SALTING-OUT ANALYSIS OF THE ACTIN-MYOSIN SYSTEM*)

By

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Introduction

The physico-chemical properties of muscle structural protein, actomyosin, have been studied by many workers. Myosin and actin were discovered by BANGA and SZENT-GYÖRGYI[1] and by STRAUB[2] respectively, but the mechanism of the interaction between these two proteins is still in doubt.[3,4] ATP induces marked changes in the physical properties of myosin B (natural actomyosin) such as viscosity, the light-scattering and sedimentation rates. As for whether these changes are due to the deformation of myosin B or its dissociation into actin and myosin, the MORALES group[5] and the TONOMURA group[6] have recently shown that the main component of myosin B is elongated by the action of ATP. But, neither the role of actin in myosin B nor the relationship between myosin B and synthetic actomyosin (made by mixing actin and myosin) has been elucidated. Usually, actin and myosin are extracted separately from whole muscle. The extraction of actin and myosin from myosin B has also been reported. A. G. SZENT-GYÖRGYI[7] separated myosin from myosin B by fractionation with KI. He also obtained actin from the residue by eliminating contaminated myosin with cold alcohol. A. WEBER[8] subjected ATP-treated myosin B to ultracentrifugation and separated myosin in the upper layer; the lower layer was rich in actin. Furthermore, taking as the criterion the degree of viscosity drop of his synthetic actomyosin in the presence of ATP, A. G. SZENT-GYÖRGYI has presented a method for the quantitative determination of actin and for the determination of its purity. Since

*) In this paper, the following abbreviations are used: ATP=adenosine triphosphate, ATP ase=adenosine-triphosphatase, EDTA=ethylene diamine tetraacetate.

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the relationship between myosin B and synthetic actomyosin is still unknown, it is doubtful whether this method is applicable to the quantitative determination of myosin B.

In order to get a better information about the nature of the interaction between actin and myosin, and, if possible, to find out a simple method for the determination of the actin-myosin ratio in natural actomyosin, the ammonium sulfate salting-out analysis was applied in the present study under several conditions. It is the purpose of this paper to report that (1) the salting-out curves of the actomyosin-pyrophosphate system indicates no dissociation of actomyosin, and that, (2) although, in the actomyosin-KI system, some physical properties show apparent dissociation of myosin B into actin and myosin, the salting-out curves suggest still an interaction, though weak, between these two components.

Materials and Experimental Procedures

All muscle proteins were prepared from rabbit skeletal muscle.

Myosin B was obtained by extraction with the WEBER-EDSALL solution for 24 hours. The extracts were precipitated at 0.1 M KCl. They were reprecipitated once or twice at 0.25 M KCl.

Actin was extracted from acetone powder prepared by STRAUB's method with some modifications.[9] The ultracentrifugal purification described by MOMMAERTS[10] was employed only once.

Myosin was prepared according to the method described by PERRY.[11] The fractional precipitation at 0.3 M KCl was carried out to eliminate actomyosin.

Salting-out analysis has followed essentially that employed by the SNELLMAN group.[12][13] A series of solution was prepared containing 0.5 M KCl or KI*), 0.1 M K-phosphate buffer of pH 7, and increasing amounts of ammonium sulfate. To 2.5 mℓ of this solution, 0.5 mℓ of protein solution was added and the mixture was allowed to stand for 18 hours**) at 5°C. Then they were filtered. The protein concentration in the filtrate was measured by colorimetry using FOLIN's phenol reagent.[14] ***)

*) In order to avoid the I_2 formation, KI solution was added a small quantity of sodium thiosulfate so that it did not interfere the protein determination.

**) Later, it was known that this lapse of time could be reduced to 2 hours or lesser.

***) This method was used for protein determination even in the presence of ultra-violet adsorbing substance. It needs a special precaution on the inhibitory action of excess ammonium sulfate on the color development.

ATPase activity was measured in a solution containing 0.05 M Tris-maleate buffer at pH 7.0, 2 mM EDTA, 0.6 M K^+, 1.2 mM ATP, at 20°C. The reaction was discontinued by the addition of trichloroacetic acid, and the mixture filtered. The inorganic ortho-phosphate liberated in the deprotenized filtrate was measured colorimetrically according to ROCKSTEIN's method.[15] All reagents were of analytical grade.

Results

The Actomyosin-KCl System.

The salting-out curves of actin and myosin were analyzed minutely by the SNELLMAN group,[12][13] and their precipitation ranges were established under the present conditions to be 16 and 34 percent respectively. Myosin could be obtained in a possibly pure state, judging from its salting-out behaviour, but actin was difficult to be obtained in a pure state even by ultracentrifugation. A peak of precipitation often appeared at 25 percent ammonium sulfate saturation (Fig. 3). The similar peak was also observed by GELOTTE,[13] who attributed it to an "undefined" component. As mentioned later, this component seems to take no part in actomyosin.

The salting-out analysis of actomyosin was also performed by many workers, but data so detailed as in the case of actin and myosin were not reported. As was observed by YAGI[16] with pecten adductor muscle, the precipitation range of myosin B varies with conditions of its preparation. So, in the present study, it was very difficult to obtain constant samples even under a carefully controlled condition. A main peak was observed at 28 to 32 percent ammonium sulfate saturation (Figs. 1, 2). This peak is considered to be correspondent to actomyosin. In many cases, another small peak was observed at 33 to 34 percent, and this seems to correspond with myosin. Likewise, synthetic actomyosin gave a diagram similar to that of myosin B, when the relative concentration of its two components was properly changed. When the quantity of actin was relatively large, the actomyosin peak appeared near 28 percent saturation. With increase in relative concentration of myosin, the precipitation range of this peak shifted to 32 percent saturation. However, no definite relation could be obtained between the precipitation range of actomyosin peak and the quantity of myosin accompanied.

The Actomyosin-Pyrophosphate System.

The addition of 2mM sodium pyrophosphate and 1 mM $MgCl_2$ to myosin B solution produced no appreciable change on its salting-out diagram. When the concentration of pyrophosphate was increased to 10 mM, a slight decrease of the actomyosin component and a corresponding increase of the myosin component were observed (Fig. 1). But the actomyosin peak did not disappear, and the actin peak was not recognized. In some cases, small peaks precipitating at 10 to 25 percent were observed. In the other cases, as shown in Fig. 1, a slight decrease was noticed in the quantity of the actomyosin peak with a corresponding increase of the myosin peak. But these slight changes were also observed in the control experiments. Therefore, these changes are thought to be within experimental errors. Conclusively, pyrophosphate did not produce any appreciable changes on the salting-

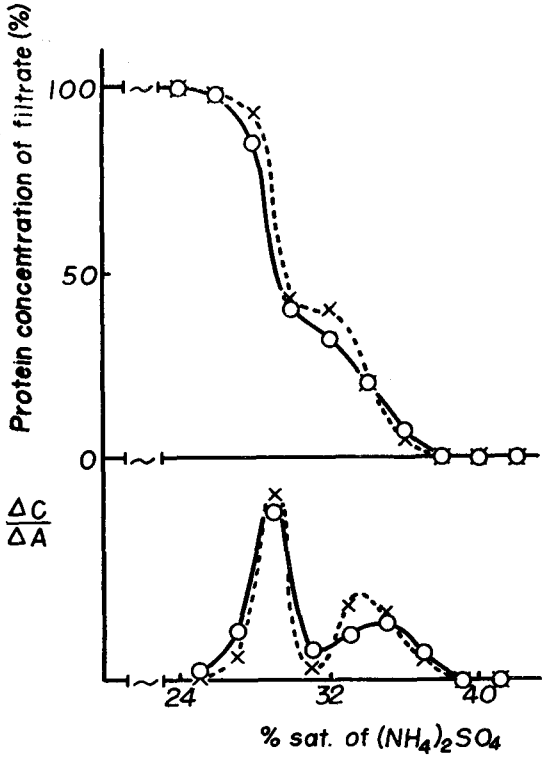

Fig. 1. Salting-out curve of myosin B in the presence of pyrophosphate.

○—○ ; control. ×—× ; pyrophosphate 10 mM + $MgCl_2$ 1 mM.

out diagram of myosin B.

The Actomyosin-KI System.

The salting-out behaviour of actin and myosin in the KI system was the very same as that in the KCl system (Fig. 3). However, the salting-out diagram of the myosin B–KI system was different from that of the KCl system (Fig. 2): the actomyosin peak did not appear at 29 percent, while only one peak was observed at 32 percent. This was correspondent to that given by an actomyosin which contained a considerably large quantity of myosin. Even in the KCl system, the sample used contained some substances precipitating before 20 percent

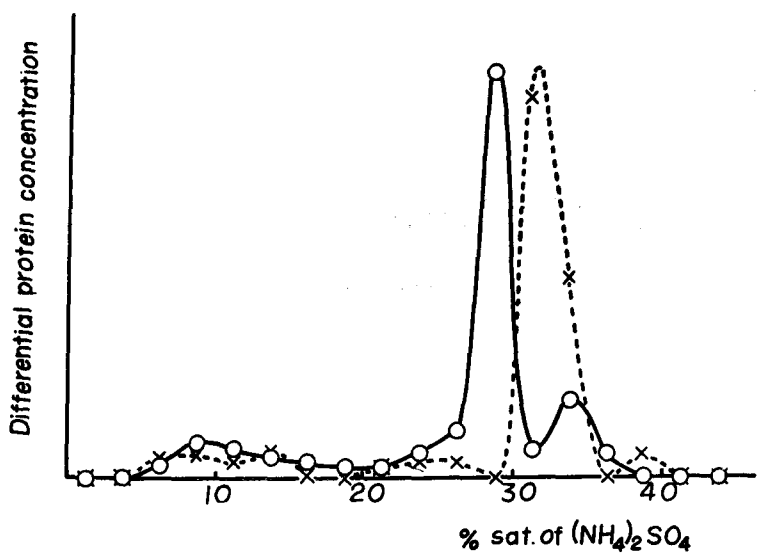

Fig. 2. Differential salting-out curves of myosin B in the KCl and KI system.
○—○ ; control. ×—× ; KI system.

ammonium sulfate saturation, but its quantity did not increase in the KI system. The actin peak was not recognized. A small peak in the KCl system which located at 25 percent remained intact in the KI system. Synthetic actomyosin having an actin-myosin ratio of about 1 : 4 showed a similar curve. Furthermore, even in the presence of a considerable amount of actin (actin-myosin ratio: about 1 : 1), only one main peak was observed (Fig. 3). This peak located at 31 percent. Generally, the larger the actin content, the less the ammonium sulfate

saturation at the precipitation range. The area of this peak was the sum of both components added. In this case also, a component precipitating at 24 percent saturation remained intact in the mixture.

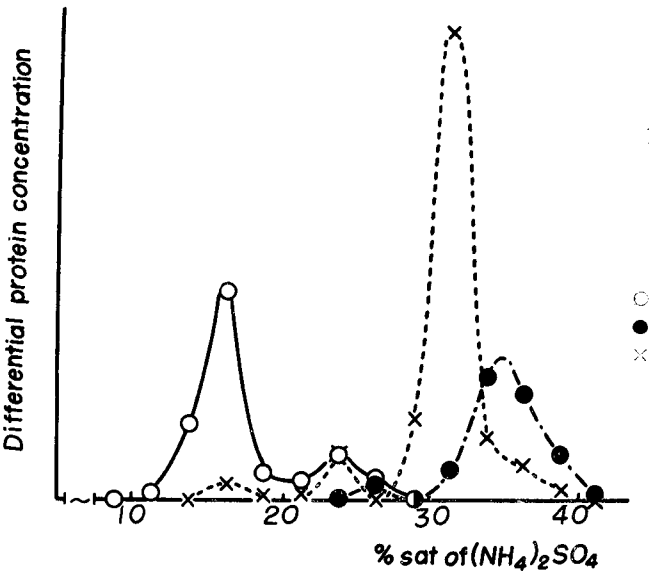

Fig. 3. Differential salting-out curves of actin, myosin and synthetic actomyosin in KI solution.

○ ○ ; actin.
●···● ; myosin.
×--× ; actin+myosin.

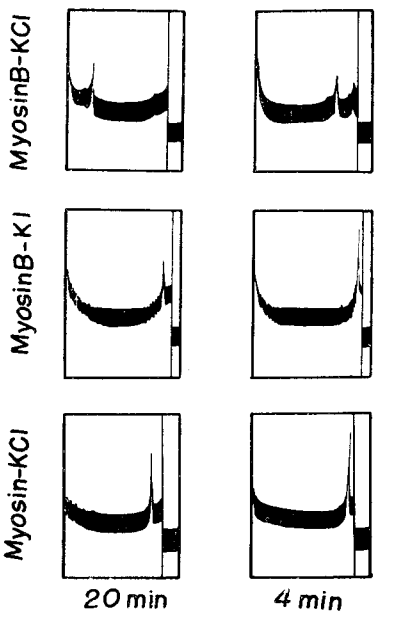

Fig. 4. Ultracentrifugal sedimentation patterns of actin-myosin system. Spinco model E, 58,780 rpm.

Upper row : myosin B inKCl, 2 mg/mℓ
Second row : myosin B in KI, 2 mg/mℓ
Third row : myosin in KI, 1.5 mg/mℓ

Ultracentrifugal Analysis.

As shown in Fig. 4, the sedimentation pattern of the myosin B–KI system was quite similar to that of the myosin B–ATP system. A similar diagram was also obtained with a synthetic actomyosin. The rapid actomyosin peak in the myosin B–KCl system disappeared and only one sharp peak was observed which sedimented with almost the same rate as that of myosin. Careful observation during the acceleration of the rotor and after the maximum speed had been reached could not reveral any other peak. That is, the peak of both F- and G-actin was not recognized.

Discussion

Before entering the discussion of the nature of the interaction between actin and myosin on the basis of their ammonium sulfate salting-out curves, some related problems should be considered.

First, the nativity of protein must be checked. For this purpose, the ATPase activity of protein in the KCl system was prealably measured. Protein was incubated under the same condition as that for salting-out analysis, except the buffer (Tris-maleate), and then diluted with the reaction mixture. EDTA was added in the reaction mixture as the activator. In parallel with this experiment, EDTA was added to the incubation mixture as the metal chelating reagent*). In both cases, no drop was noticed in the enzymatic activity of protein. Therefore, as far as the ATPase activity concerned, it should be admitted that no change occurs during the course of the salting-out analysis.

Second, the ionic effect of ammonium sulfate must be taken into consideration. Ammonium sulfate of 50 percent saturation corresponds to about 2 M. GUBA[17] reported the dissociation of actomyosin in 2 M KCl, but this seems dubious according to PORTZEHL, SCHRAMM and WEBER.[18] If any dissociation of actomyosin should be caused by the ionic effect of ammonium sulfate, the actomyosin peak would not be seen in the myosin B–KCl system, or the precipitation range of actin and myosin would be changed in the mixture, so that they unit accidentally with one another. This possibility may not be completely excluded, but it is impossible to admit that the union of the two

*) In this case the salting-out curve could not obtained since EDTA inhibit the protein determination.

different peaks always occur for different actin-myosin ratios. Therefore, in case of the KCl system, it would be reasonable to admit the existence of actomyosin complex in the reaction mixture used for salting-out analysis.

It is difficult to investigate the salting-out behaviour of the actomyosin-ATP system, since ATP is splitted by actomyosin. As is well known, like ATP pyrophosphate also induces physical changes in actomyosin sol, but it is not decomposed by the latter. In this case, therefore, actomyosin remained changed. H. MATSUMIYA *et al*[19]. reported that this change could be reversed by the decomposition of pyrophosphate present. On the basis of this fact, in the present study, the salting-out curves of actomyosin were analyzed in the presence of pyrophosphate. In some cases, the salting-out diagram of the actomyosin-pyrophosphate system revealed small peaks precipitating at 10 to 25 percent. Similar small peaks were also often observed in the control curves. Especially, the peak precipitating at 25 percent saturation remained unchanged in the KI mixture (Fig. 2, 3). The same peak was also observed in the experiment on the denaturation of myosin reported from this laboratory[20]. Therefore, this peak can be said not due to the dissociation of actomyosin. No appreciable change was recognized in many diagrams studied. If this experimental fact can not furnish a decisive counter-evidence against the dissociation theory, it would at least support the conclusion in a subsequent paper that actomyosin does not dissociate in the presence of pyrophosphate.

A. G. SZENT-GYÖRGYI separated actin and myosin from myosin B using KI. Since the salting-out curves of the actomyosin-pyrophosphate system proved to be inadequate for the quantitative estimation of the actin content in myosin B, the salting-out curves of the KI system was analyzed for this purpose. Contrary to the expectation of the author, the salting-out diagram revealed only one peak and its quantity was the sum of both components added, the precipitation ranges fluctuating to some extent according to the actin-myosin ratios. With the increase of the actin content, the precipitation range shifted to the areas low in ammonium sulfate concentration. These results would suggest the existence of a weak interaction between actin and myosin. Since F-actin is known to depolymerize in the presence of KI[7], the new peak in the actomyosin-KI system might be due to the possible formation of myosin-G-actin complex.

In the case of the actomyosin-ATP system, however, there are

many experimental results in support of the dissociation theory. The viscosity drop of actomyosin sol induced by the action of ATP was thought by the Szeged school to indicate the dissociation of this complex protein, since the reduced relative viscosity of the mixture is an additive property of both components.[21)22)] The facts that, in paralled with the decrease in viscosity, flow-birefringence[23)] and turbidity[24)] also diminish, and that the myosin component reappears in the ultracentrifugal pattern has been reported in favour of the dissociation theory. However, as will be mentioned in a subsequent paper, it would be reasonable to consider that, in this case, the main part of the complex is elongated and only a small part dissociates. Further, it may not be denied that, in the actomyosin-KI system, the interaction between actin and myosin is weakened to some extent, since A. G. SZENT-GYÖRGYI has separated actin with this system. However, in this case also, the author is of opinion that, as mentioned above, the main action of KI on actomyosin consists not in the dissociation of the latter into actin and myosin, but in its depolymerization into myosin-G-actin complex.

Summary

The salting-out diagram of the actomyosin-pyrophosphate system did not produce any appreciable change.

Although, in the actomyosin-KI system, some physical properties strongly suggest the dissociation of actomyosin, the salting-out diagram showed the remaining of an interaction, though weak, between actin and myosin. These results were discussed, regarding the method of salting-out analysis. In connection with the conclusion in a subsequent paper, the mechanism of salting-out of the actomyosin-KI system was also discussed.

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References

1) I. BANGA and A. SZENT-GYÖRGYI, Stud. from Inst. Med. Chem. Univ. Szeged **1**, 5 (1941).
2) F. B. STRAUB, Stud. from Inst. Med. Chem. Univ. Szeged **3**, 23 (1943).
3) T. C. TSAO, Biochim. et Biophys. Acta **11**, 236 (1953).
4) J. J. BLUM and M. F. MORALES, Arch. Biochem. and Biophys. **43**, 208 (1953).
5) P. H. HIPPEL, M. F. GELLERT and M. F. MORALES, Conference on the Chemistry of Muscular Contraction, 1957, Tokyo.
6) T. NIHEI and Y. TONOMURA, J. Biochem., in press; Y. TONOMURA and F. MORITA, J. Biochem., in press.
7) A. G. SZENT-GYÖRGYI, J. Biol. Chem. **192**, 361 (1951).
8) A. WEBER, Biochim. et Biophys. Acta **19**, 345 (1956).
9) H. MATSUMIYA and S. ASAKURA, *Protein, Nucleic Acid and Enzyme*, Vol. 2, No. 4 (1957).
10) W. F. H. M. MOMMAERTS, J. Biol. Chem. **198**, 445 (1952).
11) S. V. PERRY, in Methods in Enzymology, ed. by S. P. COLOWICK and N. O. KAPLAN, Vol. II, JOHNS HOPKINS Press, Baltimore, 1955, p. 582.
12) M. TENOW and O. SNELLMAN, Biochim. et Biophys. Acta **15**, 395 (1954).
13) B. GELOTTE, "Activin, a low-molecular-weight substance in the contractile element of muscle" (Thesis), Almqvist and Wiksell, Uppsala, 1954.
14) O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, J. Biol. Chem. **193**, 265 (1951).
15) M. ROCKSTEIN and P. W. HERRON, Anal. Chem. **23**, 1500 (1951).
16) K. YAGI, J. Biochem. **44**, 337 (1957).
17) F. GUBA, Stud. from Inst. Med. Chem. Univ. Szeged **3**, 40 (1943).
18) H. PORTZEHL, G. SCHRAMM and H. H. WEBER, Z. Naturforschung **5b**, 61 (1950).
19) H. MATSUMIYA, F. MORITA, S. KITAGAWA, K. YAGI and Y. TONOMURA, J. Biochem. **44**, 347 (1957).
20) T. YASUI, T. FUKUZAWA, Y. HASHIMOTO, S. KITAGAWA and A. T. SASAKI, J. Biochem. **45**, 717 (1958).
21) H. H. WEBER, Biochim. et Biophys. Acta **4**, 12 (1950).
22) W. F. H. M. MOMMAERTS, Experimental Cell Research **2**, 133 (1951).
23) M. DAINTY, A. KLEINZELLER, A. S. C. LAWRENCE, M. MIALL, J. NEEDHAM, D. M. NEEDHAM and S. C. SHEN, J. Gen. Physiol. **27**, 355 (1944).
24) W. K. JORDAN and G. OSTER, Science **108**, 188 (1948).
25) P. JOHNSON and R. LANDOLT, Nature **165**, 430 (1950).