Influence of pH and Temperature on Properties of Myosin A in Glycerol-Treated Fiber Bundles

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The effect of pH and temperature on the denaturation of myosin A in glycerol-treated fiber bundles prepared from rabbit psoas muscle was investigated by measurements of adenosine-triphosphatase activity and extractability with modified Hasselbach-Schneider solution. Myosin A in glycerol-treated fiber bundles, when placed in the medium at 3° and pH 5.5, shows little change either in its ATPase activity or in its extractability, whereas isolated myosin A and B under the same condition undergoes denaturation. When heated at 25° and pH 7.0, extractability of myosin A in the glycerol-treated fiber bundles becomes progressively less, and the properties of the extracted myosin A do not differ much from the ones before heating. The rate of the decrease in extractability is, however, much slower than that in the enzmyic activity of isolated myosin A under the same condition.

Hashimoto *et al.* (1959) reported that the quality of meat products depended greatly on the state of myosin in the raw material. Fukazawa *et al.* (1961) indicated that myosin A was an essential component of muscle in the development of the water-holding capacity of meat. In the field of muscle biochemistry, myosin A, which occupies more than 50% of the myofibrillar protein in the skeletal muscle of vertebrates, is known to play an important role as part of the contractile machine in living muscle. It is of interest that the essential protein for muscle contraction in living muscle still holds its importance in dead muscle (meat) as an essential factor for maintaining the preferable quality of meat and meat products. It is, therefore, evident that information on the denaturation of myosin A under various conditions will give a fundamental understanding on how to control the quality of meat and meat products.

The isolated myosin A has been known to be unstable and its enzymic activity easily destroyed by various factors (Blum, 1960; Ljubimova and Engelhardt, 1939; Ouellet *et al.*, 1952; Pelletier and Ouellet, 1961; Penny, 1967; Shikama, 1963; Takahashi *et al.*, 1962; Yasui and Hashimoto, 1966; Yasui *et al.*, 1958, 1960). At a fixed concentration of KCl, the denaturation of myosin A has been shown to depend on temperature and pH, and consisted of pH-independent thermal denaturation and temperature-independent acid or alkaline denaturation (Pelletier and Ouellet, 1961; Penny, 1967; Takahashi *et al.*, 1962; Yasui *et al.*, 1958, 1968). The latter reaction has been found to proceed even at low temperatures (Takahashi *et al.*, 1962; Yasui and Hashimoto, 1966).

In normal muscle, with an initially high glycogen content, the pH falls from about 7.0 to 5.4 during the course of rigor, but the rate of fall is usually so slow that the muscles on a carcass will have cooled well below body temperature before the ultimate value is attained. Despite the fact that the internal environment of the cell of the dead muscle favors the occurrence of denaturation of myosin A, it is such muscle that produces various meat products of acceptable qualities.

It has been accepted that the addition of F-actin to myosin A stabilizes myosin-ATPase, owing to the formation of actomyosin complex. The morphology of actomyosin complex in solution or in suspension is, however, entirely different from that in muscle *in situ; i.e.*, the former being the arrowhead structure and the latter being well-defined thick and thin filaments (Huxley, 1963).

To learn what happens to myosin A in the intact muscle during rigor, it is desirable to use a highly organized muscle model whose structural feature is similar to that of the intact muscle. For this reason, it was decided as a first attempt to study the denaturation of myosin A in glycerol-treated fiber bundles in which the morphological features are maintained as they were in the intact muscle (Huxley, 1963; Reedy *et al.*, 1965).

In this study, the effect of pH at low temperature and temperature at neutral pH during storage on the denaturation of myosin A in glycerol-treated fiber bundles, which are fixed to application rods at rest length, has been investigated by using extractability with modified Hasselbach-Schneider solution and adenosinetriphosphatase (ATPase) activity of the extracted myosin A as criteria. The results indicated that myosin A in glycerol-treated fiber bundles was far more stable at pH 5.5 and 3° than isolated myosin A, and was fairly stable at pH 7.0 and 25 and 35°, compared with the isolated ones, suggesting that the protein organized in the specific architecture of muscle cells was stabilized and became more resistant to denaturation than isolated molecules under severe environmental conditions.

MATERIALS AND METHODS

Reagent. The disodium salt of ATP (Kyowa Hakko Co., Japan) was used throughout these experiments. Its concentration was determined by absorption at 260 mμ in a Hitachi spectrophotometer. All inorganic and organic salts except for ATP were commercial products of the best reagent grade available.

Myosin A. Myosin A was prepared from rabbit leg and back muscle according to the method described by Perry (1957). In this paper this is referred to as "isolated myosin A."

Myosin B (Natural Actomyosin). Myosin B used was extracted from rabbit leg and back muscle for 24 hr with Weber-Edsall solution and purified by triple precipitation at 0.1 or 0.2 *M* KCl and dissolution in 0.6 *M* KCl (Szent-Györgyi, 1951). In this paper this preparation is called "isolated myosin B."

Glycerol-Treated Fiber Bundles. A fiber bundle about 1–1.5 mm in diameter was separated from rabbit psoas muscle and tied at both ends with strings to a bamboo stick (1 mm in diameter) which had been washed twice in boiling double-distilled water before use. Then the muscle was cut out; thus the fiber bundle was fixed to the stick at rest length. The fiber bundles fixed to the rod were placed in 50% glycerol solution containing 20 m*M* phosphate buffer (pH 7.0) at 0°. The next day the fluid was exchanged for fresh and precooled 50% glycerol solution with the buffer, and then it was transferred to the freezer at −20° and stored for approximately 3 months or longer before use.

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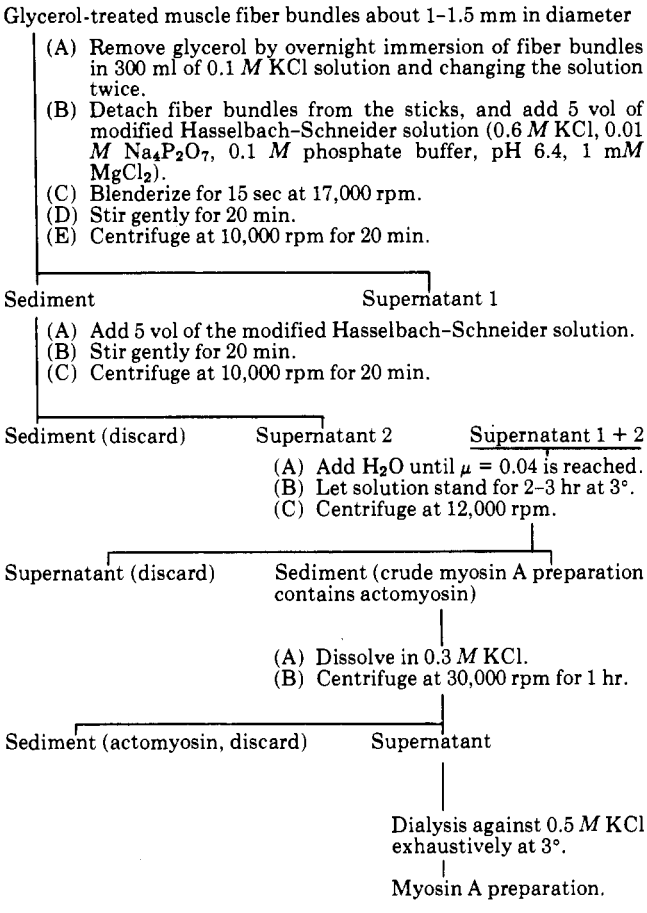

Figure 1. Extraction of myosin A from glycerol-treated fiber bundles.

Extraction of Myosin A from Glycerol-Treated Fiber Bundles. The extraction procedure is shown in Figure 1 and this myosin A preparation is designated extracted myosin A from (glycerol-treated) fibers.

Huxley and Hanson (1957) and Hanson and Huxley (1957) succeeded in selective extraction of A-band material (myosin A) from glycerol-treated muscle fiber, and their results were shown qualitatively by using interference microscopy and chemical analysis. However, the myosin A fraction thus obtained demonstrated the superprecipitation phenomenon at low ionic strength and in the presence of ATP (Figure 2), indicating the presence of a small amount of F-actin in the preparation. This contaminated F-actin which actually formed actomyosin in the preparation could be eliminated by centrifuging the crude preparation in 0.3 *M* KCl at 30,000 rpm for 1 hr (Figure 1) and the resulting preparation no longer exhibited a sign of superprecipitation upon addition of ATP (Figure 2). Turbidimetric test for superprecipitation has been the reading of absorbance changes in myosin A suspension upon addition of ATP. This was done in 1-cm cells, in the optical path of the Hitachi EP 2-type spectrophotometer, at 25°. The electrolyte medium is specified in the legend of Figure 2.

The average amount of extracted myosin A was 17 ± 2.2% (standard deviation) of the total protein in glycerol-treated fiber bundles, which was considerably less than the value (37% of total protein in glycerol-extracted muscle) reported by Hanson and Huxley (1957). However, it can be reasonably considered that the procedure to remove actomyosin as shown in Figure 1 was responsible for this difference between the two values.

To extract myosin A from myofibrils prepared from fresh skeletal back and leg muscle and psoas muscle, the following two methods were employed.

Method I. Samples were ground once with a laboratory grinder. About 10 g of each mince was blenderized for 15 sec at 17,000 rpm with 5 vol of 0.1 *M* KCl containing 39 m*M* borate buffer (pH 7.1) and 5 m*M* EDTA. The blenderized residue was collected by cetrifugation (3°) at 3000 rpm for 15 min. The sediment was washed again with the above solution and centrifuged under the same condition. About 70% of the myofibrils thus obtained were in a relaxed state when examined under the light microscope. The sediment was dispersed in 5 vol of modified Hasselbach-Schneider solution and the subsequent procedure was carried out according to that indicated in Figure 1.

Method II. The muscle mince was blenderized for 15 sec at 17,000 rmp with 5 vol of 0.1 *M* KCl. The residue was collected and washed as in the case of Method I with 0.1 *M* KCl. Almost all myofibrils obtained by this procedure were found to be in a contracted state under the microscope. The residue was dispersed in 5 vol of modified Hasselbach-Schneider solution and the subsequent procedure was the same as in Method I.

Measurement of ATPase Activity. The ATPase activity was measured at 25° in the presence of 0.5 *M* KCl, 5 m*M* $CaCl_2$, 20 m*M* tris-maleate buffer (pH 7.0), and 1 m*M* ATP. The reaction mixture was incubated at measured intervals of time, and the reaction was stopped by the addition of 10% trichloroacetic acid. The phosphate liberated was measured by the method of Martin and Doty (1949), using a Hitachi EPU-2A spectrophotometer.

Ultracentrifugal Analysis. Sedimentation studies were performed in a Hitachi UCA-1A ultracentrifuge at 55,430 rpm and 20°. After the maximum speed of rotation was reached, photographs were taken at regular time intervals.

Protein Concentration. Protein concentration was determined by the micro-Kjeldahl method or the biuret procedure standardized with Kjeldahl nitrogen determination. Ultraviolet absorption at 280 mμ ($OD_{1cm}^{1\%}$ 5.5) was also employed. The protein preparation was stored in a refrigerator maintained at 0° for no longer than 2 weeks before use.

Denaturation. The denaturation of isolated myosin A and B was studied by incubating a solution of the enzyme in a buffer of the required pH (5.5 and 7.0), ionic strength (μ = 0.1 and 0.5), and temperatures (3, 25, and 35°) and following its loss of activity with time (Yasui *et al.*, 1968). The denaturation of myosin A in the glycerol-treated fiber bundles was studied by incubating fiber bundles fixed to application rods from which glycerol had been removed by overnight immersion in 0.1 *M* KCl solution as described in Figure 1, in a buffer of the required pH (5.5 and 7.0) and temperatures (3, 25, and 35°) at μ = 0.1.

At measured intervals of time, fiber bundles were cut out of the sticks and myosin A was extracted by following the procedure shown in Figure 1. Changes in ATPase ac-

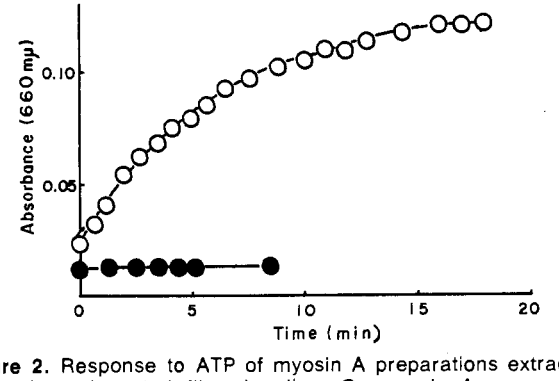

Figure 2. Response to ATP of myosin A preparations extracted from glycerol-treated fiber bundles. O, myosin A preparation which contains actomyosin shown in Figure 1; ●, myosin A preparation shown in Figure 1. Reaction mixture: 0.05 *M* KCl, 1.0 m*M* $MgCl_2$, 0.1 m*M* $CaCl_2$, 20 m*M* Tris-maleate (pH 7.0), 0.5 mg/ml of protein, and 1 m*M* ATP at 25°.

tivity, extractability, and qualitative hydrodynamic property by ultracentrifugal analysis of each sample were then examined. To study the effect of pH and temperature separately, acid denaturation was performed at pH 5.5 and 3° since at this temperature the rate of thermal denaturation was found to be very slow and only temperature-independent acid denaturation proceeded (Takahashi *et al.*, 1962; Yasui and Hashimoto, 1966). Contrary, when the pH was in the neutral region, the rate constants of the denaturation were proportional to zero power of $[H^+]$, and only pH-independent thermal denaturation played a dominant role (Takahashi *et al.*, 1962). The effect of temperature on the denaturation, therefore, was carried out at pH 7.0 and 25 and 35°. In the case of incubation at high temperatures such as at 25 and 35°, sodium dehydroacetate (DHA) of a final concentration of 2 m*M* was added to inhibit bacterial growth. The reagent in this amount had no known influence on the properties of myosin A (Yasui *et al.*, 1960).

All samples for initial activity measurements were preincubated for 5 min under the conditions mentioned above, unless otherwise noted.

RESULTS

Effect of Storage of Fiber Bundles in 50% Glycerol Solution on the ATPase Activity of the Extracted Myosin A. When fiber bundles were stored in 50% glycerol solution and myosin A was prepared from the fiber bundles as in Figure 1, ATPase activity of the prepared myosin A maintained a constant value over 14 weeks storage (Figure 3). It should be mentioned that the specific activity of the enzyme (0.2–0.3 μmol of Pi/min/mg of protein) was always found to be lower than that reported on myosin A isolated from rabbit skeletal muscle (0.3 to 0.4 μmol of Pi/min/mg of protein). This difference may be attributed to the source of the muscle used as materials, though it still leaves the question unanswered as to whether or not glycerol treatment affects the ATPase activity.

Direct extraction of myosin A from myofibrils prepared from fresh skeletal back and leg muscle and psoas muscle was performed to compare the ATPase activities of myosin A from different muscles. As shown in Table I, the activity of myosin A from the mixture of leg and back muscle always showed higher values (which agreed with those routinely observed in our laboratory) than that of the psoas muscle, which was very close to the values indicated in Figure 3. It is to be noted that the state of myofibrils before extraction (*i.e.*, contraction or relaxation of myofibrils) exerts little influence over ATPase activities of myosin A from back and leg muscle and from psoas muscle. It may, therefore, be concluded that lower activity of myosin A from psoas muscle cannot be ascribed to the glycerol treatment, but to the psoas muscle itself.

Changes in ATPase Activity and Extractability of Myosin A during Storage of Glycerol-Treated Fiber Bundles at pH 5.5 and 3°. As shown in Figure 4, the inactivation of isolated myosin A-ATPase proceeded according to the first-order law until the activity disappeared, and it was found that myosin A at high ionic strength (0.5 *M* KCl) denatured more rapidly than at low ionic strength

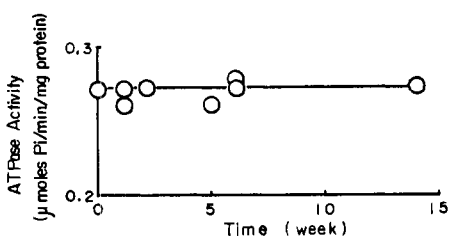

Figure 3. Effect of storage of fiber bundles in 50% glycerol solution at pH 7.0 and −20° on the ATPase activity of extracted myosin A.

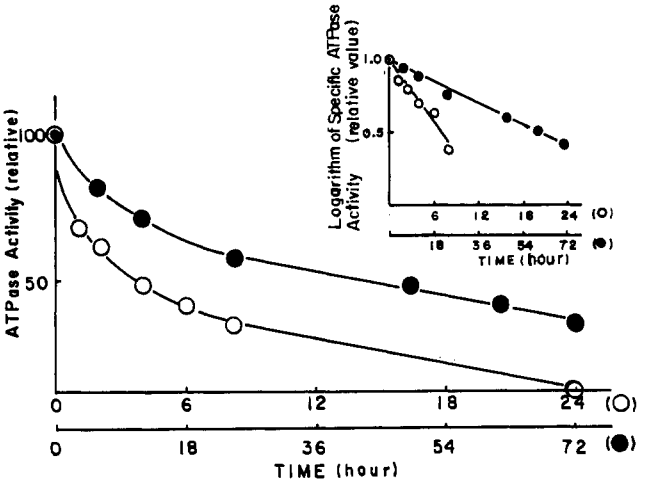

Figure 4. Changes in ATPase activity of isolated myosin A during storage at pH 5.5 and 3°. Incubation: 0.5 *M* (O) and 0.1 *M* (●) KCl, 10 m*M* Tris-maleate buffer (pH 5.5), and 1 mg of protein/ml. Reaction mixture for ATPase assay: 0.5 *M* KCl, 5 m*M* $CaCl_2$, 50 m*M* Tris-maleate (pH 7.0), and 0.2 mg of protein/ml at 25°. Inset: logarithm of the activity of myosin A as a function of time.

(0.1 *M* KCl). The apparent first-order rate constants of inactivation were 4.7×10^{-5} sec^{-1} and 3.9×10^{-5} sec^{-1} in 0.5 *M* KCl and 0.1 *M* KCl, respectively.

On the other hand, ATPase activity of isolated myosin B decreased similarly to that of myosin A to about 70% of its initial value during the stirring at pH 5.5 and 3°, and maintained the constant value thereafter (Figure 4). The loss of Ca^{2+}-activated ATPase activity of myosin B has long been studied by many (Pelletier and Ouellet, 1961; Penny, 1967; Yasui *et al.*, 1958, 1968). Yasui *et al.* (1968) pointed out that the reaction departed from the typical first-order behavior in that the rate decreased as the reaction proceeded. The extent of this effect varied greatly with the amont of F-actin added to the system and slightly with pH and ionic strength. The results shown in Figure 5 were consistent with those reported by Yasui *et al.* (1958, 1968).

The loss of ATPase activity of myosin A extracted at regular time intervals from glycerol-treated fiber bundles incubated in the same denaturing medium was found to be very slow, and about 90% of its initial activity still remained even after 72 hr of storage (Figure 5). As shown in Figure 6, the loss of extractability of myosin A indicated a similar trend to that of ATPase activity. It was not clear, however, whether the mechanism through which inactivation of ATPase occurred was the same as that for the decrease in extractability of myosin A from the glycerol-treated fiber bundles.

Changes in ATPase Activity and Extractability of Myosin A under Thermal Treatment of Glycerol-Treated Fiber Bundles. In Figures 7 and 8 inactivation curves of myosin-ATPase prepared by different methods during the storage at pH 7.0, μ = 0.1, and at 25° (Figure 5) and 35° (Figure 6) are illustrated. The inactivation of isolated myosin A proceeded until its enzymatic activity disappeared, while that of myosin B proceeded as rapidly as

Table I. Specific ATPase Activity[a] of Myosin A from Skeletal and Psoas Muscle

	Skeletal muscle	Psoas muscle
Method I[b]	0.32, 0.30	0.21, 0.25
Method II[c]	0.37, 0.35	0.24, 0.25, 0.26

[a] Activity = μmol of Pi/min/mg of protein. [b,c] See Materials and Methods section.

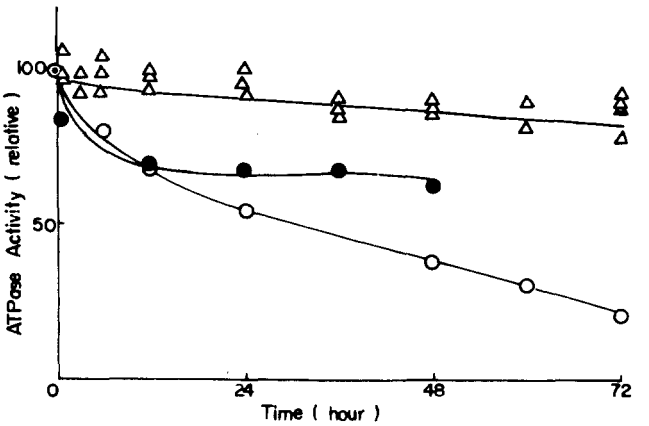

Figure 5. Changes in ATPase activity during storage in 0.1 *M* KCl at pH 5.5 and 3° of isolated myosin A (○), myosin B (●), ad myosin A extracted from glycerol-treated fiber bundles (△). Conditions for incubation and ATPase assay were the same as in Figure 4, except that the fiber bundles fixed to the application rods were placed in the incubation mixture.

myosin A at the beginning of the reaction, and the curves showed a definite tendency to approach linearity after the initial curved portion. The results are consistent with those already reported in earlier studies (Yasui *et al.*, 1958, 1968).

It was rather striking that myosin A extracted from glycerol-treated fiber bundles incubated under the same conditions indicated a great stability against thermal treatment. After 72 hr of incubation, ATPase activity still exhibited about 85% of the initial activity at 25° and about 80% even at 35° (Figures 7 and 8). On the other hand, unlike incubation at low pH value and temperature, the extractability of myosin A from glycerol-treated fiber bundles decreased with the length of time of incubation (Figure 6), though the rate of decrease in extractability was much slower than that of inactivation of isolated myosin A-ATPase; *viz.*, half-life time of the former being 60 hr at 25° and 48 hr at 35°, but that of the latter being 10 hr at 25° and 1.3 hr at 35°. Thus, it can be presumed that the denaturation of isolated myosin A examined was not directly associated with the decrease in extractability of myosin A located in the glycerol-treated fiber bundles, and that the rate of denaturation of myosin A in the fiber bundles was much slower than that of myosin A in solution or suspension. It is, therefore, likely that the major part of myosin A extractable from the fiber bundles was

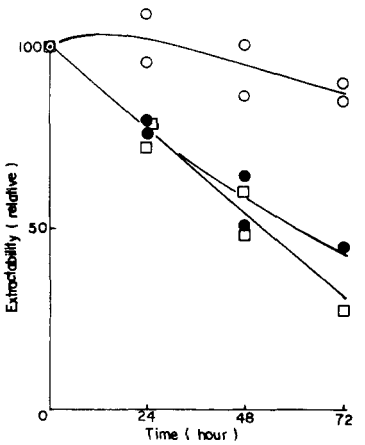

Figure 6. Change in extractability of myosin A extracted from glycerol-treated fiber bundles during storage at pH 5.5 and 3° (○), pH 7.0 and 25° (●), and pH 7.0 and 35° (□). The fiber bundles fixed to the application rods were placed in the incubation mixture. Extraction was performed according to the procedure indicated in Figure 1.

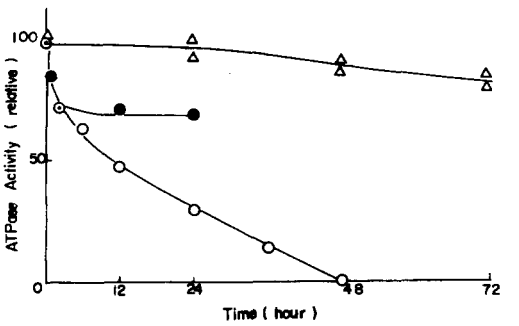

Figure 7. Changes in ATPase activity during storage in 0.1 *M* KCl at pH 7.0 and 25° of isolated myosin A (○), myosin B (●), and myosin A extracted from glycerol-treated fiber bundles (△). Conditions for incubation and ATPase assay were the same as in Figure 5, except pH and temperature.

composed of the native molecules even after 72 hr of storage of fiber bundles at 25° and pH 7.0.

Ultracentrifugal Analyses. There are suggestions that a loss of ATPase activity was accompanied with aggregation (Yasui *et al.*, 1958, 1960). Although the aggregation process was slow in rabbit skeletal muscle myosin A at 0°, the process was accelerated by thermal treatment, for the fact that peaks sedimented faster than the myosin monomer was recognized in the sedimentation pattern after 1–2 hr of incubation at 25°. After limited exposure to this temperature, Lowey and Holtzer (1957) and Johnson and Rowe (1961) have reported that the $S^0_{20,w}$ of aggregated material was 10.0 and this value was in good agreement with the calculated value for a dimer formed by side to side aggregation. An attempt was then made to investigate, by using an ultracentrifugation, the difference between the myosin A samples extracted from glycerol-treated fiber bundles before and after incubation at pH 5.5, μ = 0.1 and 3°, or at pH 7.0, μ = 0.1 and 25°. By the ultracentrifugal analysis, the degree of aggregation could be measured more unequivocally than by any other technique, since shape factors and the presence of a small amount of very large aggregates do not interfere as in light scattering.

As illustrated in Figure 9, little difference can be observed between the myosin A samples extracted from the fiber bundles before and after incubation, and the extracted myosin A revealed a sharp single peak without a sign of aggregation of high molecular weight. Sedimentation coefficients for myosin A samples examined are listed in Table II. All values are in the same order of magnitude, 5.33–

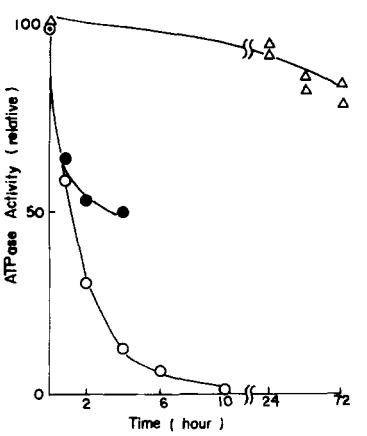

Figure 8. Changes in ATPase activity during storage in 0.1 *M* KCl at pH 7.0 and 35° of isolated myosin A (○), myosin B (●), and myosin A extracted from glycerol-treated fiber bundles (△). Conditions for incubation and ATPase assay were the same as in Figure 4, except pH and temperature.

Table II. Sedimentation Coefficients of Myosin A Extracted from Glycerol-Treated Fiber Bundles before and after Incubation

Sample no.[a]	$S_{20,w}$	ATPase activity[b]
1	5.36	0.28
2	5.60	0.29
3	5.33	0.28
4	5.60	0.27

[a] 1. Extracted myosin A before incubation. Protein concentration was 2.0 mg/ml. 2. Extracted myosin A before incubation. Protein concentration was 1.7 mg/ml. 3. Extracted myosin A after 72 hr of incubation at pH 5.5 and 3°. Protein concentration was 2.0 mg/ml. 4. Extracted myosin A after 72 hr of incubation at pH 7.0 and 25°. Protein concentration was 1.7 mg/ml. [b] ATPase activity = μmol/min/mg of protein.

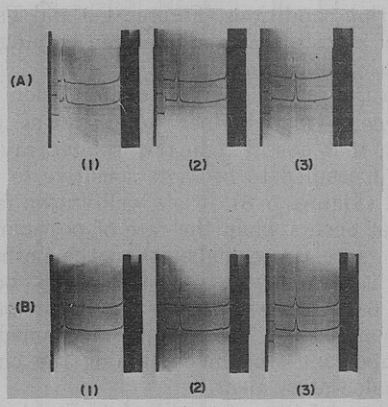

Figure 9. Sedimentation of myosin A extracted from glycerol-treated fiber bundles before and after 72 hr of incubation. (A) Plain: 2 mg/ml of myosin A in 0.5 *M* KCl extracted from glycerol-treated fiber bundles of psoas muscle. Wedge: 2 mg/ml of myosin A in 0.5 *M* KCl extracted from glycerol-treated fiber bundles of psoas muscle incubated for 72 hr in 0.1 *M* KCl and 20 m*M* Tris-maleate (pH 5.5) at 2–3°. Pictures were taken at 15(1), 39(2), and 72(3) min after the speed of 55,430 rpm was reached at 20°. Angle was 65°. (B) Plain: 1.7 mg/ml of myosin A in 0.5 *M* KCl extracted from glycerol-treated fiber bundles of psoas muscle. Wedge: 1.7 mg/ml of myosin A in 0.5 *M* KCl extracted from glycerol-treated fiber bundles of psoas muscle incubated for 72 hr in 0.1 *M* KCl, 2 m*M* DHA, and 20 m*M* Tris-maleate (pH 7.0) at 25°. Pictures were taken at 12(1), 39(2), and 57(3) min after the speed of 55,430 rpm was reached at 20°. Angle was 65°.

5.60, irrespective of the pretreatment of the fiber bundles. The plot of $1/S_{20,w}$ against concentration of myosin A gives a nearly linear function, and the following equation was proposed (Godfrey and Harrington, 1970): $1/S_{20,w} = 0.165 + 0.0169\,Ci$ (mg/ml).

The values in Table II fall in line with that of native myosin A already reported, when we intrapolate them to the curve of $1/S_{20,w}$ *vs.* protein concentration described above. Furthermore, ATPase activity of myosin A extracted from the fiber bundles showed little change either before or after incubation in the denaturation medium (Table II).

DISCUSSION

Our investigations have confirmed the difference in specific activity between myosin A samples extracted from rabbit skeletal muscle consisting of leg and back muscle and the one from rabbit psoas muscle (Table I). Recent studies (Barany, 1967; Barany *et al.*, 1965) have established that the skeletal muscle can be classified into two types, red (slow) and white (fast) muscles, and that the specific ATPase activity of myosin A from the former is, generally, lower than that from the latter. Our results, therefore, may stem from the fact that the ratio of red fibers to white fibers in psoas muscle was higher than that in the mixture of back and leg muscles.

Nevertheless, it was of interest that the ATPase activity of myosin A in glycerol-treated muscle fiber was found to keep its initial activity unchanged over 3 months of storage in 50% glycerol solution at pH 7.0 and −20° (Figure 3), thus reconfirming the importance of glycerol-treated muscle fibers as muscle model.

It has been tacitly recognized that contractile proteins in muscle model such as glycerol-treated fiber bundles were relatively stable in comparison with the isolated myosins. The results shown in Figures 3–8 present the clear-cut evidence for this, as far as myosin A in the fiber bundles was concerned. The myosin A extracted from the fiber bundles at certain time intervals under the denaturing conditions behaved quite differently from the isolated myosin A and B in solution or suspension (Figures 4, 5, 7, and 8).

As reported by Takahashi *et al.* (1962) and Yasui *et al.* (1958), at neutral pH region the effect of temperature-dependent denaturation was dominant, while at low pH and temperature, temperature-independent acid denaturation played a dominant role. From this point of view, it was presumed that the data shown in Figures 5 and 6 correspond with acid denaturation and those in Figures 6–8 correspond with thermal denaturation.

Myosin A in the fiber bundles appeared to be stable at pH 5.5 and 3° and showed a slight decrease in its extractability as well as in ATPase activity, even after 72 hr of storage. It was, however, fairly sensitive to temperature, and its extractability decreased to 30–40% of its initial level after 72 hr of storage at 25 and 35° (Figure 6). The specific ATPase activity of extractable myosin A, in this case, maintained more than 80–85% of its initial value (Figures 7 and 8), showing a sharp contrast to changes in activity of the isolated myosin A and B studied under the same experimental conditions.

The extractability, in M-KCl, of the actomyosin from pale watery meat was less than 50% of that meat allowed to go into rigor at lower temperatures (Bendall and Wismer-Pedersen, 1962). It has been suggested that this arose because of the deposition of denatured sarcoplasmic proteins on the myofilaments, without necessarily involving the denaturation of actomyosin itself (Bendall and Wismer-Pedersen, 1962). Later, Scopes (1965) showed that in watery meat most of the proteins retained their native form except for the specific protein.

Although this idea was an attractive hypothesis, there still remained the question whether or not the myosin B and myosin A of watery meat were denatured. Unfortunately this cannot be directly investigated by heating the washed myofibrils (sacoplasm-free) under identical conditions, because the fibrils then tend to clump together into ill-defined aggregates of different sizes. Penny (1967) studied the denaturation of myosin A itself under the conditions of temperature and pH encountered in practice, that is, pH from 5.3 to 6.1 and temperatures from 33 to 43°. He found that the loss of ATPase activity and solubility were both first-order and pH-dependent reactions. Myosin A, however, became insoluble only when heated within a narrow range of pH. Nevertheless, it was not possible to say from Penny's experiments how myosin A would behave in the muscle.

Since our experiments have been designed to extract myosin A itself from the fiber bundles from which a considerable amount of sarcoplasmic proteins had been eliminated, and to study temperature-independent acid denaturation and pH-independent thermal denaturation separately, it may be said that results presented here can help develop the information bridging the gap between investigations on the biochemistry of muscle during the rigor and on the denaturation of myosin *in vitro*. In addition, the results in this paper indicate that only the native form of myosin A is extractable from the fiber bundles exposed to

the denaturing conditions (Figures 4-9 and Table II). The condition of myosin A in the fiber can be judged by investigating the loss of extractability.

Although the rate of thermal denaturation of myosin A in the glycerol-treated fiber bundles was considerably slower than that observed in the isolated myosin A (Figures 6-8), it seemed to be more sensitive to temperature than to pH (Figures 5-8). It is a well-known fact that pale watery meat occurs when the rate of postmortem pH fall is three or more times higher than the normal rate and the muscle is not allowed to cool below 38° before the pH falls below 6.0 (Briskey and Wismer-Pedersen, 1961; Wismer-Pedersen, 1959). The results mentioned above will give an important clue in explaining the change in the quality of pale watery meat.

The greater stability of myosin A in the muscle over the isolated molecules was not fully determined. It is, however, very likely that, in muscle undergoing rigor in conditions of high temperature and low pH, the highly organized contractile proteins of myofibrils could be more resistant to environmental changes due to the restriction of thermal motion of the molecules. Fusion of molecules which caused aggregation and masking of active sites observed on myosin systems (Kawakami *et al.*, 1971) would be expected to take place locally in the rigid built-in system (Huxley, 1963; Reedy *et al.*, 1965) such as glycerol-treated fiber bundles. This expectation was borne out by the results in Figures 6-8, as well as Figure 9 and Table II, which show only that the native form of myosin A was extracted, in spite of the total decrease in extractability.

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Model Studies Regarding the Internal Corrosion of Tin-Plated Food Cans. III. On the Binding of Tin(II) Ions and Iron(II) Ions by Sulfur-Containing Amino Acids

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Stability constants of chelates formed by tin(II) and iron(II) ions with *l*-cysteine hydrochloride and *dl*-penicillamine were determined by pH titrations at 25° and in 0.1 *M* KCl. With Fe(II), cysteine, as well as penicillamine, forms stable complexes of the type ML and ML_2 at pH values above 6. With Sn(II), the ligands form only ML chelates; however, complex formation proceeds already at pH values above 2. Indications are that in all instances hydroxo complexes are formed at high pH values. However, these reactions were not investigated in any detail due to the onset of precipitation. The results are discussed in relation to the well-known reversal in polarity of the tin-iron couple observed in cans containing low-acid, protein-rich food materials.

It is generally believed that the protection offered by tin to steel at the interior surface of tin-plated food cans is a sacrificial one. Contrary to its position in the electrochemical series of the elements, which would require tin to be cathodic to iron, the metal becomes the anode in the local electrolytic cells that are set up when tin plate is in contact with the liquid medium of the food product. In such instances the protective coating (tin) will undergo slow even corrosion, whereas the base metal (iron), exposed under the pores or cracks that exist in the coating, acts as the local cathode (Hoar, 1934; Kohman and Sanborn, 1928a,b,c; Lueck and Blair, 1928a,b; Mantell and King, 1927; Mantell and Lincoln, 1926).

While there has been no exact agreement as to the cause of the observed change in polarity (*cf.* Hartwell, 1951; Hoare *et al.*, 1965; Koehler, 1961) it appears that one major contributing factor is the ability of the liquid medium to bind free stannous ions (Hoar, 1934). From simple thermodynamic considerations alone it can be readily shown that the electrode potential of tin has to become more negative than that of iron as soon as the ratio of the concentration of stannous ions to ferrous ions, $[Sn^{2+}]/[Fe^{2+}]$, is smaller than the value 5×10^{-11}, *i.e.*, free Sn(II) ions are present only in extremely minute amounts. This is of course a consequence of the concen-

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