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.

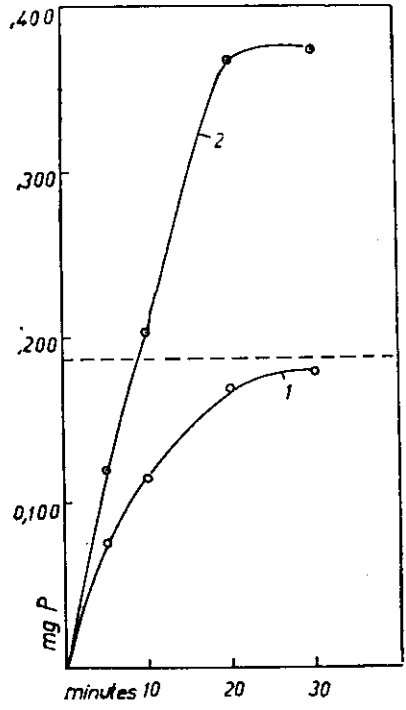

Fig. 1. Liberation of phosphate from ATP by the washed muscle suspension (curve 1), and washed muscle suspension + aqueous extract (curve 2). The dotted line represents the quantity of P corresponding to the splitting of one phosphate group from ATP.

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

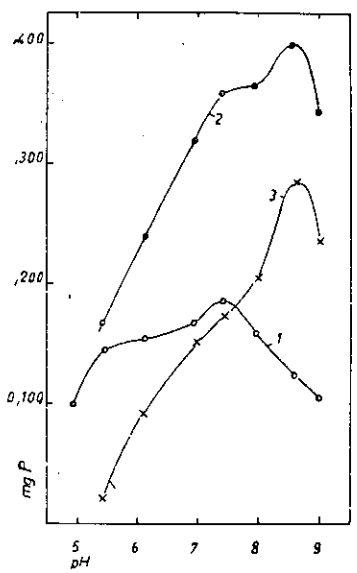

Fig. 2. The action of pH on the liberation of phosphate when only one phosphate group (curve 1), and when two phosphate groups are split off from ATP (curve 2). Curve 3 results by subtracting curve 1 from curve 2 and thus reprecents the activity pH curve of the oqueous extract.

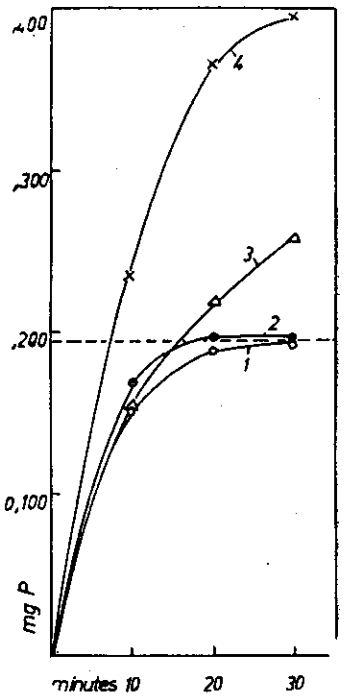

Fig. 3. The rate of liberation of phosphate from ATP by the muscle suspension (curve 1). Curve 2 represents the same in the presence of Mg, curve 3 in the presence of the aqueous extract and curve 4 in the presence of Mg and the aqueous extract together. The dotted line represents the quantity of P corresponding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