specific substrate provides a potent tool for this type of analysis.

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REFERENCES

Bender, M. L., and Glasson, W. A. (1960), *J. Am. Chem. Soc. 82*, 3336.
Benditt, E. P., and Arase, M. (1958), *J. Histochem. Cytochem. 6*, 431.
Benditt, E. P., and Arase, M. (1959), *J. Exptl. Med. 110*, 451.
Bergmann, M., and Fruton, J. S. (1942), *Advan. Enzymol. 2*, 49.
Burstone, M. (1959), *Am. J. Clin. Pathol. 28*, 429.
Caplow, M., and Jencks, W. P. (1962), *Biochemistry 1*, 883.
Davis, B. J. (1959), *Proc. Soc. Exptl. Biol. Med. 101*, 90.
Dreyer, W. J., Wade, R. D., and Neurath, H. (1955), *Arch. Biochem. Biophys. 59*, 145.
Gomori, G. (1939), *Proc. Soc. Exptl. Biol. Med. 42*, 23.
Gomori, G. (1953), *J. Histochem. Cytochem. 6*, 469.
Hein, G. E., and Neimann, C. (1961), *Proc. Natl. Acad. Sci. U.S. 47*, 1341.
Hein, G. E., and Niemann, C. (1962), *J. Am. Chem. Soc. 84*, 4495.
Lagunoff, D. (1962), *J. Histochem. Cytochem. 11*, 114.
Lagunoff, D., and Benditt, E. P. (1963), *Ann. N. Y. Acad. Sci. 103*, 185.
Lagunoff, D., Benditt, E. P., and Watts, R. (1962), *J. Histochem. Cytochem. 10*, 672.
Laidler, K. J. (1958), The Chemical Kinetics of Enzyme Action, London, Oxford, p. 148.
Marshall, J. M. (1954), *Exptl. Cell Res. 6*, 240.
McDonald, C. E., and Balls, A. K. (1956), *J. Biol. Chem. 221*, 993.
Mead, J. A. R., Smith, J. N., and Williams, R. T. (1963), *Biochem. J. 61*, 569.
Moss, D. W. (1960), *Clin. Chim. Acta 5*, 283.
Nachlas, M. N., and Seligman, A. M. (1949), *J. Natl. Cancer Inst. 9*, 415.
Neurath, H., and Hartley, B. S. (1959), *J. Cellular Comp. Physiol. 54* (Suppl. 1), 179.
Neurath, H., and Schwert, G. W. (1950), *Chem. Rev. 46*, 69.
Pearse, A. G. E. (1960), Histochemistry: Theoretical and Applied, 2nd ed., Boston, Little, Brown.
Ravin, H. A., and Seligman, A. M. (1954), *J. Biol. Chem. 208*, 1.
Robinson, D. (1956), *Biochem. J. 63*, 39.
Rotman, B. (1963), *Proc. Natl. Acad. Sci. U. S. 47*, 1981.
Siekevitz, P., and Palade, G. E. (1958), *J. Biophys. Biochem. Cytol. 4*, 203.
Speigler, L. (1953), *J. Org. Chem. 18*, 1292.
Sturtevant, J. M. (1959), *J. Cellular Comp. Physiol. 54* (Suppl. 1), 190.
Takamatsu, H. (1939), *Trans. Soc. Pathol. Japon.* (now *Nippon Byori Gakkai Kaishi*) *29*, 492.
Van Duren, B. L. (1963), *Chem. Rev. 63*, 325.
Wallace, R. A., Kurtz, A. N., and Niemann, C. (1963), *Biochemistry 2*, 824.

Studies on Structure and Enzymatic Activity of Myosin
Relationship between Conformations and Adenosine Triphosphatase Activity of Myosin and H-Meromyosin in Urea*

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To understand the nature of the enzymatic action of myosin on the basis of its structural specificity, the relationship between ATPase activity and conformational changes induced by urea treatment of myosin A and H- and L-meromyosin was investigated. Conformational changes were studied by means of optical rotation, viscosity determinations, and ultracentrifugation. L-Meromyosin, which has a high percentage of α-helix content, rapidly transforms to a coiled form in urea and regains its original structure upon removal of urea. The H fragment of myosin A, on which ATPase activity is localized, has a less-ordered structure and responds to urea treatment more slowly, although the conformational change finally induced cannot be reversed. These rapid and slow, reversible and irreversible responses of L- and H-meromyosins to urea exist as a complex in myosin A. Loss of ATPase activity of myosin A caused by urea may be owing to (1) oxidation of SH groups at the active site in the presence of metal ion, (2) disintegration of myosin A initiated by the unfolding and subsequent dissociation of L component, and (3) irreversible changes in the secondary structure of the H component. The partial recovery of ATPase activity by urea-treated myosin after removal of urea might be attributed to the refolding ability of L-meromyosin.

Dissociation of the myosin A molecule into three apparently identical subunit chains, in concentrated urea (Small *et al.*, 1961) and guanidine-HCl (Kielley and Harrington, 1960; Young *et al.*, 1962; Woods *et al.*, 1963), has been demonstrated. Upon dilution or removal of these denaturing agents by dialysis, re-formation of the original multistranded structure of myosin A occurred to an appreciable degree; however, no Ca^{2+}-activated ATPase activity was found. With enzymes, such as RNAase (White, 1961) and lysozyme (Imai *et al.*, 1963), a clearer relationship was found between their primary and secondary structures and their enzymatic activities; i.e., these enzymes, denatured in 8 M urea, regain both their structure and activities upon the removal of urea. The mechanism of enzymatic action of

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these enzymes may be quite different from that of myosin A and, moreover, urea may affect these enzymes differently. Nevertheless, it seems pertinent at the present time to extend this kind of study to myosin with the idea that its biological activity can be understood ultimately on the basis of its structural specificity.

The secondary and tertiary structures of myosin were correlated with its enzymatic activity by studying the optical rotation, ultracentrifugal pattern, viscosity, and ATPase activity of myosin A in moderate concentrations of urea solution.

Materials and Methods

Myosin A was extracted from rabbit back muscle with Guba-Straub solution for 15 minutes at 5°. Myosin A can be dissolved in 0.5 M KCl solution and precipitated by dilution with water to 0.05 M KCl solution. Purification was performed by repetitive precipitation and redissolution three times. For the elimination of myosin B as a contaminant in the solution, only the fraction soluble in 0.25 M KCl was taken from the first precipitate, and the final preparation of the purified myosin A was stored in 0.6 M KCl.

H- and L-Meromyosins were prepared by the method of Szent-Györgyi (1953) using twice-crystallized trypsin and soybean-trypsin inhibitor obtained from the Worthington Biochemical Corp. Myosin A dissolved in 0.6 M KCl–0.02 M Tris, *p*H 7.2, was treated with trypsin for 10 minutes at 25° and dialyzed for 10 hours against 20 volumes of 0.02 M Tris buffer, producing a precipitate. After centrifugation the supernatant was taken as the H-meromyosin fraction and the sedimented precipitate was redissolved in 0.6 M KCl solution to give the L-meromyosin fraction. The ATPase activities of myosin A and H-meromyosin under standard conditions (0.6 M KCl, 0.05 M Tris, 1 mM $CaCl_2$, 25° and *p*H 8) were near 5 and 8 μmoles P_i/sec per g of protein, respectively. The reagents used here were "reagent grade" obtained from WAKO Pure Chemical Co. (Osaka). Except for urea, which was recrystallized once, the reagents were used without further purification.

SH *Groups*.—Owing to the greater purity and solubility of *p*-mercuribenzoate (Sigma Chemical Co.), this compound was employed for SH titration, instead of usual *p*-mercuribenzoate. Spectrophotometrically, SH groups were determined by comparing the absorbancy at 250 mμ for mixed and unmixed samples of protein and *p*-mercuribenzoate solutions as directed by Boyer (1954).

Optical Rotation.—Measurements of optical rotation were made with a Nihonbunko automatic recording polarimeter ORD/UV-5 between the wavelengths of 600 and 290 mμ, using a xenon lamp as light source. Approximately 50 minutes were required for scanning this wavelength range. The optical path of the cell was 5 cm and the concentration range of the proteins was 3–4 mg/ml.

The refractive index of each solvent was determined by using a Zeiss differential refractometer and comparing it with the refractivity of known concentrations of sucrose solutions.

The content of excess right-handed α-helix in the samples was estimated from the b_0 term of the Moffitt-Yang equation.

$$[\alpha] = \left(\frac{100}{M_0}\right)\left(\frac{n^2+2}{3}\right) \times \frac{a_0\lambda^2_0}{\lambda^2 - \lambda^2_0} + \frac{b_0\lambda^4_0}{(\lambda^2 - \lambda^2_0)^2}$$

where n is the refractive index of the solvent, M_0 represents the average molecular weight of a single residue (estimated to be 117), and λ_0 was taken as a constant equal to 2140 A as suggested by Tonomura *et al.* (1963).

Ultracentrifuge.—Sedimentation studies were made with a Spinco Model E ultracentrifuge at 59,780 rpm at 10°. The bar angle was fixed at 55° and, after the maximum speed of rotation was reached, five photographs were taken at 4-minute intervals. The protein concentrations were 3.5 mg/ml for H-meromyosin and 2.5 mg/ml for myosin A.

Viscosity.—Viscosity of solutions was measured with an Ostwald viscometer of 5-ml capacity having a flow time of 145.2 seconds for 0.02 M Tris buffer at 25°. The flow times for 5 ml of untreated H-meromyosin (8.1 mg/ml), L-meromyosin (3.1 mg/ml), and myosin A (7.5 mg/ml) solutions were 181.2, 173.0, and 367.3 seconds, respectively. Relative viscosities were calculated by taking the ratios of the flow time of solutions and solvents without considering the kinetic energy correction.

Protein Concentration.—The concentration of proteins was estimated spectrophotometrically by the Folin-phenol method (Lowry *et al.*, 1951).

Urea Treatment of Protein.—Various amounts of 8 M urea solution were added to the protein solutions at 22° to give the desired concentration of urea. Both rapid and slow methods for diminishing urea concentration were employed because of possible differences in the mode of refolding dissociated chains, depending on the speed of elimination of urea. The rapid dilution of urea to approximately 0.5–0.3 M had virtually no effect on myosin. Slow elimination of urea was accomplished by dialysis with constant gentle stirring against buffer for 20–24 hours at 2–5°. It is expected that the effect of urea on myosin can be eliminated within a few minutes after dialysis of the treated sample under the given conditions (see Fig. 2). Even the highest concentration of urea tested (6 M) might not be sufficient to cause complete dissociation of the myosin molecule into subunit chains (Small *et al.*, 1961). However, this concentration seems to induce a drastic conformational change in the protein structure, because (1) the ATPase activities of myosin A and H-meromyosin were lost rapidly at 25°, (2) the drop in viscosity of the myosin solution reached a maximum in 4–5 M urea, and (3) in 4 M urea destruction of the helical structure of myosin A and H- and L-meromyosin was almost complete as judged by the optical rotation measurement.

ATPase Activity.—ATPase activity was measured in the presence of 2 mM ATP, 0.01 M Ca^{2+}, *p*H 7.2 at 25°, unless noted otherwise. The buffers used were 0.02 M Tris-HCl or Tris-histidine for H-meromyosin and for myosin A, 0.6 M KCl or 0.5 M tetramethylammonium chloride was added. ATPase activity measurement of undialyzed urea-treated samples was completed within 4 minutes after stopping the reaction by dilution with buffers. The amount of inorganic phosphate (P_i) liberated from ATP was determined by the Fiske-Subbarow method (1929) using ascorbic acid as a reducing agent. For the phosphate analysis, the protein concentration in the medium was adjusted to approximately 0.15 mg/ml for H-meromyosin and 0.5 mg/ml for myosin A in order to obtain suitable color development. Activity was expressed as μmoles P_i liberated per unit weight of enzyme (gram) per unit time (sec).

Results

ATPase Activity of Urea-treated Myosin and H-Meromyosin.—Re-examination of the effect of urea on myosin ATPase reported by Small *et al.* (1961) revealed that the rate of decrease in ATPase activity depends greatly on the kinds of existing ions and *p*H of the

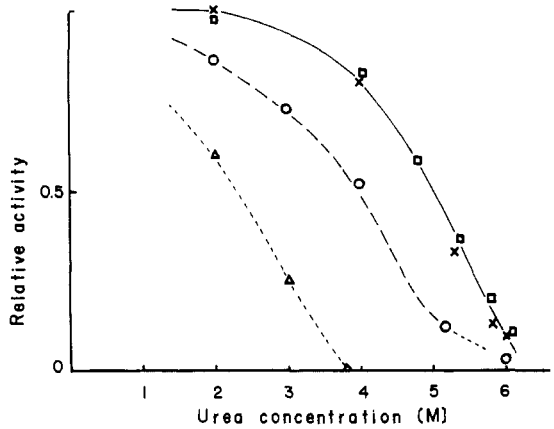

FIG. 1.—Relative ATPase activities of urea-treated myosin A and H-meromyosin. O, myosin A in 0.5 M tetramethylammonium chloride, undialyzed; ×, myosin A in 0.5 M tetramethylammonium chloride, dialyzed; △, myosin A in 0.6 M KCl, undialyzed; □, H-meromyosin in 0.02 M Tris. The undialyzed and dialyzed samples showed the same activity; for other conditions, see text. Protein concentrations: for the urea treatment, myosin A, 6–3 mg/ml; H-meromyosin, 4–2 mg/ml; for the ATPase measurement, myosin A, 0.6–0.3 mg/ml; H-meromyosin, 0.2–0.15 mg/ml. Urea treatment was performed at 22°, *p*H 8, in the presence of 1 mM SO_3^{2-} for 10 minutes. Immediately after the dilution of urea with respective buffers, activity measurement was made for undialyzed samples. For the slow removal of urea, dialysis was performed at 5° for 22 hours against the buffer.

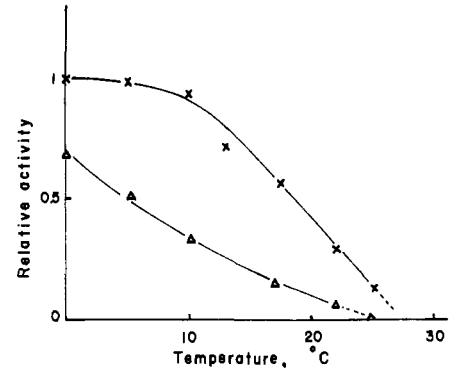

FIG. 2.—Relative ATPase activities of urea-treated myosin A and H-meromyosin—effect of temperature. △, myosin A in 0.5 M tetramethylammonium chloride; ×, H-meromyosin in 0.02 M Tris. Treatments were performed in 5 M urea in the medium indicated, in the presence of 1 mM SO_3^{2-}, *p*H 8, for 10 minutes. Samples then were diluted and their activities were measured immediately. Protein concentration: for the urea treatment, myosin A, 6 mg/ml; H-meromyosin, 4 mg/ml; for the ATPase measurement, myosin A, 0.5 mg/ml, H-meromyosin, 0.2 mg/ml.

medium, as well as the temperature and the concentration of urea. Urea treatment of myosin in 0.6 M KCl in the presence of substrate or the reducing agents, SO_3^{2-} or β-mercaptoethanol, had only a slight protective effect for the ATPase active site. After treatment with urea, myosin ATPase activity was preserved to a greater extent in 0.5 M tetramethylammonium chloride than in 0.6 M KCl, and still better in 0.5 M tetramethylammonium chloride containing reducing agent (1 mM SO_3^{2-}), as shown in Figure 1.

The ATPase activity of H-meromyosin in 0.02 M Tris (*p*H 7.2) was more resistant to the denaturing effect of urea than was the enzymatic activity of myosin A. However, in 0.6 M KCl containing urea, H-meromyosin quickly lost its ATPase activity to the same degree as did myosin A. This seems to indicate that K^+ accelerates the destruction by urea of the enzymatic site(s) on myosin A and H-meromyosin. At low temperature, below 10°, 5 M urea hardly affected ATPase activity of H-meromyosin while similar treatment of myosin A showed a considerable effect (Fig. 2).

Reversibility of ATPase.—As reported by Young *et al.* (1962), the ATPase of guanidine-HCl-treated myosin A showed no sign of recovery after diminishing the guanidine concentration by dialysis against 0.5 M KCl–0.05 M Tris buffer, even when it was treated and dialyzed in the presence of substrate and reducing agents. The present experiment has demonstrated that although approximately 80% of myosin A–ATPase activity was diminished in 0.5 M tetramethylammonium chloride at moderate concentrations of urea (up to 4–5 M for 10 minutes at 22°) a partial recovery was obtained after the elimination of urea by dialysis against 0.5 M tetramethylammonium chloride for 22 hours. The results obtained are shown in Figure 1. Treatment at concentrations higher than 6 M urea resulted in no renewal of ATPase activity. On the contrary, the urea-treated H-meromyosin, both diluted and dialyzed, showed essentially the same activity (see legend, Fig. 1). It appears that H-meromyosin, which showed strong resistance against the effect of urea, did not recover its prior ATPase activity during the dialysis procedure; i.e., denaturation caused by urea treatment was irreversible. However, H-meromyosin treated with urea in the presence of 1 mM ATP and Mg^{2+} retained more than 90% of its original activity when urea was removed by slow dialysis (Fig. 3).

SH Groups.—The number of free SH groups in myosin A and H-meromyosin, determined by titration, were decreased by urea treatment. It was also found that the number of SH groups depends on the conditions in the medium, e.g., ionic strength, kind of ions, urea concentration in which the proteins are dissolved, and the presence or absence of reducing agents (Table I). The oxidation of SH groups also occurred during dialysis if reducing agent (1 mM SO_3^{2-} or β-mercaptoethanol) was not present in the dialysis fluid. In 0.6 M KCl, reducing agent was not effective in protecting SH

TABLE I

NUMBER OF *p*-MERCURIBENZOATE-TITRATABLE SH GROUPS IN 10^5 G OF MYOSIN A AND IN 10^5 G OF H-MEROMYOSIN[a]

Treatment	Medium	In Absence of Reducing Agent	In Presence of SO_3^{2-} (10^{-3} M)
Myosin A			
Control	0.6 M KCl	7.8	8.0
Urea treated (5.3 M)	0.6 M KCl	4.9	5.3
	0.5 M Tetramethylammonium chloride	6.6	7.0
Urea removed	0.6 M KCl	3.8	4.3
	0.5 M Tetramethylammonium chloride	3.2	6.9
H-Meromyosin			
Control	0.02 M Tris	9.4	10.4
Urea treated (5.3 M)		5.5	9.2
Urea removed		5.8	10.1

[a] Reducing agent, Na_2SO_3, 1 mM. The treatment was performed approximately 10 minutes in the medium indicated. For the removal of urea, dialysis was performed in the buffer for 22 hours at 5°. The values shown are the average of three experiments.

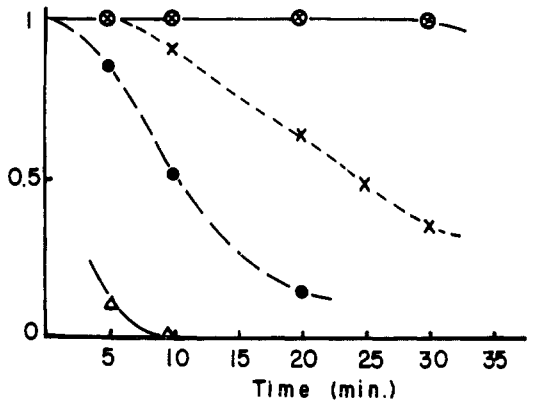

FIG. 3.—Relative ATPase activities of myosin A and H-meromyosin, time dependence of 4 M urea treatment. ●, myosin A in 0.5 M tetramethylammonium chloride; Δ, myosin A in 0.6 M KCl; ×, H-meromyosin in 0.02 M Tris. Protein concentrations as in Fig. 2. At the end of each treatment period urea was diluted and activity measurements were made immediately. ⊗, H-meromyosin, treated with 4 M urea in the presence of 1 mM Mg^{2+} and ATP, followed by dialysis against buffer for 22 hours.

groups from oxidation, while in 0.5 M tetramethylammonium chloride the number of intact SH groups in myosin was well preserved. The results obtained are shown in Table I.

Conformational Change Estimated from Optical Rotatory Dispersion.—α-Helical content of myosin A, H-meromyosin, and L-meromyosin of the native, 4 M urea–treated, and urea-removed materials, are listed in Table II. The values obtained for the native proteins

TABLE II
CONTENT OF α-HELIX IN MYOSIN A AND MEROMYOSINS[a]

	Native		Urea-treated (%)	Urea Removed (%)
Myosin A	59.0		34.2	57.5
H-Meromyosin	51.6		36.9	37.2
L-Meromyosin	89.3	(*p*H 8)	19.2	75.7
		(*p*H 6)	41.5	

[a] Per cent of helical content was estimated by dividing b_0 terms of the Moffitt-Yang equation by 580. The treatment was performed with 4 M urea for approximately 10 minutes in the medium indicated. For the removal of urea, dialysis was performed in the buffer for 22 hours at 5°. Protein concentrations: myosin A, 6 mg/ml; H-meromyosin, 4.5 mg/ml; L-meromyosin, 3 mg/ml. Media: myosin A and L-meromyosin, 0.6 M KCl, 0.02 M Tris; H-meromyosin, 0.02 M Tris. The values shown are the average of five experiments.

agree reasonably well with those reported earlier by Cohen and Szent-Györgyi (1957) and Tonomura *et al.* (1963). Apparently the helical content of myosin A in 0.5 M tetramethylammonium chloride obtained by dividing b_0 by 580 (Tonomura *et al.*, 1963), does not differ from that in 0.6 M KCl.

A conformational change of L-meromyosin induced at concentrations greater than 4 M urea (*p*H 8 at 22°) was so rapid that it seemed to reach equilibrium within a few minutes, as represented by the rapid change in $-[\alpha]_{400}$ (Fig. 4). At *p*H 6 the action of urea on the unfolding of L-meromyosin was less effective. This fact is consistent with the strong *p*H dependence for the depolymerization rate of L-meromyosin, as shown by Szent-Györgyi and Borbiro (1956). The corresponding process for H-meromyosin was slow and moreover, when the urea treatment was performed in

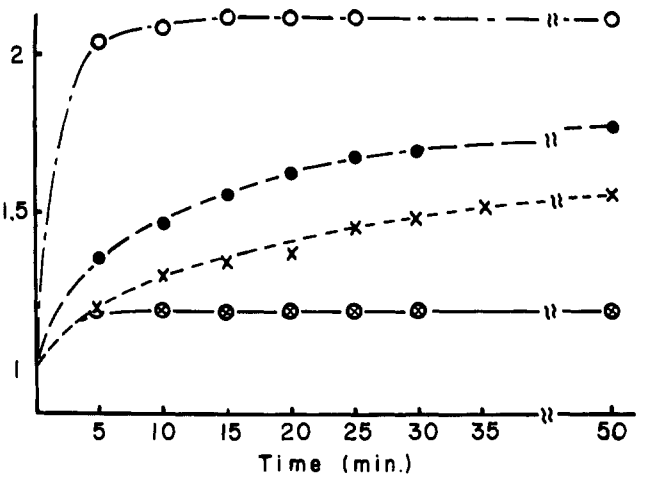

FIG. 4.—Relative change of specific rotation at 4000 A in 4 M urea, at 22°, pH 8. ●, myosin A in 0.5 M tetramethylammonium chloride–0.02 M Tris; ○, L-meromyosin in 0.6 M KCl–0.02 M Tris; ×, H-meromyosin in 0.02 M Tris; ⊗, H-meromyosin with 1 mM Mg^{2+} and ATP. Protein concentrations: myosin, 6 mg/ml; H-meromyosin, 4.5 mg/ml; L-meromyosin, 3 mg/ml.

the presence of Mg^{2+} and ATP (1 mM each), the slow phase of the $-[\alpha]_{400}$ increment was almost completely suppressed as shown in Figure 4. The response of myosin A to urea treatment was intermediate in both speed and magnitude of change between that of H-meromyosin and L-meromyosin.

The degree of recovery of the original structure of urea-treated myosin and L-meromyosin upon removal of urea, as judged by the optical rotation, was excellent, while that of H-meromyosin was poor (Table II). This result corresponds to a stronger resistance against the effect of urea and poorer recovery of structure and ATPase activity observed with H-meromyosin.

Sedimentation Patterns.—Under the same conditions as those for the measurements of ATPase and optical rotation, the original one peak of the sedimentation pattern of myosin A splits into at least two peaks. The original pattern could not be restored even after the elimination of urea by dialysis. These patterns have similarities with the patterns of myosin in guanidine-HCl obtained by Young *et al.* (1962). The slower component sediments at a speed almost the same as that of the original material. The proportion of the two peaks differ depending on the concentration of urea, time of treatment, and temperature. Appearance of the faster-sedimenting peak indicates the formation of an aggregated state of myosin in urea which is not reversed readily by dialysis. There is no apparent difference in the sedimentation patterns of myosin treated with urea in 0.6 M KCl and in 0.5 M tetramethylammonium chloride (Fig. 5). When the treatment is performed in 0.5 M tetramethylammonium chloride, a considerable degree of Ca^{2+}-activated myosin ATPase activity was noted, while this was not the case in 0.6 M KCl. Similarly, two peaks were observed in the sedimentation pattern of urea-treated H-meromyosin (Fig. 5), although the second peak obtained is broader in area and sediments at a faster rate compared to the second peak obtained for myosin.

Viscosity.—The relative viscosity of myosin A in solution dropped almost instantaneously to one-tenth its original value by the treatment with 5 M or higher concentrations of urea. This was followed by a gradual increase until a maximum value was reached. The maximum value was subject to variation depending upon the urea concentration. Figure 6 shows the time

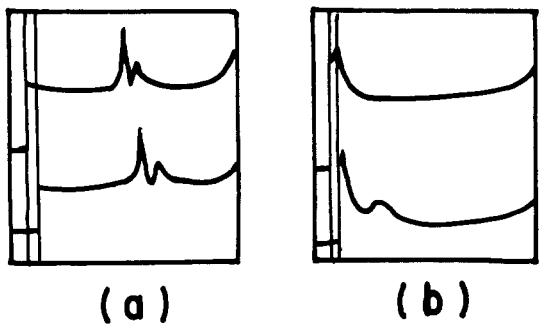

FIG. 5.—Sedimentation patterns of myosin A and H-meromyosin. (a) Myosin A treated with 4 M urea for 10 minutes at 22° and removed by dialysis. Protein concentration, 4.5 mg/ml. Top, in 0.5 M tetramethylammonium chloride–0.02 M Tris, *p*H 7.2; bottom, 0.6 M KCl–0.02 M Tris. (b) H-Meromyosin, 4 M urea-treated for 10 minutes at 22°, and urea removed by dialysis. Protein concentration, 3.5 mg/ml. Top, control, in 0.02 M Tris, *p*H 7.2; bottom, urea-treated, in 0.02 M Tris. Conditions for the treatment and dialysis as in Fig. 1.

course for the viscosity changes of myosin A, L-meromyosin, and H-meromyosin induced by 4 M urea, which might represent the rapid dissociation of myosin into its subunits and the slow aggregation of the dissociated chains. The removal of urea from the urea-treated myosin by slow dialysis brought back the viscosity to its original value although the sedimentation patterns indicated that an aggregated fraction of molecules might be present in the solution. It is probable that the restoration of gross structure of urea-treated myosin A, as well as the recovery of α-helical content, proceeded during the removal of urea by dialysis.

A diphasic response for the viscosity change of L-meromyosin induced by urea was also observed. In this case, however, only a gradual decrease in viscosity was observed after the initial sharp drop, as shown in Figure 6. The viscosity of H-meromyosin was essentially unaffected by the urea treatment. With both L- and H-meromyosin, after the initial drop, a slow increase in viscosity, which might reflect the formation of an aggregate, was not observed.

DISCUSSION

Urea has been commonly employed by many workers as one of the denaturing agents for the study of structure and function of enzymes, although the nature of its action is not yet clearly understood. In the case of myosin, urea induces the unfolding of the stranded structure, a dissociation of the molecules into subunit chains, and also depolymerization of L-meromyosin (Szent-Györgyi and Borbiro, 1956). Guanidine-HCl has a similar and seemingly more pronounced effect on myosin. Urea (12 M, 40°) is needed to induce complete dissociation of subunit chains of myosin A (Small *et al.*, 1961) while 5 M guanidine-HCl can achieve the same effect (Kielley and Harrington, 1960). The specific levorotation of myosin solutions increases abruptly to a terminal level after treatment with guanidine-HCl (Young *et al.*, 1962). A slower change in the levorotation of myosin is observed in urea (Fig. 4) (Small *et al.*, 1961). However, the time course of the viscosity change and the complex sedimentation patterns are quite similar for moderate treatments of myosin with either guanidine-HCl or urea. These facts may suggest that both reagents act on myosin by the same mechanism, but that the effectivities of their action is different. In the present experiment urea, because of its slow reactivity, has been chosen as a denaturing

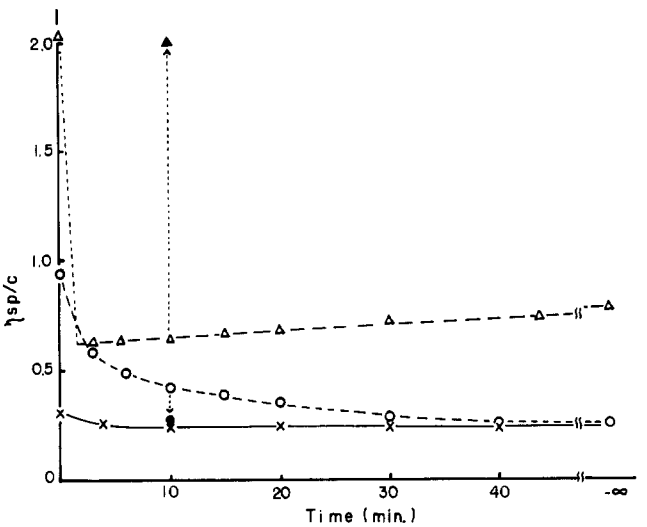

FIG. 6.—Time dependence of viscosity change of myosin A and L- and H-meromyosin in 4 M urea. Δ, myosin A; ○, L-meromyosin; ×, H-meromyosin. Protein concentrations: myosin A, 2.4 mg/ml; H-meromyosin, 8.1 mg/ml; L-meromyosin, 3.1 mg/ml. ▲, ● indicate the reduced viscosities of myosin and L-meromyosin, respectively, after the removal of urea by slow dialysis at 5° for 22 hours.

agent to follow the correlation and reversibility of structural changes and enzymatic activity. Moreover, the use of guanidine-HCl introduces large amounts of electrolytes and these may have some effect upon the active site in myosin (Kielley and Harrington, 1960).

It is difficult to elucidate the mechanism of urea action on enzymatic activity. It may act directly on the primary site or through the conformational change of the active center. Since only a small fraction of the whole molecule makes up the active site, several per cent change of helical content, which seems to be within the experimental error, corresponds to a drastic change in enzymatic activity (Tonomura *et al.*, 1963). Inactivation of myosin-ATPase activity by urea might be achieved, in part, by the oxidation of the SH group, which is believed to be responsible for the enzymatic action. The formation of intermolecular disulfide linkages might result in distortion of the structure of the enzymatic site. Urea treatment of myosin decreases the number of free SH groups in the molecule and this decrease can be prevented by the presence of reducing agent in the system (Table I). In addition, the ATPase activity is preserved also in the presence of reducing agent. It was shown by Tonomura and Furuya (1960) that urea accelerates the inactivation of *p*-mercuribenzoate-treated myosin ATPase which may indicate that the SH group is sensitive to urea.

There are considerable differences in the effect of urea on the secondary structure of H- and L-meromyosin as shown in the present experiment. H-Meromyosin has low α-helix content, related to its high proline-residue content ($29/10^5$ g, Kominz *et al.*, 1954), and shows slow response to urea treatment and poor recovery of its original structure upon the removal of urea (Table II and Fig. 4). The stabilization of the secondary structure of H-meromyosin may not be attributed to intramolecular hydrogen bonding but to some other mechanism which is insensitive to urea and sensitive to temperature or metal ions. To some extent this assumption may be applied to the secondary structure of the ATPase-active site, because there is a good parallelism between ATPase activity and structural changes of H-meromyosin represented by the

change of $-[\alpha]_{400}$. Both ATPase activity and structural changes induced by urea were depressed by the formation of enzyme-substrate complex between H-meromyosin, ATP, and Mg^{2+}. Cu^{2+}, which is supposed to have a strong interaction with H-meromyosin, also stabilizes the secondary structure of H-meromyosin.[1] This would mean that the conformation of the active site, or H-meromyosin itself, has a less rigid structure originally and its conformation may be determined by the interaction between specific groups in the site and cofactors in the medium. Differences in the action of several denaturing agents on myosin A, viz., urea, guanidine-HCl, and LiCl, all of which cause depolymerization of L-meromyosin in the same manner (Szent-Györgyi and Borbiro, 1956), may originate from variation in the interactions of these agents with a specific group in the H-meromyosin component.

The rapid and drastic changes in the optical rotation, $-[\alpha]_{400}$, and viscosity of urea-treated L-meromyosin solution which reflect both changes of conformation in the individual subunit chains and that of the gross structure, contrast sharply with the corresponding behavior of H-meromyosin. The loss of myosin A–ATPase and α-helix content in urea and the partial recovery after the removal of urea may be attributed to the behavior of the L fraction of myosin A. Disintegration of the molecule initiated in the L component of myosin A leading to distortion and/or destruction of the active site located on the H moiety. Therefore protection of the enzymatic active site of myosin A by the substrate in the presence of urea was less effective.

The slow rise in the viscosity of myosin in urea solutions, which took several hours to reach the final value, was not observed with L-meromyosin. Recovery of the original viscosity after the removal of urea was also not clearly observed with L-meromyosin in spite of the fact that its helix content was recovered to a considerable extent[2] (Fig. 6, Table II). L-Meromyosin shows no tendency to form aggregates in urea and a small capacity to reassociate the dissociated subunit chains, although the individual chain regains its helical structure upon the removal of urea. The aggregation of intact myosin in urea might be attributed to the nature of the H component to form intermolecular bridge as indicated by the appearance of a fast sedimenting component in the sedimentation pattern of urea-treated H-meromyosin and that from which urea was removed (Fig. 5). There is also a possibility that in intact myosin a factor may be present which accelerates the reassociation of dissociated chains and that this factor may be inactivated by tryptic digestion. Recently, Maruyama[3] has suggested the presence of a factor, in so-called natural actin, which inhibits the formation of the network structure of F-actin. Similarly, there might be such a factor, in the case of myosin, might be play a role in the reassociation of the dissociated subunit chains.

[1] Hotta, K., unpublished data.

[2] Szent-Györgyi and Borbiro (1956) have observed the reversible-viscosity response of L-meromyosin against urea if the treatment was not prolonged (<5 min). This is an observation incompatible with the results obtained in the present work, but differences in the length of treatment and the method of elimination of urea (slow dialysis, dilution, or quick dialysis) could have led to the different results.

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References

Boyer, P. D. (1954), *J. Am. Chem. Soc., 76,* 4331.
Cohen, C., and Szent-Györgyi, A. G. (1957), *J. Am. Chem. Soc. 79,* 248.
Fiske, C. H., and Subbarow, Y. C. (1929), *J. Biol. Chem. 81,* 629.
Imai, K., Takagi, T., and Isemura, T. (1963), *J. Biochem. (T kyo) 53,* 1.
Kielley, W. W., and Harrington, W. F. (1960), *Biochim. Biophys. Acta 41,* 401.
Kominz, D. R., Haugh, A., Symonds P., and Laki, K. (1954), *Arch. Biochem. Biophys. 50,* 148.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.
Small, P. A., Harrington, W. F., and Kielley, W. W. (1961), *Biochim. Biophys. Acta 49,* 462.
Szent-Györgyi, A. G. (1953), *Arch. Biochem. Biophys. 42,* 305.
Szent-Györgyi, A. G., and Borbiro, M. (1956), *Arch. Biochem. Biophys. 60,* 150.
Tonomura, Y., and Furuya, K. (1960), *J. Biochem. (Tokyo) 48,* 899.
Tonomura, Y., Sekiya, K., and Imamura, K. (1963), *Biochim. Biophys. Acta 69,* 296.
White, F. H., Jr. (1961), *J. Biol. Chem. 236,* 1353.
Woods, E. F., Himmelfarb, S. H., and Harrington, W. F. (1963), *J. Biol. Chem. 238,* 2374.
Young, M. D., Harrington, W. F., and Kielley, W. W. (1962), *J. Biol. Chem. 237,* 3116.

[3] Maruyama, K., personal communication.