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Note on the work of

F. Bruno Straub

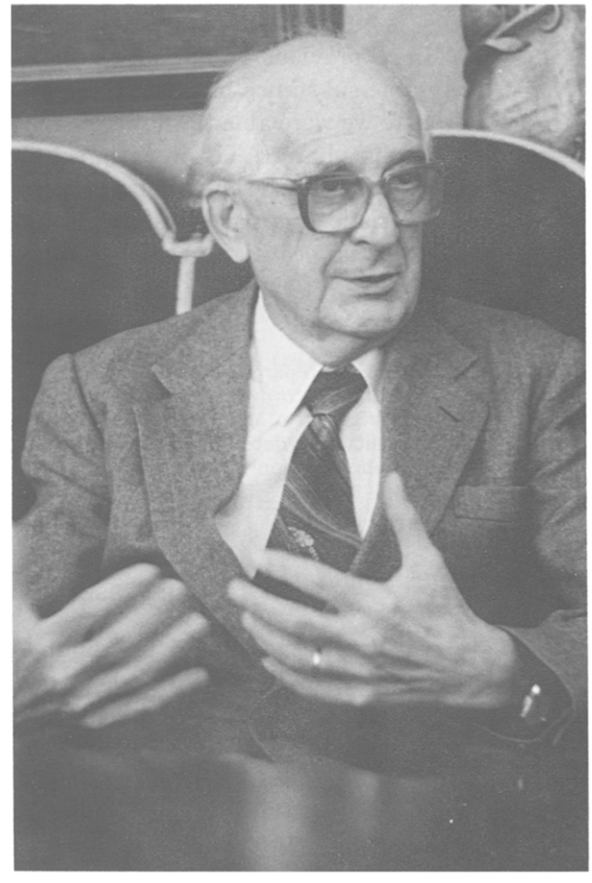
Institute of Enzymology, Hungarian Academy of Sciences, Budapest (Hungary)

concerning 'Adenosine triphosphate. The functional group of actin'
by F.B. Straub and G. Feuer
Biochim. Biophys. Acta 4 (1950) 455–470

Professor F. Bruno Straub worked with Albert Szent-Györgyi between 1932 and 1945, at Szeged University. Later he became professor at Szeged University and Budapest University and went on to become director of both the Biological Research Centre and Institute of Enzymology of the Hungarian Academy of Science.

Reference to Professor Straub's work is made in the essay prepared by S.V. Perry entitled 'The interaction of actin and myosin 40 years ago'.

At present, Professor Straub is President of the Presidential Council of the Hungarian People's Republic.



F. Bruno Straub

Correspondence: F.B. Straub, Institute of Enzymology, Hungarian Academy of Sciences, Karolina u. 29, Budapest XI, Hungary.

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ADENOSINETRIPHOSPHATE THE FUNCTIONAL GROUP OF ACTIN*

by

F. B. STRAUB AND G. FEUER

Institute of Medical Chemistry, University of Szeged (Hungary)

INTRODUCTION

In a previous communication we have reported the presence of a heat-stable, dialysable substance in actin, the removal of which leads to the inactivation of actin[1]. Recently we were able to show[2] the regular presence of a considerable amount of adenosine-triphosphate (ATP) in actin solutions. It will be shown in the present paper that ATP is actually the functional group of actin, firmly bound to the protein. The most characteristic property of actin, its polymerization in presence of salts, is connected with the disappearance of ATP.

METHODS

Actin was prepared according to our modified method[1]. Myosin, crystalline myosin and myosin-B were prepared according to the methods of this laboratory[3]. The ATP routinely employed was supplied by the firm MAGYAR GYÓGYSZER, Budapest.

Viscosity was determined—if not otherwise stated—in Ostwald viscometers as specified in our earlier communication[1].

RESULTS

I. THE PRESENCE OF ATP IN GLOBULAR ACTIN

1. It has been shown that a quinine-treated hypodynamic frog heart (summer frogs) regains its normal amplitude when treated with a 1 : 40 – 1 : 100 diluted actin solution (0.01–0.004 mg protein per ml) in the continued presence of quinine[2]. This reaction is very specific for ATP and ADP. The quinine-treated frog heart responds equally well to ATP and ADP in a concentration of 0.5 μg/ml and the results show that either of them is present. But it is impossible to decide which of the two substances is responsible for the effect.

2. A specific method for the determination of ATP in minute quantities has been recently found[4]. The method was actually worked out in order to determine ATP in actin solutions. The principle of the method is as follows: the viscosity of an actomyosin solution is decreased on addition of a small amount of ATP, but the viscosity soon rises again, on account of the decomposition of ATP. The time required for this rise of viscosity depends on the amount of ATP added. The reaction is very specific for ATP

* This work was carried out with the aid of a grant from the HUNGARIAN SCIENTIFIC COUNCIL.

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and can be utilized for the estimation of 10–30 μg ATP, even in the presence of large amounts of inorganic phosphate. The test may be carried out on a trichloroacetic acid filtrate after neutralization with solid sodium bicarbonate. It is best to use myosin-B instead of actomyosin, in a concentration of 1 mg/ml, dissolved in 0.5 *M* KCl, containing 0.01 *M* phosphate buffer of p_H 7.4 and 0.001 *M* $MgSO_4$.

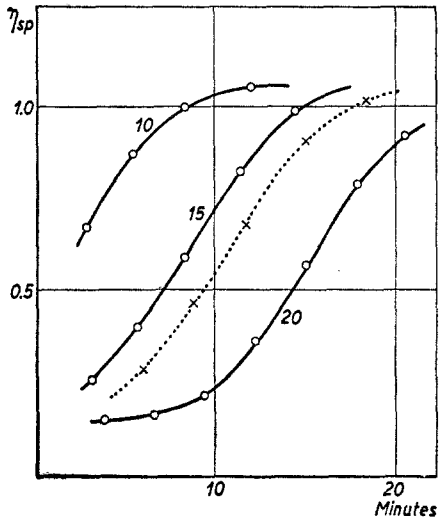

Fig. 1. Determination of ATP. The unknown solution was mixed with B myosin as described in the text, in a total volume of 2.5 ml. Zero time is the moment of mixing. Curves marked 10, 15, and 20 were obtained by mixing 10, 15 and 20 μg ATP respectively with the same amount of myosin-B at time O. The unknown is evaluated to contain 16.5 μg ATP.

Fig. 1 shows the result of one such estimation of ATP, where the viscosity-time curve (at 0°) of an unknown is compared with those of known quantities of ATP using reprecipitated myosin-B. It is essential that the myosin-B should be reprecipitated by dilution, in order to remove myokinase, the presence of which would disturb. This enzyme transforms adenosinediphosphate into ATP.

By the above method we have determined the ATP content of a great number of actin solutions, prepared from different lots of acetone-dried rabbit muscle. Some typical results are shown in Table I.

As seen from Table I, the ATP content of actin is somewhat variable. The variation may be due to the possible variation of free ATP, this fraction being perhaps only an admixture (see Section III). On the other hand, some data suggest the possibility that a fraction of the actin-ATP may have been lost during the process of isolation of actin. If the molecular weight of actin is 70 000, then the above data would mean that one protein molecule contains 1.5–2 molecules of ATP. We believe 2 molecules of ATP in one molecule of globular actin to be the most probable figure.

TABLE I

Preparation No.	mg N/ml	mg ATP/ml	% ATP in protein
1	1.07	0.073	1.09
2	1,09	0.083	1.21
3	0.78	0.055	1.14
4	0.53	0.045	1.35
5	0.58	0.053	1.47
6	0.68	0.054	1.28
7	0.85	0.056	1.10
8	0.95	0.051	0.86
9	0.90	0.066	1.18

3. Although both of the above indirect methods are highly specific for ATP, we have sought confirmation of our findings by the direct isolation and analysis of ATP from actin solutions. The process of isolation makes use of the directions of DOUNCE *et al.*[5] for the preparation of pure ATP from rabbit muscle.

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90 g acetone-dried rabbit muscle, as prepared in our method of the preparation of actin[1], were extracted with 1 800 ml distilled water at room temperature for 20 minutes. The mixture was filtered with suction and the residue extracted for the same length of time with another 900 ml distilled water. The combined filtrates (1 850 ml) were treated with 185 ml 20% trichloroacetic acid at 0°. After standing for one hour, the protein precipitate was removed by filtration and the clear solution was neutralized with 10% NaOH. 5 ml of 20% Hg-acetate (in 0.2% acetic acid) were then added for every 100 ml of solution, the reaction of the solution at this point being p_H 6. After standing in the cold room for several hours, the mercury precipitate was centrifuged off, washed in the centrifuge with a 0.5% Hg-acetate solution, and then suspended in 120 ml water and H_2S was led into the suspension. The HgS precipitate was centrifuged off, washed with 20 ml water and the combined supernatant was aerated. The solution was then neutralized to p_H 7 and then 7 ml *M* Ba-acetate was added. The Ba precipitate was centrifuged off and redissolved by the addition of *N* acetic acid solution. The p_H of the resulting solution was p_H 3. 0.5 ml 20% Hg-acetate were then added and the mixture left to stand for several hours. The Hg precipitate was collected, washed, treated with H_2S and aerated essentially in the same way as described above. After the removal of H_2S the solution was brought to p_H 6 by the addition of dilute NaOH, and the resulting precipitate, containing colloidal sulphur and some ATP, was discarded. After the addition of a few drops of a *M* Ba-acetate solution, the mixture was brought to p_H 7.0. The resulting precipitate was centrifuged and washed in turn with 2 changes of ice-cold water, then with 50%, 75% and absolute alcohol, and finally with two changes of ether. Dried at 35° in a desiccator over $CaCl_2$ the substance showed the following composition:

Analysis of ATP isolated from actin solution.

	Found	Theoretical for $Ba_2ATP.4H_2O$ %
N (KJELDAHL)	7.07	8.2
P inorganic (FISKE–SUBBAROW)	0.42	0.0
P 7 minutes hydrolysis ,,	6.55	7.26
P total after wet ashing ,,	9.40	10.88
ribose (MEJBAUM)	17.6	17.9

According to these results, the substance contains inorganic impurities, but represents mainly ATP. From the analytical data the following ratios are calculated:

$$P_7 : P_{total} = 2.05 : 3 \quad \text{Theory: } 2 : 3$$
$$P_{total} : N = 2.87 : 5 \quad \text{Theory: } 3 : 5$$
$$P_{total} : \text{ribose} = 2.6 : 1 \quad \text{Theory: } 3 : 1$$

When compared with a sample of ATP prepared according to the method of DOUNCE *et al.*[5], our substance gave qualitatively and quantitatively the same effect on actomyosin.

The overall yield in pure ATP was 16.4 mg from 90 g acetone-dried muscle. Determination of the ATP content of the original actin solution according to the method of PETTKÓ AND STRAUB[4] showed the presence of 90 mg ATP in the original actin solution. The yield is thus 18%.

II. THE CHANGE IN ATP CONTENT DURING POLYMERISATION

According to the evidence to be presented in Section IV, the presence of ATP prevents the denaturation of actin under conditions which remove actin-bound ATP. Experiments to be described in this section show that ATP is not only a stabilizing factor but it actually takes part in the process of polymerization.

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1. When actin polymerizes on addition of any salt, a significant portion (40–80% of the ATP it contains disappears.

Polymerization is followed by determining the rise of viscosity of the actin solutions. Part of the same solution as was used for the determination of viscosity was kept at the same temperature, and samples were removed at various intervals after the addition of the salt (Zero time of polymerization). These samples were used for ATP determination according to PETTKÓ AND STRAUB[4]. Such experiments are recorded in Fig. 2 and 3. The relation between rate of polymerization and rate of ATP disappearance was studied under a variety of conditions, *viz.*, at different temperatures and using different salts. In all cases polymerization and disappearance of ATP run parallel.

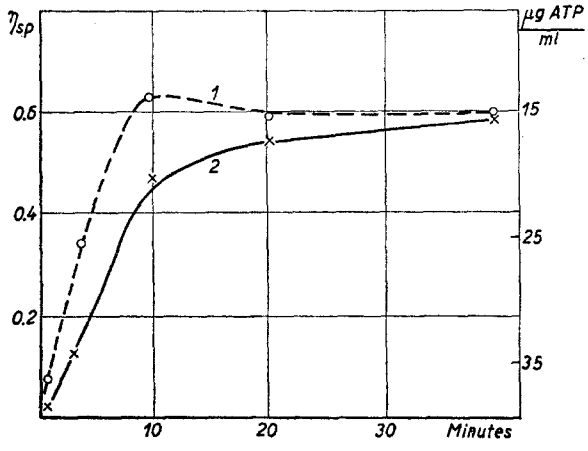

Fig. 2. Polymerization of actin at 24° C in presence of 0.1 *M* KCl. Viscosity measured at p = 60 Hg mm. o---o viscosity, x---x ATP content.

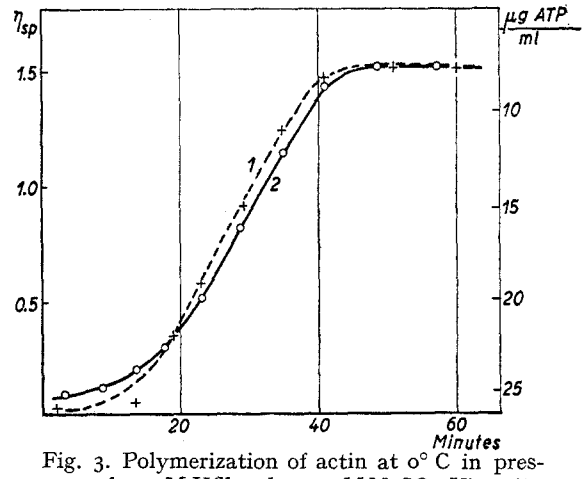

Fig. 3. Polymerization of actin at 0° C in presence of 0.1 *M* KCl and 0.001 *M* $MgSO_4$. Viscosity measured in an Ostwald viscometer. o---o viscosity, x---x ATP content.

We have been unable to decide by our present methods which of the two processes—polymerization or disappearance of ATP—is the primary one. But so much can be definitely stated that the two processes are interrelated. Discounting the possibility that the two processes can be separated, it can be assumed that the two phenomena are expressions of one and the same thing: when actin polymerizes ATP disappears, and when actin-ATP is decomposed, the actin polymerizes.

2. In order to study the fate of the disappearing ATP, we have examined the possibility of the *formation of inorganic phosphate.* Actin solutions often contain surprisingly large amounts of inorganic P (apparently formed from organic esters during the acetone treatment of the washed muscle residue). Thus it is impossible to determine the small amount of inorganic phosphate that might be formed during polymerization. So we have studied this problem on dialysed actin preparations. As described later in this paper, actin can be dialysed only in the presence of added ATP, but the ATP present after dialysis is mostly bound to the protein, like the ATP present in crude actin solutions.

Globular actin solution was dialysed against 0.001 *M* $NaHCO_3$ containing 4 mg % ATP and 4 mg % ascorbic acid for 4 days, the outer fluid being changed every 24 hours. 30 ml of the dialysed actin, which contained 0.019 mg ATP/ml, were mixed with 120 ml 0.1 *M* KCl containing 0.001 *M* $MgSO_4$ at 8° C. Polymerization was followed by measuring the viscosity of the solution at 8° C., whereas from the bulk of the solution kept at the same temperature samples were deproteinized at intervals with trichloroacetic acid. True inorganic P, according to the method of LOWRY AND LOPEZ[6] and ATP according to PETTKÓ AND STRAUB[4] were determined on this filtrate.

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The results of this experiment are shown in Fig. 4. 0.011 mg ATP/ml has disappeared and 0.0008 mg inorganic P/ml were formed during polymerization, calculated in terms of the original actin solution. The ratio of ATP disappeared: inorganic phosphate formed = 1 : 1.18.

The method of LOWRY AND LOPEZ was used in determining inorganic phosphate in order to be sure thet no labile phosphate ester, *e.g.*, phosphocreatine, is formed. It appears that the process is a true dephosphorylation and not a transphosphorylation. A phosphate transfer from ATP to the protein is most likely, but if this takes places, the phosphate group must be subsequently split off from the protein during polymerization or during deproteinization.

3. As ATP disappears and inorganic phosphate is formed during polymerization in a ratio of approximately 1 : 1, the *formation of adenosinediphosphate* must be assumed. There is no specific reaction for the determination of adenosinediphosphate (ADP) in presence of ATP. It was observed by PETTKÓ AND STRAUB[2] that globular and polymerized actin restore the amplitude of the hypodynamic frog heart in the same degree. As the quinine-treated frog heart reacts equally well to ADP and ATP this is consistent with the possibility of the formation of ADP during polymerization. However, the frog heart method is not accurate enough to yield clear-cut evidence on this point.

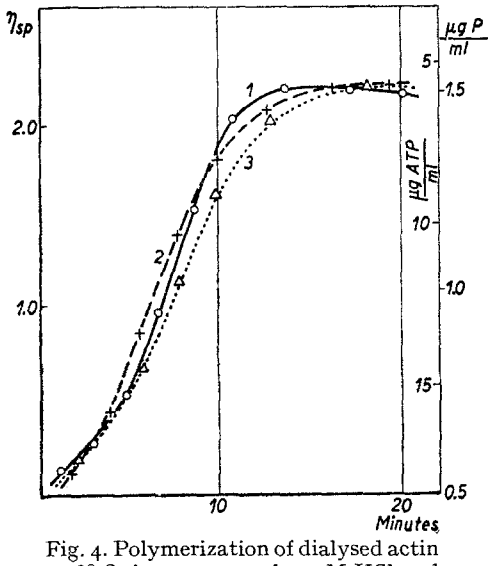

Fig. 4. Polymerization of dialysed actin at 8° C. in presence of 0.1 *M* KCl and 0.001 *M* $MgSO_4$. Curve 1: viscosity, curve 2: ATP content, curve 3: inorganic phosphate content.

More reliable evidence is secured in the following way. An actin solution is used in which ATP has nearly completely disappeared after polymerization. The trichloroacetic acid filtrate of polymerized actin is neutralized. On a portion of this solution ATP is determined first, the rest of the solution is mixed with purified myokinase (KALCKAR[7]), and after 30 minutes at room temperature myokinase is destroyed by the addition of trichloroacetic acid, then the solution is neutralized again and used for ATP determination. It is found that after incubation with myokinase the ATP content increases.

ATP content of actin before polymerization: 19.8 μg/ml
ATP content of actin after polymerization: 9.4 ,,
ATP content of filtrate of polymerized actin incubated for 30 minutes with myokinase: 21.4 ,,

Experiments are now in progress to show that the ADP formed during polymerization is present also in a bound form.

In view of the above evidence, we conclude that the *polymerization of actin is connected with the simultaneous formation of ADP and inorganic phosphate from the ATP present in actin.* In other words, globular actin is ATP-actin; ADP-actin, if formed, is in the fibrous form, *i.e.* polymerized.

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III. THE ATP OF GLOBULAR ACTIN IS BOUND TO PROTEIN

Actin is prepared from fresh rabbit muscle under sharply defined conditions. The insoluble residue containing actin is extracted 3 times with salt solutions of varying composition and then dried with acetone. It is therefore possible that ATP is present in actin solutions only as a contaminant, and even its regular presence may mean nothing more than that the actin preparations are too uniform. The following experiments were devised to show that the ATP present in actin solutions is not in a free state but bound to protein.

We have found that a purified potato apyrase (or myosin, or hexokinase in presence of glucose), when added to globular actin, can destroy only a small fraction of the ATP present in the solution. If, however, actin is first denatured by boiling its solution for 10 minutes and then incubated with these enzymes, the ATP it contains is completely utilized.

Polymerized actin also contains some ATP, although only a fraction of that present in the globular state (see Section II). This ATP in polymerized actin is in the free state, as can be shown by similar enzymic studies. The experiments to be recorded below were performed with a purified potato apyrase.

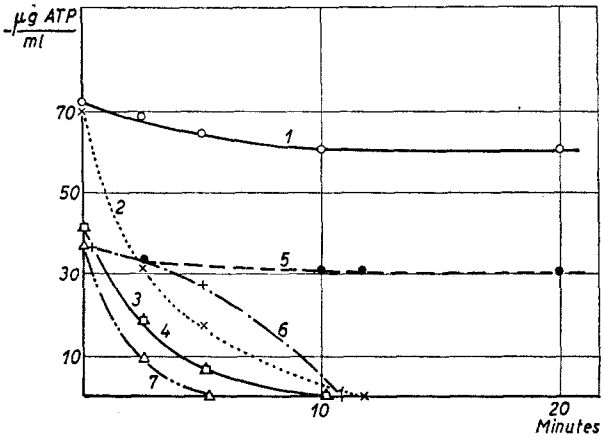

Fig. 5. Effect of dilute apyrase on the ATP content of actin. Curve 1: globular actin, 72 μg ATP. Curve 2: 72 μg free ATP. Curve 3: polymerized actin, 41 μg ATP. Curve 4: 41 μg free ATP. Curve 5: globular actin (No. 2), 36 μg ATP. Curve 6: boiled globular actin (No. 2) 36 μg ATP. Curve 7: 36 μg free ATP.

Apyrase was purified according the method of M. SZÉKELY (unpublished). The dialysed final product (5 ml), obtained from 1 kg potatoes, had a very high activity: 0.01 ml of the solution when incubated with 0.04 mg ATP at 0° C. decomposed 50% of it in 3 minutes. The action of this enzyme on actin-ATP was studied at 0° C. and p_H 7 by determining the concentration of ATP by the method of PETTKÓ AND STRAUB[4].

In the experiment, the results of which are shown in Fig. 5, curves 1–4, an actin was used which contained a rather high fraction of its ATP unchanged even in the polymerized state. This preparation was used to enable us to study the state of ATP in both globular and polymerized actin on one and the same preparation.

In similar experiments another sample of actin was denatured by boiling the globular actin solution for 10 minutes and then the availability of its ATP for enzymic action was studied (Fig. 5, curves 5–7).

These experiments were set up in the following way

	1	2	3	4	5	6	7
Actin No. 1 ml	2.0	—	2.0	—	—	—	—
Actin No. 2 ml	—	—	—	—	2.0	—	—
Actin No. 2 boiled, ml	—	—	—	—	—	2.0	—
M KCl + 0.01 *M* $MgSO_4$ to polymerize actin	—	—	0.2	0.2	—	—	—
1 : 10 diluted apyrase, ml	0.1	0.1	0.1	0.1	0.1	0.1	0.1
ATP solution, ml	—	2.0	—	2.0	—	—	2.0

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ATP was added in experiments 2, 4 and 7 in a volume of 2 ml containing exactly the same amount of ATP as that found in 2 ml of the corresponding actin solution in experiment 1, 3 and 5, respectively, *i.e.*, 72, 41 and 36 μg ATP.

Identical samples of the above mixtures were incubated at 0° C. for various length of time. At the desired moment the reaction was stopped by the addition of 0.2 ml 20% trichloroacetic acid, and ATP was determined on the neutralized filtrate by the method PETTKÓ AND STRAUB[4]. The results are shown in Fig. 5, expressed as μg ATP per experiment.

It is clear from these results that whereas only a small fraction of the ATP present in globular actin is available to the enzyme (Fig. 5, curves 1 and 5), that present in polymerized actin (curve 2) and in denatured actin (curve 6) is destroyed at a rate similar to the destruction of free ATP.

Our studies on the dialysis of actin have revealed that ATP can be removed from actin by prolonged dialysis. The combination between ATP and protein must therefore be a dissociation equilibrium. Therefore it is to be expected that the unavailability of actin-ATP to enzymic action is only a quantitative difference, and if a higher concentration of apyrase is used, bound ATP will be removed and broken down. As the presence of ATP is necessary for the polymerization of actin, incubation of actin with an excess of potato apyrase should lead to an actin which does not polymerize. That this is actually the case is shown by the results of the following experiment (see Fig. 6). 1.5 ml actin was incubated with 0.1 ml apyrase for 20 minutes at 24° C. After this time 0.25 ml of salt solution (M KCl + 0.01 M $MgSO_4$) was added, the solution made up to 2.5 ml, and the rate of polymerization was followed in the viscometer at 8° C. A sample of the same actin, not treated with apyrase, served as control. The activity of the apyrase solution employed was the following: 1 ml of the apyrase solution split 0.192 mg P from 4 mg ATP in 4 ml in 3 minutes at 30° C. Protein content of apyrase solution 0.2 mg/ml. We are indebted to Miss M. SZÉKELY for this purified apyrase preparation.

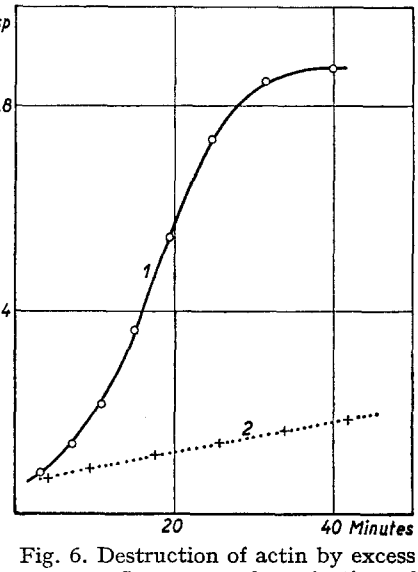

Fig. 6. Destruction of actin by excess apyrase. Curve 1: polymerization of control actin solution; curve 2: polymerization of the same actin solution first treated with a strong apyrase solution.

IV. EFFECT OF REMOVAL OF ATP ON THE PROPERTIES OF ACTIN

In our previous communication[1] we described two simple methods by which actin becomes inactivated:

1. dialysis;

2. precipitating actin from a dilute solution and washing the precipitate 3 times with acetate buffer (0.01 M) of p_H 5.

In each case actin is progressively inactivated. When during and after these treatments an aliquot of the actin solution is mixed with salt, it is found that the viscosity of the solution does not rise to the level which a similar sample of the untreated actin would reach. After a dialysis of 72 hours against 0.001 M $NaHCO_3$ or after washing the

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isoelectric precipitate three times as described[1], the viscosity of the solution will show practically no rise on addition of salt, *i.e.*, it has lost its ability to polymerize.

As already described[1], both types of inactivation can be prevented if a boiled muscle extract is added to the solution, *i.e.*, when actin is dialysed against a dilute muscle extract or muscle extract is added to the acetate buffer used for the washing of the isoelectric precipitate. In both cases the degree to which actin is able to retain its native properties, *i.e.*, its ability to polymerize (measured by the viscosity of the solution after the addition of salt) is proportional to the amount of muscle extract added.

It should be stated again that it is impossible to reactivate the protein once it has lost its properties. Addition of muscle extract to dialysed actin fails to reactivate it[1].

In order to determine the nature of the active substance present in muscle extracts, we have attempted its isolation, using for its quantitative estimation the following method:

A solution of actin containing 1.5 mg protein was diluted with distilled water to 7.5 ml, treated with 0.15 ml of 1 *M* acetate buffer of p_H 4.6, and left to stand in a water bath at 37° C. The precipitate was then centrifuged off and resuspended in 7.5 ml 0.01 *M* acetate buffer of p_H 5, and then again put into a water bath of 37° C. for 10 minutes. The latter procedure was repeated twice more, and finally the precipitate was dissolved by adding 0.4% $NaHCO_3$ with one drop of phenol red until the p_H was raised to 7. The solution was then made up to 1.5 ml. 1 ml of this solution was then mixed with 2.5 mg myosin, the mixture was made up to 2.5 ml so that it should contain 0.5 *M* KCl, and the viscosity of the resulting actomyosin was determined first at 0° C. After the addition of 0.1 ml 1% pyrophosphate the viscosity was determined again. From these data the actin content could be calculated according to STRAUB[8].

When a muscle extract, or a fraction prepared from it, was investigated, a series of such tubes were taken, one of which was treated exactly as described above. The other tubes received various amount of the extract, the same amount of extract being added to every washing. The result of such a series is shown in Fig. 7, where the effect of a boiled extract (2 volumes of water) of washed horse skeletal muscle is shown. The actin content after the treatment was evaluated and the result expressed as % native actin after 3 washings.

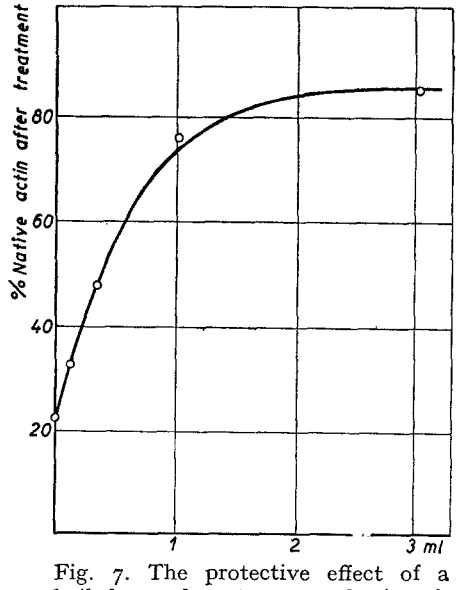

Fig. 7. The protective effect of a boiled muscle extract on the inactivation of actin during the isoelectric washing of precipitated actin.

Using this method of assay we have been able to determine the relative potency of fractions prepared from muscle extracts. Following the activity of these fractions, we have finally isolated a substance which proved to be ATP. The activity of ATP in preventing the loss of actin is shown in Fig. 8.

A very dilute solution of ATP is able to prevent the inactivation of actin during dialysis. This is shown in Fig. 9. Samples of the same actin solution were dialysed at 0° C. against 0.001 *M* $NaHCO_3$ containing 0.04 mg/ml ascorbic acid and different concentrations of ATP (5 ml actin and 100 ml outer fluid), the outer fluid being changed every 12 hours. After 3 days of dialysis, salt solution was added to the actin solution so that it should contain

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0.1 *M* KCl and 0.001 *M* $MgSO_4$, and it was left to stand at room temperature for several hours. The viscosity of the solutions was then measured.

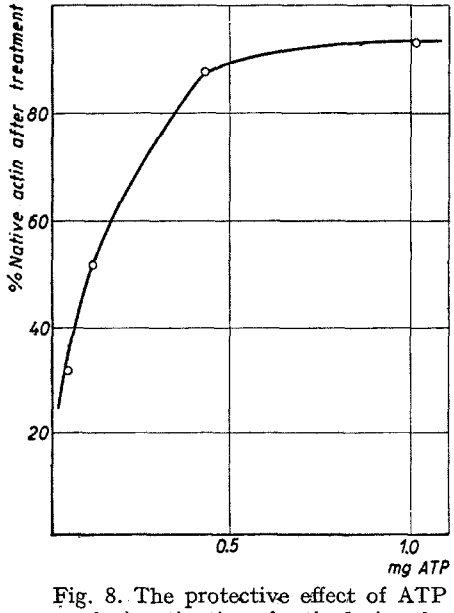

Fig. 8. The protective effect of ATP on the inactivation of actin during the isoelectric washing of precipitated actin.

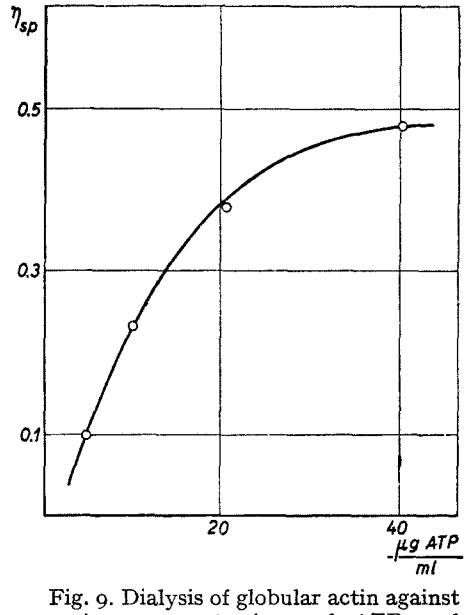

Fig. 9. Dialysis of globular actin against various concentrations of ATP and 4 mg % ascorbic acid. Ordinate: viscosity of the actin solution after the addition of salt.

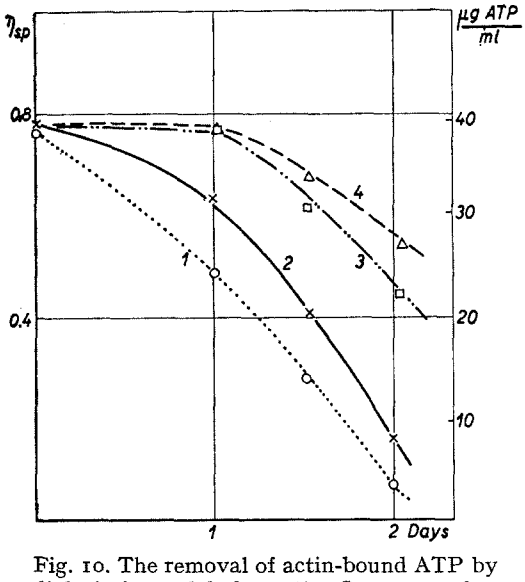

Fig. 10. The removal of actin-bound ATP by dialysis from globular actin. Curves 1 and 2: without ascorbic acid. Curve 1: viscosity of actin polymerized at the time marked on the abscissa. Curve 2: ATP content of actin. Curves 3 and 4: dialysis in presence of 4 mg % ascorbic acid. Curve 3: viscosity of actin polymerized at the time marked on the abscissa. Curve 4: ATP content of actin.

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According to the above experiments the inactivation of actin during dialysis and isoelectric washing must be due to the loss of ATP. This can be shown to be actually the case by the following experiment. 12 ml actin solution were dialysed against 250 ml 0.001 *M* $NaHCO_3$ at 0° C. and at intervals samples were removed. On part of this sample ATP was determined, and the rest was polymerized by the addition of KCl and $MgSO_4$. It can be seen from the results of such an experiment (Fig. 10) that the amount of ATP still present in the actin solution and the ability of actin to polymerize change progressively and similarly during dialysis.

We have observed that the presence of reducing substances, *e.g.*, ascorbic acid[1], will prevent to some extent the inactivation of actin during the above-mentioned treatments. This effect is explained by the results of the experiment shown in Fig. 10, curve 3 and 4. In this case the same actin solu-

tion that was used for dialysis in the experiments of Fig. 10, curve 1, was dialysed against a 0.001 *M* $NaHCO_3$ solution containing 0.04 mg ascorbic acid/ml. ATP content of the actin solution and the ability of actin to polymerize was determined likewise at intervals. The results show that the loss of ATP from the actin solution is greatly reduced by the presence of ascorbic acid. This explains why ascorbic acid (or other reducing substance) alone, when used in sufficiently high concentration, can prevent the inactivation of actin as well as a small amount of ATP. Addition of ATP produces a specific effect: it prevents the dissociation of the actin-ATP complex. The action of ascorbic acid is not specific: it increases the strength of the protein-ATP bond.

V. REVERSIBILITY OF POLYMERIZATION

The above experiments led to the possibility of the reversible depolymerization of fibrous actin. As actin is in a globular from when its solution is free from salts, it might be expected that dialysis of the polymerized actin would lead to depolymerization. In view of the above results it is clear that dialysis of polymerized actin should be made against a dilute ATP solution (in presence of ascorbic acid) in order to prevent inactivation.

Example: An actin solution was polymerized and then dialysed at 0° C. against 0.001 *M* $NaHCO_3$ containing 4 mg% ATP and 4 mg% ascorbic acid. The outer fluid was changed every 24 hours. The viscosity of the solution was measured every day on samples diluted threefold with distilled water. After 6 days the viscosity of the solution had dropped to $\eta_{sp} = 0.22$. On addition of salt (final concentration: 0.1 *M* KCl and 0.001 *M* $MgSO_4$), the viscosity of the same solution rose to $\eta_{sp} = 2.17$, *i.e.*, the solution contained most of the actin in the globular form.

ADP is unable to replace ATP in the dialysis experiment. When globular or polymerized actin was dialysed against 0.001 *M* $NaHCO_3$ containing 4 mg % ADP and 4 mg % ascorbic acid, the actin solution obtained after 4–5 days of dialysis was nearly completely inactivated, *i.e.*, it did not polymerize on addition of salts.

When dialysed against ATP, fibrous actin depolymerizes and acquires again bound ATP. That ATP is again bound to the protein is shown by the fact that on addition of salts this ATP behaves in the same way as that of the original actin solution; it disappears during polymerization. The process of dialysis is thus the complete reverse of polymerization: when actin depolymerizes bound ATP appears and conversely, when bound ATP appears and there is no salt present, actin depolymerizes.

In support of this concept, we are able to show one further piece of interesting evidence.

Actin is prepared according to our method from an acetone dried powder by extraction with distilled water. We have followed the ATP content of the various fractions during the preparation of actin. The following results were obtained (Table II).

It appears from these results that the ATP found in actin solutions after extracting the dry muscle with distilled water is formed during the process of extraction, only a small fraction, if any, being present in the dry preparation.

As ATP was determined on trichloroacetic acid filtrates, the objection can be made that the extraction of ATP was incomplete from the dried muscle. That this is not the case is indicated by the following experiment.

Acetone-dried muscle was mixed with 20 volumes distilled water and after only 2 minutes of contact the mixture was rapidly separated on a Buchner funnel. From the

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TABLE II

	mg ATP/g fresh muscle
Rabbit muscle minced	2.88
Mince extracted with phosphate-saline. Residue	0.12
Residue washed with Na_2CO_3 and pressed out. Residue	0.018
Residue washed with Na_2CO_3–$NaHCO_3$ mixture and pressed out. Residue	0.014
Residue treated twice with acetone and dried	0.005
Dry residue extracted with 20 volumes of distilled water, actin solution not separated	0.028

solution, containing actin, samples were removed at short intervals and after treatment with trichloroacetic acid ATP was determined on the filtrate. Zero time of this experiment is the time of mixing distilled water with the dry muscle. The results are shown

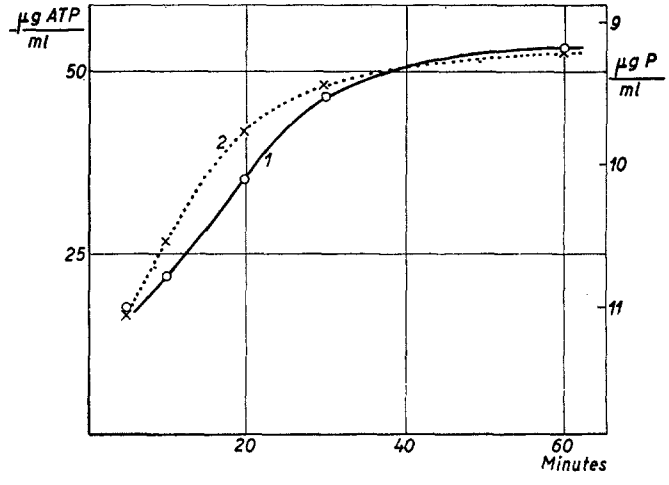

Fig. 11. Formation of ATP and disappearence of inorganic phosphate in actin solution immediately after the extraction of actin from the dried muscle. Curve 1: ATP content; curve 2: inorganic phosphate content.

in Fig. 11 together with the change in the inorganic P content, which in this particular actin preparation was low enough to allow the detection of the decrease of inorganic P during the formation of ATP.

The evidence shows that the ATP found in actin is produced during and after the extraction of the protein from the dried muscle residue. Addition of distilled water decreases the salt content and dilutes actin to such an extent that depolymerization occurs. As the dry preparation contains practically no ATP, the actin in it must be in the polymerized state. No enzyme can be detected in actin solutions which would produce a high energy phosphate bond. The energy needed for the formation of ATP must therefore have been furnished by the depolymerization of the protein.

DISCUSSION

The first important conclusion we have arrived at is that actin contains ATP in

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a bound form. As the addition of a reducing substance increases the strength of this bond, it is most likely that ATP is bound to easily oxidizable groups, most likely to masked SH groups. In previous studies we have shown[1] that actin contains 0.21% Ca, the ratio of mol ATP bound: atoms Ca bound being approximately 1 : 2. As Ca forms stable complexes with ATP, it is reasonable to suppose that Ca is responsible for the bond between protein and ATP. We believe that actin is a coordination complex in which Ca forms a bond both with the protein and with the ATP.

PETTKÓ AND STRAUB[2] have shown that skeletal muscle contains about 0.3 mg ATP (or ADP) per g fresh muscle in a bound form, inaccessible to enzymes. The method employed in their studies was that of the quinine-treated frog heart's increase of amplitude on addition of a boiled muscle extract. The method is very specific for ATP and ADP but does not differentiate between these two substances. The viscosimetric method outlined in the present paper and to be described in more detail[4] enables us to show that the substance present in minced muscle after several hours and not split by its enzymes is ADP. ADP therefore occurs in muscle in a bound form.

It is very interesting to recall the observation[2] that heart muscle contains about the same amount of bound ADP as skeletal muscle, although the ATP content of the fresh heart muscle is only $^1/_4$–$^1/_3$ of that present in fresh skeletal muscle.

These findings are easily explained now in terms of the data of the present paper. The major part of the proteinbound ADP is that bound to actin. The amount of actin per g fresh rabbit muscle was estimated by BALENOVIC AND STRAUB[9] to be 23 mg. This estimate is only an approximation, obtained by a complicated method. According to the findings described in the present paper 0.2–0.3 mg ADP are expected to be bound to this amount of actin in one g rabbit muscle. The agreement between this figure and that obtained by the frog heart method is very suggestive.

2. In actin, ATP is necessary not only for the stabilization of the actin, it actually takes part in the mechanism of polymerization; it is a real prosthetic group, taking part in the process specific for the protein. Polymerization is connected with the dephosphorylation of ATP and depolymerization seems to be connected with the reversal of this change, *i.e.*, with the resynthesis of the protein-ATP complex. It is interesting to draw attention to the parallel existing between glycogen synthesis and the polymerization of actin: the phosphorylated monomer (glucose-1-phosphate or globular (ATP-) actin) polymerizes with the formation of inorganic phosphate to form the dephosphorylated polymer (glycogen or fibrous (ADP-) actin).

We wish to attach special emphasis to the apparently non-enzymic nature of the ATP breakdown during the polymerization of actin. To sum up the evidence presented above:

1. Actin has no adenosinetriphosphatase activity, either in the presence or absence of salts. Added ATP is not split at all.

2. Only part of the total ATP is used up during polymerization and the rest remains untouched. Although only a small fraction of the total ATP is in a free state in globular actin, that remaining in polymerized actin is entirely free. Thus, only that fraction which is *unavailable* for enzymes is split during the process of polymerization.

3. The change in ATP occurs only during polymerization, *i.e.*, simultaneously with the change of form of the protein molecule. Polymerization under a diversity of conditions proceeds with widely varying velocity, but it always coincides with the change in the ATP content.

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4. The process is not a transphosphorylation, as true inorganic phosphate appears. ATP disappears from dialysed actin solutions which are free from low molecular impurities.

The possibility remains that the protein of actin is phosphorylated and an unstable phosphoproteid is formed, which yields up its phosphate group when it polymerizes or when it is treated with trichloroacetic acid.

Although the molecular weight of actin is not precisely known, globular actin appears to belong to the 70000 class. In this case about 2 molecules of ATP are attached to 1 molecule of protein. When one of these ATP molecules is dephosphorylated, this would produce a net loss of negative charge at a definite point of the protein molecule. It is conceivable that this decrease of negativity makes it possible for the protein to attach itself to another actin molecule. The complex thus formed would still have one or two ATP side-chains, which in turn—after dephosphorylation—would initiate further association. The autocatalytic nature of the polymerization of actin is probably due to the greater lability of actin-bound ATP when present at the end of a chain of actin molecules. ASTBURY *et al.*[10] conclude in their studies on the electronmicroscopy and X-ray analysis of fibrous actin that the molecules are aggregated in a very regular fashion: "always in the same way and with atomic precision". The above picture of polymerization would be sufficient to explain this amazing regularity.

The great advances made during the past 20 years in researches on enzyme actions have immensely contributed to our knowledge concerning metabolism. Yet it is increasingly obvious that all phenomena of life cannot be explained merely by supposing the presence of enzymes whenever a change is observed. We believe that the efforts to view muscle contraction as an enzymic process rest on misconceived ideas. That chemical changes do occur and that these changes are abolished when the underlying fabric of proteins is denatured is no proof at all that the reactions are of enzymic nature. We believe that enzyme action is a borderline case of a more general phenomenon: proteins are able to catalyse chemical changes, from which energy can be utilized or not. In practically all cases of isolated enzymes which catalyse exergonic reactions, the energy liberated is dissipated and cannot be utilized. Moreover, the term enzyme is applied only to catalysts from which the product of the reaction is dissociated, thus allowing for the turnover of a great number of substrate molecules. The enzymes isolated are those which are easily detached or not attached at all to the protoplasmic structure. These enzymes are probably not meant at all to produce utilizable energy; they might be only safety valves which remove surplus material. On the other hand it is possible that the known, isolated enzymes are artefacts, some important part of them being lost during the process of extraction.

The example of actin teaches quite a different lesson. A protein-bound substrate is changed and at the same time a change of form of the protein molecule occurs, the change of form being reversible when the chemical change is reversed. Further work in our laboratory is now in progress to show that a mechanical change which leads to a change in the protein molecule would in turn cause a change in chemical composition, *i.e.*, doing mechanical work on fibrous actin which produces depolymerization would generate chemical energy in producing actin-bound ATP from ADP.

3. One great problem of actin chemistry remains still unsolved, and that is the mode of physiological depolymerization. The only feasible method to attain depolymerization is that described in the present paper: dialysis against dilute ATP solution. Polymerized

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actin contains no bound ATP as shown in the present paper. Yet, when it is dialysed against ATP, it is found to contain again bound ATP. The process is therefore the following one: during dialysis, as the salts are removed, ATP replaces the ADP that actin contains. When the concentration of salt becomes sufficiently low, actin-ATP will no longer be transformed to actin-ADP and it will thus become stabilized in the form of globular actin.

From what has been said above, the physiological depolymerization of actin should be the phosphorylation of actin-bound ADP. This process might be the connecting link between contraction and the enzyme system furnishing the energy for it.

Actin-ATP is not accessible to enzymes, as shown above. It is quite reasonable to suppose that actin-bound ADP will behave in the same way, *i.e.*, the phosphorylation of ADP through enzyme action may not take place at all. Then the possibility seems closer that actin-ATP is resynthesized by mechanical forces. It is important to call attention to the observation described above, that actin-ATP is resynthesized when the acetone-dried muscle is simply extracted with distilled water. It is very unlikely that any enzymic process of energy-yielding character occurs during the extraction. It looks more as if the change of form of the actin molecule from fibrous to globular (which in this case is produced by the sudden dilution) results in the synthesis of ATP.

4. The term "mechano-chemical coupling" has been lately often used to designate a rather vague concept and a totally unknown mechanism of transfer of energy from an "ultimate chemical change" into a mechanical structural element. Regardless of whether later research will prove or not that the reversible polymerization of actin has anything to do with muscle contraction, the phenomena described in this paper would give the term "mechano-chemical coupling" some real substance: mechano-chemical coupling cannot be separated into two fractions, one component being responsible for the chemical change and another for the mechanical one. Actin seems to be an instrument of mechano-chemical coupling, the correct use of which is at present obscure.

5. Electron microscope pictures of the micro-structure of muscle reveal that the fibrous proteins of the contractile elements are situated parallel to the fibre axis. As PERRY *et al.*[11] have pointed out, the synaeresis of actomyosin threads cannot lead to a shortening of the muscle fibre if the above interpretation of such pictures is accepted.

For this reason, intramolecular changes are again sought for to explain the mechanism of muscle contraction. A recent theory of RISEMAN AND KIRKWOOD[12] examines the possibility, first suggested in a rather unfortunate context by K. H. MEYER[13], that the change of electric charge is the cause of shortening. As a result of an approximate calculation, RISEMAN AND KIRKWOOD suggest that if 100 electric charges in an actomyosin fibre of MW = 10^6 are changed simultaneously, a substantial change of the elastic modulus of the muscle would follow. They suggest that the phosphorylation of the hydroxyaminoacids through the phosphate split off from ATP by myosin-adenosinetriphosphatase is the cause of such a change in electric charge. Although there is some evidence that myosin is phosphorylated when it decomposes ATP (SZÖRÉNYI AND CHEPINOGA[14]), other evidence is contrary (BANGA AND SZENT-GYÖRGYI[15]), and the point remains to be proved. Although we have as yet no direct proof that the polymerization of actin occurs during contraction, the outstanding properties of actin make it certainly suitable for such a rôle. Then polymerization of actin would produce enough change in the electric charge of the actomyosin molecule (decrease of negative charge on the actomyosin, due to dephosphorylation) to produce a drastic change in the electrostatic

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structure of the molecules of muscle fibres. The change of ATP-actin to ADP-actin would produce a change repeated at every 50 Å along the length of the individual actomyosin fibres.

6. A final, more general aspect of the present work is the probable importance of protein-bound ATP or ADP in proteins other than actin. Although it is generally accepted and experimentally proved that ATP is an energy reservoir on which the cell draws whenever energy is needed to perform chemical or mechanical work, there is no certainty about the mechanism by which the energy of ATP is actually utilized. It is quite probable that the energy liberated from ATP through dephosphorylation by myosin has nothing to do with energy transformations. It seems more probable that a structural mechanism such as actin-ATP is used for this function, where the energy produced is poured directly into the protein molecule in the form of a change of molecular structure.

The adenylic acid present in phosphorylase and probably in other enzymes (KRITSKII[16]), might be the remnant of such a mechanism. The bound ADP found by PETTKÓ AND STRAUB[2] in liver and kidney might be the structural mechanism performing chemical, perhaps secretory work in these organs.

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SUMMARY

1. Globular actin contains adenosine triphosphate in a bound form, Ca ions and SH groups being perhaps responsible for the bond.

2. When actin polymerizes to the fibrous form, ATP is transformed to ADP and inorganic phosphate is formed. The change is not catalysed by any enzyme.

3. Polymerized actin contains no bound ATP. It can be depolymerized when dialysed against a very dilute ATP, in which case a globular actin is produced that contains again ATP in a bound form.

4. The ATP–ADP change is interrelated with the globular–fibrous change of the protein molecule.

5. The importance of actin-bound ATP and its change during the change of form of the actin molecule is discussed in relation to the theory of muscle contraction.

6. The probable importance of protein-bound ATP and ADP in systems other than actin is discussed.

RÉSUMÉ

1. L'actine globulaire contient de l'acide adénosinetriphosphorique (ATP) sous une forme liée; ce sont probablement les ions de Ca et les groupes –SH qui sont responsables de cette liaison.

2. Quand l'actine est polymérisée sous une forme fibreuse, l'ATP est transformé en acide adénosinediphosphorique (ADP) et un phosphate inorganique est formé. Cette transformation n'est catalysée par aucun enzyme.

3. L'actine polymérisée ne contient pas d'ATP lié. En la dialysant contre un ATP très dilué, elle peut être dépolymérisée; dans ce cas, une actine globulaire est produite, contenant de nouveau de l'ATP sous une forme liée.

4. La transformation ATP–ADP est étroitement liée à celle de la molécule protéinique passant de l'état globulaire à l'état fibreux.

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5. L'importance de l'ATP lié à l'actine et de sa transformation au cours de la modification de la molécule d'actine est discutée en relation avec la théorie de la contraction musculaire.

6. L'importance probable de l'ATP et de l'ADP liés à une protéine dans d'autres systèmes que celui de l'actine est discutée.

ZUSAMMENFASSUNG

1. Globuläres Aktin enthält Adenosintriphosphat (ATP) in gebundener Form. Die Bindung geschieht höchstwahrscheinlich durch Ca-Ionen und SH-Gruppen.

2. Während der Polymerisation von Aktin wird ATP, unter Entstehung von anorganischem Phosphat zu ADP abgebaut. Der Prozess wird nicht durch ein Enzym katalysiert.

3. Polymerisiertes Aktin enthält kein gebundenes ATP. Während der Dialyse gegen eine stark verdünnte Lösung von ATP kann es depolymerisiert werden. In diesem Falle entsteht ein globuläres Aktin, das wiederum gebundenes ATP enthält.

4. Die Umwandlung von ATP zu ADP ist mit der globulär-fibrösen Veränderung des Eiweisses eng verbunden.

5. Die Bedeutung des an Aktin gebundenen ATP und seiner Umwandlung während der Umgestaltung der Struktur des Aktins wird bezüglich der Theorie der Muskelkontraktion besprochen.

6. Die mögliche Bedeutung der Anwesenheit von eiweissgebundenem ATP und ADP in anderen Systemen wird besprochen.

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