Actin. II.*

by

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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 double 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.

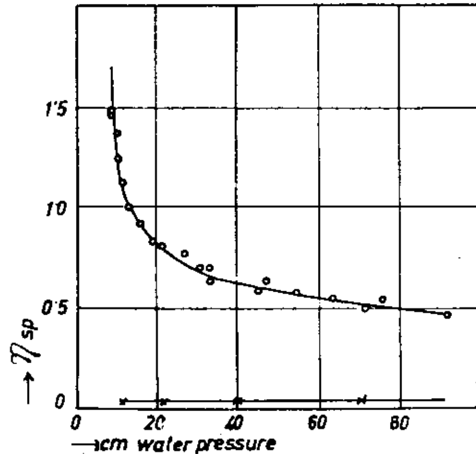

Fig. 1. Viscosity of inactive and active actin at different pressures. o——o 2 mg/ml active actin in 0,2 *M* KCl. ×——× 3 mg/ml inactive actin in dist. water at 0°.

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.

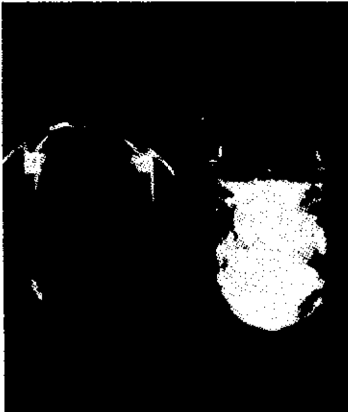

Fig. 2. Double refraction of actin solutions. Photographs taken through crossed nicols. Left: inactive actin. Right: the same solution after activation.

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 (1/4 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

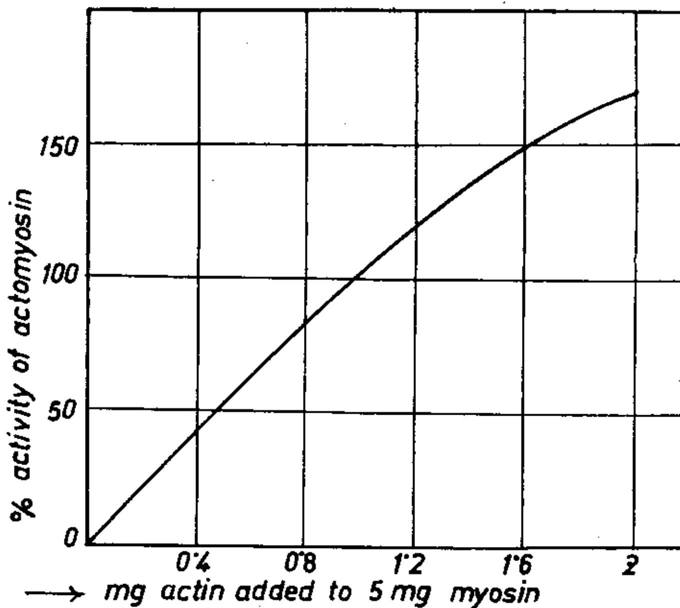

Fig. 3.

* 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.

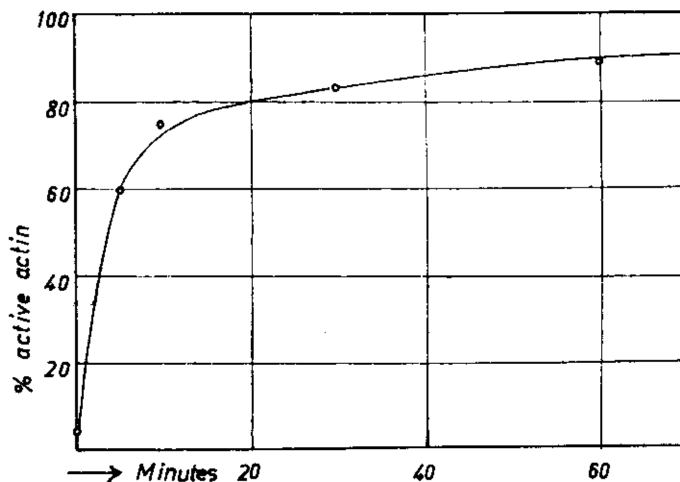

Fig. 4. Activation time curve in 0,02 *M* NaF at pH 6,5 and 35,5°.

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

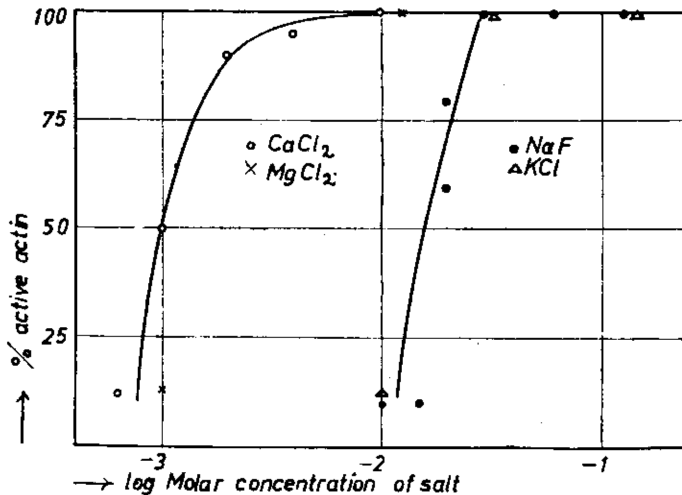

Fig. 5. Effect of concentration of salt on the activation of actin. 2,5 mg/ml actin incubated at 35.5° with salt of the concentration indicated on the abscissa. pH 7,4. The % active actin content was determined after 60 minutes incubation.

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.

References.

1. *F. B. Straub,* These studies **2,** 3 (1942).
2. *A. Szent-Györgyi,* These studies **3** (1943).