Krishna, N. R., Huang, D. H., Glickson, J. D., Rowan, R., & Walter, R. (1979) *Biophys. J. 26*, 345–366.
Laiken, S. L., Printz, M. P., & Craig, L. C. (1969) *Biochemistry 8*, 519–526.
Leichtling, B. H., & Klotz, I. M. (1966) *Biochemistry 5*, 4026–4037.
Levy, G. C., & Lichter, R. L. (1979) *Nitrogen-15 NMR Spectroscopy*, Wiley-Interscience, New York.
Linderstrøm-Lang, K. (1955) *Spec. Publ.–Chem. Soc. No. 2*, 1–20.
Live, D. H., Agosta, W. C., & Cowburn, D. (1977) *J. Org. Chem. 42*, 3556–3561.
Live, D. H., Wyssbrod, H. R., Fischman, A. J., Agsota, W. C., Bradley, C. H., & Cowburn, D. (1979) *J. Am. Chem. Soc. 101*, 474–479.
Llinas, M., & Klein, M. P. (1975) *J. Am. Chem. Soc. 97*, 4731–4737.
Llinas, M., Klein, M. P., & Nielands, J. B. (1973) *J. Biol. Chem. 248*, 915–923.
Llinas, M., Horsley, W. J., & Klein, M. P. (1976) *J. Am. Chem. Soc. 98*, 7554–7558.
Llinas, M., Wilson, D. M., & Klein, M. P. (1977) *J. Am. Chem. Soc. 99*, 6846–6850.
Markowski, V., Posner, T. B., Loftus, P., & Roberts, J. D. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 1308–1313.
Meraldi, J. P., Hruby, V. J., & Brewster, A. I. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 1373–1377.
Molday, R. S., & Kallen, R. G. (1972) *J. Am. Chem. Soc. 94*, 6739–6745.
Molday, R. S., Englander, S. W., & Kallen, R. G. (1972) *Biochemistry 11*, 150–158.
Nikiforovich, G. V., Leonova, V. I., Galaktionov, S. G., & Chipins, G. I. (1979) *Int. J. Pept. Protein Res. 13*, 367–373.
Philson, S. B., & Bothner-By, A. A. (1980) in *Peptides: Structure and Biological Function* (Gross, E., & Meienhofer, J., Eds.) pp 209–212, Pierce, Rockford, IL.
Pletcher, T. C., Koehler, S., & Cordes, E. H. (1968) *J. Am. Chem. Soc. 90*, 7072–7076.
Saito, H., & Nukada, K. (1971) *J. Am. Chem. Soc. 93*, 1072–1076.
Saito, H., Nukada, K., Kato, H., Yonezawa, T., & Fukui, K. (1965) *Tetrahedron Lett. 2*, 111–117.
Saito, H., Tanaka, Y., & Nukada, K. (1971) *J. Am. Chem. Soc. 93*, 1077–1081.
Scheinblatt, M. (1966) *J. Am. Chem. Soc. 88*, 2123–2126.
Scheinblatt, M. (1970) *J. Am. Chem. Soc. 92*, 2505–2509.
Tu, A. T., Bjarnasson, J. B., & Hruby, V. J. (1978) *Biochim. Biophys. Acta 533*, 530–533.
Urry, D. W., & Walter, R. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 956–958.
Vinogradov, S. N., & Linnell, R. H. (1971) *Hydrogen Bonding*, Van Nostrand Reinhold, New York.
Welch, W. H., & Fasman, G. B. (1974) *Biochemistry 13*, 2455–2466.
Wittbold, W. M., Fischman, A. J., Ogle, C., & Cowburn, D. (1980) *J. Magn. Reson. 39*, 127–135.
Woodward, C. K., & Hilton, B. D. (1979) *Annu. Rev. Biophys. Bioeng. 8*, 99–127.
Wyssbrod, H. R., Ballardin, A., Schwartz, I. L., Walter, R., Van Binst, G., Gibbons, W. A., Agosta, W. C., Field, F. H., & Cowburn, D. (1977) *J. Am. Chem. Soc. 99*, 5273–5276.

Structural Changes in Synthetic Myosin Minifilaments and Their Dissociation by Adenosine Triphosphate and Pyrophosphate†

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ABSTRACT: Morphologically similar short myosin and rod filaments (minifilaments) have been prepared in 10 mM Tris–citrate buffer, pH 8.0, in the absence of other myosin or rod forms. Both minifilament systems are dissociated in the same manner in the presence of ATP or pyrophosphate. Identical binding of these ligands to myosin and rod minifilaments suggests that myosin heads play no role in substrate-induced destabilization of the minifilaments. The effects of ATP and pyrophosphate on minifilaments are similar to their dissociating effect on synthetic filaments [Harrington, W. F., & Himmelfarb, S. (1972) *Biochemistry 11*, 2945–2952], thus justifying their use in conformational studies in lieu of filaments. In view of their small size and homogeneity, the minifilaments constitute an appropriate material for such studies. The binding of pyrophosphate to myosin and rod minifilaments decreases their α-helical content, as measured by circular dichroism. No change in the secondary structure of subfragment 1 and light meromyosin is observed upon binding of pyrophosphate, but substantial changes (10%) are detected in subfragment 2. The structural changes in myosin, possibly relevant to contraction, are localized in the subfragment 2 region of the molecule. These results emphasize the importance of charge interactions in the functional behavior of thick filaments.

Electrostatic interactions within the myosin filament have been considered by many authors to be an important element of contractile events. In a contraction model developed by Morales & Boots (1953) and Morales (1955), the negative charge of adsorbed ATP molecules would lead to an extension of a segment of the myosin heavy chain. Following cleavage of the terminal phosphate, the local charge would be reduced, resulting in contraction of the chain and consequent force generation. The search for corresponding conformational changes in myosin induced by ATP binding and cleavage or by interaction with the competitive inhibitor, pyrophosphate, produced no evidence in support of any transitions except those

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localized on the myosin head (Gratzer & Lowey, 1969; Godfrey & Harrington, 1970; Seidel & Gergely, 1971). In view of the experimental difficulties and the ambiguities in interpreting measurements on myosin filaments, the conformational studies were confined to monomeric myosin in high ionic strength conditions (0.5 M KCl).

It is possible that an alteration in the structure of the myosin molecule is blocked in the presence of high concentrations of monovalent salt or, alternatively, that cooperative interactions in the filament are required to generate conformational changes. Indeed, experiments carried out with myosin filaments or muscle fibers implicate charge interaction in causing detectable structural changes. Rome (1967) has shown that changes in charge balance in glycerinated muscle can of themselves lead to a lateral contraction or expansion of the filament lattice. More recently, Thomas et al. (1975) and Mendelson & Cheung (1976) have observed that the rotational mobility of myosin heads in synthetic filaments is greatly increased upon raising the pH from 6.8 to 8.3. These findings are complemented by the cross-linking studies of Sutoh et al. (1978a), who detected a release of myosin heads from the filament surface over the same pH range.

Direct titrations of synthetic myosin filaments with nucleotides and pyrophosphate have revealed dissociation of the polymeric structure upon binding of one to two ligand molecules per monomeric protein unit (Harrington & Himmelfarb, 1972). Similar dissociation of rod filaments has suggested that the destabilization of myosin polymers is caused by substrate binding to the rod segment of the molecule. These observations raised the possibility that the specific binding of nucleotides or pyrophosphate induces conformational changes in myosin pertinent to contraction. The intriguing aspect of such a possibility is that the model of contraction by Tsong et al. (1979) indeed predicts a limited α-helix–coil transition in myosin. Conclusive detection of such changes in the filament system would be rather difficult.

Recently, we have described a new and organized structure of myosin—the minifilaments (Reisler et al., 1980). Myosin minifilaments are composed of 16–18 molecules, have a bipolar appearance corresponding to the bare central region of the thick filaments, and can be stabilized in the absence of monovalent salt. Because of their relatively small and homogeneous size (about 0.3 μm long), minifilaments constitute the most appropriate material for investigating conformational changes in an organized myosin system.

In the work to be presented below, we have investigated the dissociation of minifilaments by ATP and pyrophosphate. These experiments were carried out as a part of our studies on the properties of myosin minifilaments, particularly in relation to those of regular filaments. In these experiments, we demonstrate that myosin minifilaments can be, indeed, used in lieu of filaments. In order to compare myosin and rod systems, we have prepared rod minifilaments which morphologically resemble the myosin minifilaments. Using these well-defined systems, we show that ATP and pyrophosphate binding profiles are identical for myosin and its rod segment. Our goal of measuring conformational changes related to binding of substrates to myosin has been pursued by comparing the CD spectra of myosin and rod minifilaments in the presence and absence of pyrophosphate. As a result of these, as well as the control experiments, we assign the change observed upon binding of pyrophosphate to structural transition in the subfragment 2 region of the molecule.

Materials and Methods

Distilled water and analytical grade reagents were used in all experiments. ATP, α-chymotrypsin, and phenylmethanesulfonyl fluoride were obtained from Sigma Chemical Co. (St. Louis, MO).

Preparation of Proteins. Rabbit myosin was prepared and stored as described previously (Godfrey & Harrington, 1970). Before use, myosin was exhaustively dialyzed against 0.6 M KCl, 10 mM sodium phosphate (pH 7.0), and 0.1 mM NaN_3 and clarified by centrifugation at 100000g for 2 h. No attempt was made to remove the C protein on a DEAE ion-exchange column. Subfragment 1 (S-1)[1] was obtained by α-chymotryptic digestion of myosin in 0.12 M NaCl, 1 mM EDTA, and 10 mM sodium phosphate (pH 7.0). Digestion and purification followed previously described procedures (Weeds & Taylor, 1975). Prior to use, S-1 was further purified by fractionation between 43 and 57% saturated ultrapure ammonium sulfate.

Myosin rod was prepared with α-chymotrypsin by using the same conditions employed for the subfragment 1 preparations (Weeds & Taylor, 1975). The rod was recovered from proteins precipitated at low ionic strength after chymotryptic digestion of myosin filaments (in 0.12 M NaCl) and purified by the procedure of Harrington & Burke (1972).

Heavy meromyosin (HMM) was prepared by chymotryptic digestion of myosin in 0.6 M NaCl, 1 mM $CaCl_2$, and 10 mM sodium phosphate (pH 7.0), as described by Weeds & Taylor (1975).

Light meromyosin (LMM) was recovered from proteins precipitated at low ionic strength during the course of HMM preparation. The LMM segment was purified by using the same conditions and procedures as employed in the rod preparation (Harrington & Burke, 1972).

The high molecular weight subfragment 2 (S-2)[2] (120000) was prepared from chymotryptic HMM by the method of Sutoh et al. (1978b). The purity of all protein preparations was verified by sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

Preparation of Synthetic Minifilaments and Filaments. Solutions of myosin minifilaments were prepared by a two-step dialysis of monomeric myosin (in 0.5 M KCl and 10 mM phosphate, pH 7.0) into a Tris–citrate buffer at pH 8.0 (Reisler et al., 1980). Prior to the first dialysis against 5 mM sodium pyrophosphate (pH 8.0), the protein concentration was adjusted to about 5 mg/mL. After equilibration with pyrophosphate, the protein was clarified by centrifugation at 80000g for 1 h and redialyzed against 10 mM Tris–citrate buffer (pH 8.0). The final minifilament preparation in Tris–citrate buffer was reclarified by centrifugation (27000g for 20 min). Myosin filaments were prepared according to Josephs & Harrington (1966) by dialyzing monomeric myosin into 0.13 M KCl and 0.025 M Tris, pH 8.0.

Minifilaments of myosin rods were generated by the same procedure as myosin minifilaments.

We were unable to form a homogeneous system of LMM minifilaments under the conditions used in the preparation of myosin and rod minifilaments. Although centrifugation of the LMM system following its equilibration with Tris–citrate buffer (pH 8.0) resulted in a solution of very low turbidity, electron micrographs of such preparations revealed polymorphic forms of LMM assembly. However, because of their low turbidity, LMM solutions could be used in CD studies.

[1] Abbreviations used: HMM, heavy meromyosin; LMM, light meromyosin; S-1, subfragment 1; S-2, subfragment 2.

[2] Throughout the text, subfragment 2 refers to the high molecular weight (120000) preparation by the methods of Weeds & Pope (1977) and Sutoh et al. (1978).

Concentration Determinations. Concentrations of myosin, rod, LMM, and their polymer solutions were determined spectrophotometrically. The polymeric systems were dissociated to monomeric proteins by dilution to 0.5 M KCl. The following extinction coefficients were employed: $E_{280}^{1\%} = 5.55$ cm^{-1} for myosin (Godfrey & Harrington, 1970); $E_{280}^{1\%} = 2.00$ cm^{-1} for rod (Harrington & Himmelfarb, 1972); $E_{280}^{1\%} = 3.29$ cm^{-1} for LMM (Young et al., 1964); $E_{280}^{1\%} = 6.5$ cm^{-1} for HMM; $E_{280}^{1\%} = 7.5$ cm^{-1} for S-1. The concentration of S-2 was determined by biuret and Lowry measurements. The concentration of ATP was obtained by using the molar extinction coefficient $\epsilon_{M,260} = 15.4 \times 10^3$.

Centrifugation Experiments. The sedimentation velocity experiments were carried out at rotor speeds of (30–40) $\times 10^3$ rpm and temperatures close to 20 °C in a Spinco Model E analytical ultracentrifuge. These relatively high rotor speeds can be employed in sedimentation studies of myosin minifilaments since they do not dissociate under the pressure generated in the centrifuge (Reisler et al., 1980). Sedimentation of minifilaments in the presence of ATP was possible since the triphosphate nucleotide was not hydrolyzed by myosin in Tris–citrate buffer in the absence of KCl (Reisler et al., 1980). In all cases, double-sector cells, 12 mm in path length, were employed. The calculated sedimentation coefficients were reduced to standard conditions of water at 20 °C. Determinations of myosin "monomer–dimer" concentration in the presence of minifilaments, whenever made by centrifugation, followed the procedure of Harrington & Himmelfarb (1972).

Spectrophotometric Experiments. Optical densities of polymer solutions, at room temperature, at 310 nm, were recorded with a Beckman Model 25 spectrophotometer. The turbidity of the myosin and rod minifilaments obeyed Beer's law over the protein concentration range 0.01–0.3%. The turbidity of LMM solutions in Tris–citrate buffer (pH 8.0) was linear with protein concentration in the range 0.03–0.2%. Stock solutions of ATP and pyrophosphate were made in Tris–citrate buffer and added in small volumes to protein solutions. Appropriate corrections were made for the dilution effect. In the case of addition of Mg^{2+} to the ATP or pyrophosphate–myosin systems, additives were mixed prior to addition to the protein.

The fraction of minifilaments in equilibrium with monomer–dimer species was derived from turbidity measurements at 310 nm. Turbidity readings in the absence of ATP, pyrophosphate, or KCl were considered to correspond to a minifilament fraction of 1.0 and the readings in the presence of 0.5 M KCl to a zero fraction of minifilaments. Spectrophotometric estimates of minifilaments and monomer–dimer fractions agreed well with the centrifugation determinations.

Binding Analysis. The cooperative binding to ATP to minifilaments, as detected through their dissociation, can be analyzed according to the Hill equation:

$$\bar{\nu} = \frac{K[A]^n}{1 + K[A]^n}$$

where $\bar{\nu}$ is the average number of ligand A bound per macromolecule, K is the association constant, and n is the Hill coefficient. Following Harrington & Himmelfarb (1972), we assume that the fraction of binding sites occupied by substrate (ATP or pyrophosphate) and responsible for dissociation corresponds to the fraction of monomer–dimer in solution. This leads to a formulation (Harrington & Himmelfarb, 1972):

$$\frac{\theta}{1-\theta} = \frac{[\text{sites occupied}]}{[\text{sites vacant}]} = \frac{C_{\text{monomer–dimer}}}{C_{\text{minifilament}}}$$

and

$$\frac{\theta}{1-\theta} = K[A]^n$$

Circular Dichroism. CD measurements in the far-UV were carried out with a modified Jasco J-10 spectropolarimeter, at room temperature, employing a 1-mm path cell. When the effect of pyrophosphate on the secondary structure of myosin and its fragments was tested, considerable care was taken to ensure precise dilution of protein samples. In a typical experiment, 2-mL samples of 0.1 mg/mL protein stock solution were mixed in parallel with 0.1 mL of solvent and 0.1 mL of stock pyrophosphate solution, and their CD spectra were compared. At this protein concentration, the turbidity of minifilament solutions was negligible, and no scattering corrections were required. Differential absorption data were converted to a mean residue ellipticity, $[\theta]$ (deg cm^2/dmol), assuming the value of 114 for the mean residue molecular weight of amino acids. The α-helical content was calculated as described by Greenfield & Fasman (1969).

Electron Microscopy. Electron micrographs were taken at an operating voltage of 80 kV on a Philips 400 microscope. Samples were negatively contrasted by a double-layer carbon procedure described by Lake (1979).

Results

Dissociation of Myosin and Rod Minifilaments by ATP. Earlier work has shown that synthetic myosin filaments are dissociated in the presence of low concentrations (1–5 mM) of ATP, ADP, pyrophosphate, and relevant analogues (Harrington & Himmelfarb, 1972). The dissociation of filaments could be monitored spectrophotometrically by relating the observed turbidity at 310 nm (OD_{310}) to the concentration of the polymeric species or by examining the velocity sedimentation of myosin filaments in the presence of nucleotides. Analysis of the ATP-induced dissociation of myosin polymers was somewhat complicated by the potassium-stimulated hydrolysis of triphosphate nucleotide. Since the resulting diphosphate nucleotide was less effective than ATP in dissociating synthetic filaments, partial or complete regeneration of polymers occurred with ATP hydrolysis. Although adding Mg^{2+} to the myosin–ATP system could slow the hydrolysis process, it also depressed filament dissociation (Harrington & Himmelfarb, 1972).

The hydrolysis of ATP by myosin is effectively blocked in the myosin minifilament system since potassium is eliminated from the solvent composition. The minifilaments are highly homogeneous and stable in Tris–citrate buffer in the absence of monovalent salt (Reisler et al., 1980).

In Figure 1, we compare the changes in OD_{310} of the filament (in 0.13 M KCl and 0.025 M Tris, pH 8.0) and minifilament systems after addition of ATP. The initial decrease in the concentration of regular filaments (decreased OD_{310}) is gradually reversed with time due to the hydrolytic reaction (Harrington & Himmelfarb, 1972). It will be noted that minifilaments differ in three aspects from the filament system. Solutions of minifilaments have lower initial turbidity, are dissociated to a smaller extent by similar levels of ATP, and, due to the absence of Ca^{2+}, Mg^{2+}, and K^+, they do not hydrolyze ATP. The lower turbudity of minifilaments results from their size being smaller than that of filaments. Their relative stability in the presence of 2 mM ATP (Figure 1) could be caused either by a diminished nucleotide binding to myosin in the absence of KCl or be a reflection of strong binding interactions within the polymeric structure. Control

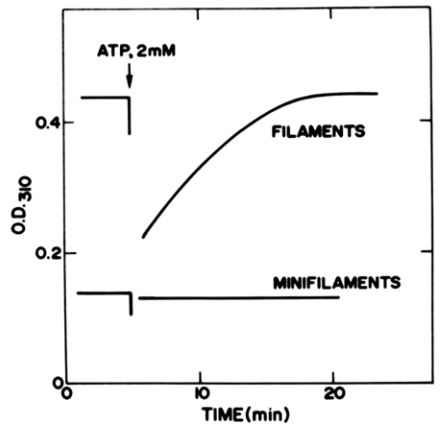

FIGURE 1: Turbidity changes in myosin filament (0.13 M KCl and 0.025 M Tris, pH 8.0) and minifilament (10 mM Tris–citrate, pH 8.0) systems following addition of ATP to a final concentration of 2 mM. ATP solution was adjusted to pH 8.0 before addition. Protein concentration 2 mg/mL; temperature 23 °C.

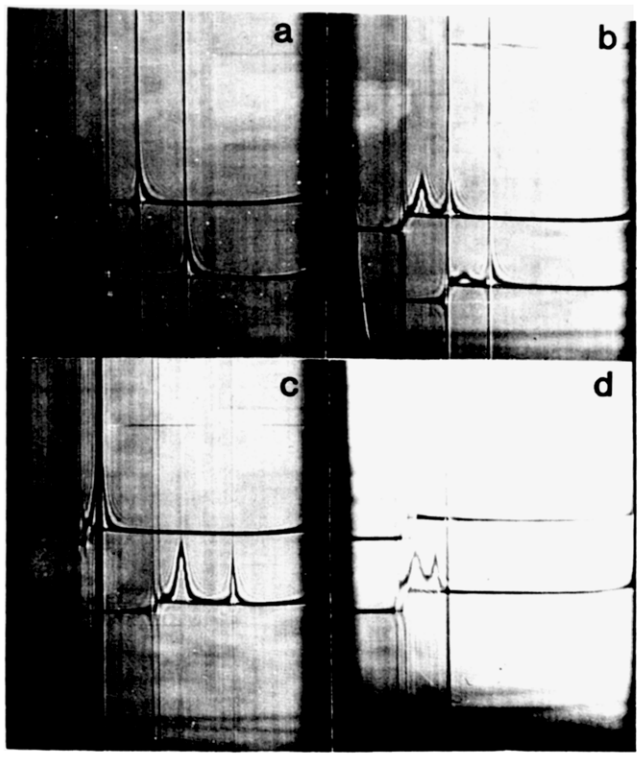

FIGURE 2: Velocity sedimentation patterns of synthetic myosin (a–c) and rod (d) minifilaments in the presence of ATP. Solvent is 10 mM Tris–citrate buffer, pH 8.0. Temperature 20–21 °C, protein concentration 3 mg/mL, rotor speeds 33 000 (a–c) and 39 000 rpm (d), bar angle 70°. (a) Myosin minifilaments in the absence of ligand; time of centrifugation 32 min, $s_{20,w}$ = 17.0 S (lower, regular cell); myosin minifilaments plus 2 mM ATP, $s_{20,w}$ = 15.8 S (upper, wedge cell). (b) Protein as in (a) plus 4 (lower, regular cell) and 6 mM ATP (upper, wedge cell). The repsective sedimentation coefficients, $s_{20,w}$, for the slow and fast peaks are 7.6 and 19 S for the regular cell and 7.3 and 19.8 S for the wedge cell. (c) Protein as in (a) plus 8 (lower, regular cell) and 12 mM ATP (upper, wedge cell). The respective sedimentation coefficients of the slow and fast peaks are 6.7 and 22.5 S for the regular cell and 6.8 S for the wedge cell. (d) Rod minifilaments (upper, wedge cell, $s_{20,w}$ = 9.6 S) and rod minifilaments plus 8 mM ATP (lower, regular cell, $s_{20,w}$ of the slow peak = 4.5 S).

ATP titrations of regular filaments (in 0.13 m KCl and 0.025 M Tris, pH 8.0) have been carried out in order to distinguish between these two simplest alternative explanations. A Hill plot of the filament dissociation pattern yields a Hill coefficient n = 2.3 [in agreement with Harrington & Himmelfarb (1972)] and an association constant $K = 5 \times 10^5$. A similar association constant ($K = 2 \times 10^6$) characterizes the binding of ATP to minifilaments.

Table I: Effect of 5 mM Pyrophosphate on the α-Helical Content of Myosin Fragments in Tris–Citrate Buffer[a]

protein	state of aggregation	Δ(α helix)[b] (%)	Δ$[\theta]_{221}$[c] (%)
myosin	minifilament	5 (71)	6 (24 600)
rod	minifilament	8 (95)	9 (34 700)
LMM	polymer	0 (90)	2 (33 300)
S-1	monomer	0 (34)	2 (14 700)
S-2	monomer	10 (85)[d]	11 (31 500)[d]

[a] CD measurements were carried out as described under Materials and Methods. All proteins were dissolved in 10 mM Tris–citrate buffer, pH 8.0 (at room temperature), and their spectra were recorded at room temperature. Each entry in the table represents an average of 3–5 measurements. [b] The α-helical content was calculated from ellipticity values at 209 nm according to Greenfield & Fasman (1969). Difference data [Δ(α helix)] represent the change in the α-helical content of proteins due to the addition of pyrophosphate. The reproducibility of these measurements was ±1%. The numbers in parentheses refer to the α-helical content of proteins in the absence of pyrophosphate (reproducibility ±3%). [c] Changes in the mean residue ellipticity at 221 nm due to the addition of pyrophosphate. The numbers in parentheses refer to ellipticities in the absence of pyrophosphate. Reproducibility of these estimates was the same as above. [d] The estimates of α helix and $[\theta]_{221}$ quoted for the S-2 fragment are less reliable because of the error margin involved in calorimetric determinations of protein concentration.

The sedimentation pattern of myosin minifilaments consists of a hypersharp polymer band with $s_{20,w}$ = 17.0 S and does not reveal the presence of any monomeric species (Figure 2a). This pattern agrees with our previous observations (Reisler et al., 1980). Although in the presence of 2 mM ATP the polymer band appears to be unchanged (Figure 2a), it sediments at a slower rate ($s_{20,w}$ = 15.8 S). It seems unlikely that this decrease in the sedimentation coefficient of minifilaments is due to their partial dissociation into somewhat smaller structures. The entire pattern of ATP-induced dissociation is that of a two-state system with monomer and/or dimers in equilibrium with minifilaments (Figure 2). Intermediate species are either absent or present at very low concentration. The observed change may be caused by a primary charge effect on the sedimentation of minifilaments. An alternative interpretation of the decreased sedimentation coefficient in the presence of 2 mM ATP could be provided by evoking conformational transitions in the minifilament structure. Detection of a pyrophosphate effect on the α-helical content of myosin (Table I) adds credibility to such speculation.

At higher ATP concentrations (>2 mM), myosin minifilaments dissociate into slowly sedimenting species with $s_{20,w} \approx 7$ S (Figure 2b,c). This sedimentation coefficient is higher than the one characteristic of myosin monomers ($s_{20,w}$ = 6 S) and could signify the presence of a considerable fraction of dimers in equilibrium with monomers. With increasing ATP leavels (from 4 to 12 mM), the $S_{20,w}$ value of the slow peak decreased from 7.6 to 6.7 S. The polymer boundary sediments in all cases at the rates of myosin minifilaments (Reisler et al., 1980). The apparent absence of intermediate dissociation products (Figure 2) justifies the simplified representation of minifilament depolymerization in terms of a two-state system: minifilaments and monomer–dimers. This, in turn, simplifies the spectrophotometric analysis of ATP binding to minifilaments.

Rod minifilaments, prepared by the same method as myosin minifilaments, behave in the presence of ATP like their myosin counterparts. Schlieren patterns of rod solutions containing ATP reveal two peaks: the minifilaments with $s_{20,w}$ = 9.6 S

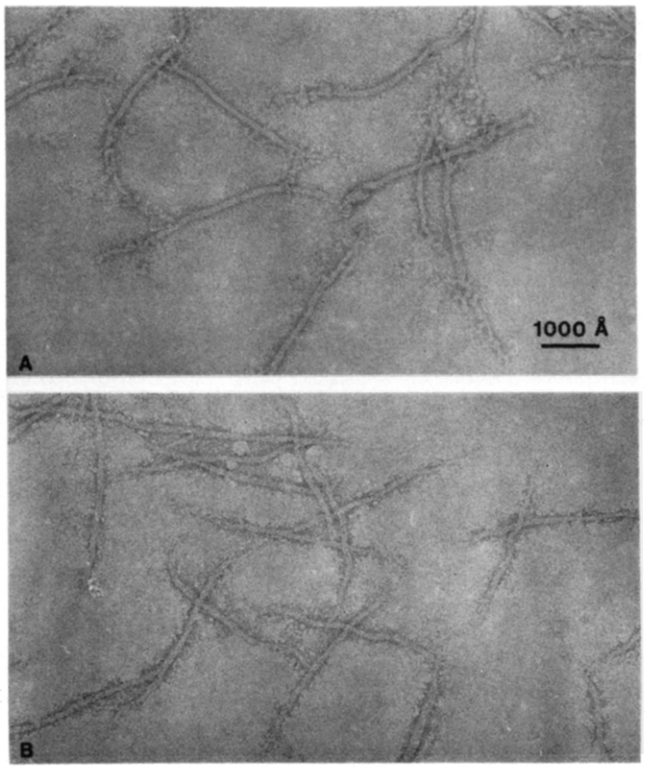

FIGURE 3: Electron micrographs of fields of myosin minifilaments (A) and of rod minifilaments (B). Both fields are taken at the same magnification.

and dissociated species with $s_{20,w}$ = 4.5 S (Figure 2d).

Electron micrographs (Figure 3) of rod minifilaments mimic those of myosin minifilaments (except for the projections due to myosin heads). Both have the same average length and diameter; both distribute over a rather narrow range of sizes. A similar conclusion, about the narrow size distribution of rod minifilaments, can be reached on the basis of sedimentation velocity runs (Figure 2). The appearance of LMM polymers, prepared in the same solvent system, is markedly different. Electron microscopy observations reveal polymorphic forms of LMM assemblies distributed over a wide range of sizes.

Binding of ATP and Pyrophosphate to Minifilaments. The structural similarity of rod and myosin minifilaments validates a direct comparison of their dissociation by nucleotides and pyrophosphate. Spectrophotometric titrations of myosin and rod minifilaments with ATP reveal cooperative decrease in the turbidity of these systems and their eventual complete dissociation at 10–15 mM substrate concentrations (figure 4). Similar titration curves, shifted toward higher ligand concentrations, were obtained with pyrophosphate. This contrasts with the decrease in the turbidity observed when ATP is added to LMM solutions, a decrease that is completed at about 6 mM ATP (Figure 4). However, as noted above, LMM does not assemble into minifilaments, and there is no structural basis for its quantitative comparison with the myosin and the rod systems.

The turbidity measurements of the myosin and rod minifilaments can be related to the concentration of these species in solution [See Materials and Methods; see also Harrington & Himmelfarb (1972)]. The apparent weight fractions of minifilaments, derived from data shown in Figure 4, are plotted in Figure 5 vs. the logarithm of substrate concentration. Strikingly, myosin and rod minifilaments are dissociated to the same extent at any given level of ATP. Similar representation of the pyrophosphate-induced dissociation of myosin (or rod) minifilaments shows a clear shift toward higher

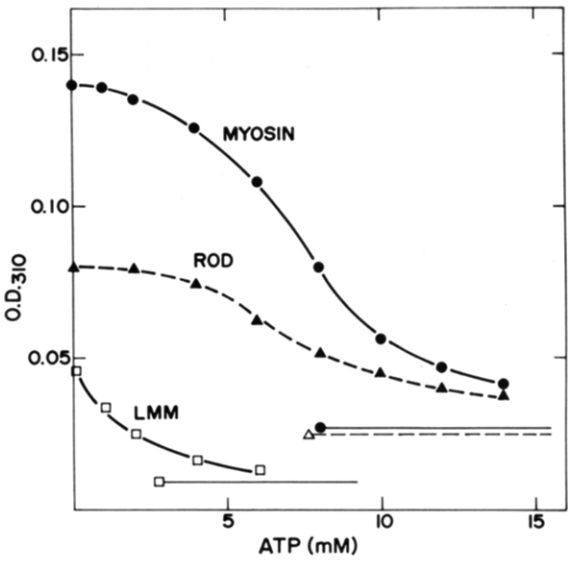

FIGURE 4: Turbidity changes in myosin (●) and rod (▲) minifilaments and in LMM polymers (□) as a function of added ATP. ATP solutions were adjusted to pH 8.0 before addition. Solvent is 10 mM Tris–citrate buffer (pH 8.0). Protein concentrations are the following: 2 mg/mL; rod, 2 mg/mL; LMM, 1.5 mg/mL.

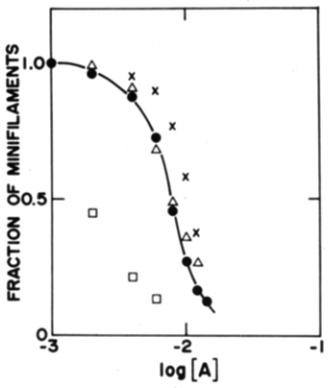

FIGURE 5: Fraction of minifilaments vs. logarithm of ligand concentration. Myosin (●) and rod (△) minifilaments and LMM (□) polymer data are taken from Figure 4 and refer to titrations of protein solutions with ATP. The dissociation profile of myosin minifilaments by pyrophosphate (×) was obtained under identical experimental conditions as those in the legend to Figure 4.

substrate concentrations but does not change the dissociation curve profile (Figure 5). This is not the case with LMM solutions, for which the transition from polymeric to dissociated units upon binding of ATP is considerably less cooperative. The Hill plot for myosin and rod minifilaments is shown in Figure 6. The two systems are essentially indistinguishable and can be represented by the same linear dependence of log $[\theta/(1-\theta)]$ on log [ATP] with a Hill coefficient of $n = 3$. Hill plots obtained for minifilament dissociation by pyrophosphate are linear over the entire range of substrate concentrations and show a complete overlap between the myosin and rod systems and a somewhat higher binding cooperativity ($n = 3.7$). Since the binding of substrates to the "dissociation" sites on myosin has the same characteristics for rod and myosin, the presence of high affinity binding sites for ATP and pyrophosphate on the myosin heads seems to have no effect on the dissociation of minifilaments by these substrates.

Effect of MgATP and Magnesium Pyrophosphate. Harrington & Himmelfarb (1972) noted that addition of equimolar amounts of Mg^{2+} greatly depressed the ATP-dependent depolymerization of filaments. Similarly, the dissociation of myosin and rod minifilaments by ATP and pyrophosphate is

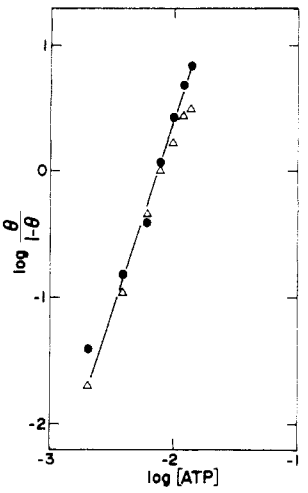

FIGURE 6: Hill plot for analysis of cooperativity of ATP binding to myosin (●) and rod (△) minifilaments. The ratio $\theta/(1-\theta)$ (sites occupied/sites vacant) was obtained as described under Materials and Methods.

much reduced in the presence of Mg^{2+}. The fraction of minifilament vs. log [A] profile (similar to those in Figure 5) is shifted with MgATP toward higher substrate concentrations (0.25 log unit). About the same shift (0.3 unit on the log [A] scale) has been found in control titrations of myosin filaments with ATP and MgATP. From observations like this, Harrington & Himmelfarb (1972) deduced that the filament dissociation depends on a specific ion and not simply on the ionic strength. More striking evidence against a simple ionic strength effect can be derived from minifilament titrations with KCl (at pH 8.0). Salt concentrations up to 40 mM do not induce any significant changes in the minifilaments (within 10–30 min) whereas higher concentrations of KCl (0.04–0.2 M) lead to growth of filaments (Reisler et al., 1980; C. Oriol-Audit, J. A. Lake, and E. Reisler, unpublished experiments). Clearly, the net effects of ATP and KCl on the minifilament system are diametrically opposite and cannot be reconciled with changes in ionic strength alone.

Structural Changes in Myosin Induced by Pyrophosphate Binding. The dissociation of myosin minifilaments (and filaments) by nucleotides and pyrophosphate involves their specific binding to the myosin rod. Harrington & Himmelfarb (1972) estimated this binding to be at the level of one to two molecules of substrate per myosin molecule. It was of interest to investigate whether the binding of charged substrates and the consequent dissociation of organized myosin structures are coupled to conformational changes in the rod segment.

Minifilaments, unlike regular filaments, are well suited for conformational studies because of their homogeneity and relatively low turbidity. In terms of their secondary structure and the entire far-UV profile, they are indistinguishable from myosin monomers (obtained by 50-fold dilution of minifilaments into 0.5 M KCl and 0.025 M Tris-HCl, pH 8.0, solvent). However, dissociation of minifilaments by pyrophosphate is accompanied by a significant decrease in the α-helical content of myosin and rod (Table I). The CD changes in Table I have been observed upon addition of pyrophosphate to a final concentration of 5 mM, but almost identical changes can be detected also in the presence of 10 mM pyrophosphate. At the lower substrate concentration, the minifilament fraction for both myosin and rod is estimated from turbidity titrations to be 0.9; at the higher pyrophosphate level (10 mM), the polymer fraction is about 0.6. Thus, it appears that CD measurements reveal structural changes in the rod segment which are caused by partial occupation of pyrophosphate binding sites and are not directly related to minifilament dissociation. Charge effects play an important role in eliciting these conformational changes. Addition of magnesium pyrophosphate (5 or 10 mM) to minifilaments does not affect their CD spectrum. The fact that a larger CD effect is noted with rods than with myosin is consistent with the finding that S-1 structure is not perturbed by pyrophosphate binding (Table I).

The spectral changes induced in myosin and rod minifilaments at low pyrophosphate concentrations (1 mM) are within the experimental accuracy of these measurements (±1%). An equally small perturbation of myosin and rod (1–2%) is caused by addition of high levels of pyrophosphate (10 mM) to the monomeric form of these proteins (in 0.5 M KCl and 0.025 M Tris-HCl, pH 8.0).

As shown in Figure 4, LMM polymers in Tris–citrate buffer are dissociated at relatively low concentrations of ATP and pyrophosphate. However, the α-helical content of LMM does not decrease with pyrophosphate binding to polymers (Table I) or monomers of this fragment. The largest CD changes are noticed with the S-2 fragment dissolved in Tris-citrate buffer (Table I). Clearly, pyrophosphate binding sites must be located on this fragment, and their occupation leads to a substantial destabilization of the protein.

Detection of CD changes caused by the binding of ATP to minifilaments or to myosin fragments is not possible because of the large UV absorption by the nucleotide. At the spectroscopically accessible ATP levels (10^{-4} M), no perturbation of the CD pattern in the far-UV can be detected.

Discussion

The present study has established that myosin minifilaments, consisting of 16–18 molecules and resembling the central part of thick filaments (Reisler et al., 1980), constitute a valid and attractive system for studying conformational changes in assembled myosin. In order to prove this thesis, we have examined the dissociation of minifilaments by ATP, pyrophosphate, and their metal complexes and compared the properties of the minifilament–substrate system with those of the previously described filament–substrate system (Harrington & Himmelfarb, 1972). We have chosen this particular set of experiments for several reasons: (i) The assembly "potential" of myosin is confined to its rod segment. Consequently, in any new assembly form of myosin, we should inquire into the environment and properties of the rods. (ii) The dissociation of filaments by ATP and relevant analogues is a direct probe of their binding to the rod segment of myosin (Harrington & Himmelfarb, 1972). (iii) The sensitivity of the binding measurements is increased many fold through the effect of the bound ligand on the polymer stability. (iv) We wished to find out whether the substrate-induced changes in the polymer stability could be related to or be a consequence of structural perturbation of the protein.

The binding of the same substrates to the high-affinity sites located on the myosin heads is irrelevant to minifilament dissociation. Unambiguous demonstration of this fact has been made possible with the preparation of rod minifilaments which morphologically resemble the myosin minifilaments. As a side point, it is interesting to note that the growth-terminating mechanism operates in the assembly of rod as well. Rod minifilements not only have the same appearance but also have the same general width and length as their myosin counterparts. This similarity forms the structural basis for directly comparing their substrate binding, and other properties. Futhermore, both types of minifilaments are dissociated by ATP and pyrophosphate in the same manner; i.e., these sub-

strates bind to both structures with the same affinity and the same level of cooperativity. Together, the combined evidence of the structural and functional identity in both types of minifilaments argues strongly that the essential features of minifilament assembly and structure, as well as their dissociation, reside in the portion of the myosin molecule common to both. Hence, it is extremely unlikely that myosin heads play any detectable role in ligand-induced destabilization of the minifilaments. The relative importance of LMM and S-2 segments in maintaining the minifilament structure could not be assessed since LMM does not form structures comparable to minifilaments.

The results of our ATP and pyrophosphate titrations of myosin minifilaments reveal their striking similarity to the regular filaments. Like filaments, the minifilaments are progressively dissociated with the increasing substrate concentrations. This dissociation yields a two-state system of monomer–dimer in equilibrium with minifilaments and with no apparent presence of intermediate species. Thus, such a pattern of dissociation resembles the previously described depolymerization of filaments by ATP, pyrophosphate, etc. (Harrington & Himmelfarb, 1972). The binding of ATP and pyrophosphate to minifilaments is highly cooperative and is characterized by approximately the same Hill coefficients and binding constants as in the case of filaments (Figures 5 and 6 and the text). Also, in analogy to filaments, Mg^{2+} complexes of ATP or pyrophosphate are much less efficient in dissociating the minifilaments than the free substrates. In view of these common properties and behavior of myosin filaments and minifilaments, we may expect that binding of ATP or pyrophosphate to myosin triggers similar events in both structures. Furthermore, extrapolating from the filament system (Harrington & Himmelfarb, 1972), we may assume that these two assembled forms of myosin dissociate because of repulsive electrostatic interactions. Such interactions are induced by the binding of 1–2 substrate molecules to the low-affinity binding sites on each myosin (rod) molecule. Thus, if conformational changes accompany the above dissociation, they are expected to occur within the rod segment of the molecule.

Direct CD comparisons of rod filaments and monomers could not have been undertaken in the past because of considerable turbidity and heterogeneity of the rod and myosin assemblies. However, the minifilament systems are largely free of such complications and can be checked against the monomeric proteins. Although we have not detected any changes in the α-helical content of myosin and rod upon dissociation of their minifilaments (by adding 0.5 M KCl), this result is not conclusive. It could be interpreted to mean that in the high-salt environment (0.5 M KCl), which favors the monomeric state of the protein, the S-2 structure is stabilized by monovalent cations.

In the absence of KCl, when the dissociation of minifilaments is affected by pyrophosphate, the observed structural changes are unambiguous. A well-defined and reproducible decrease in the α-helical content of myosin and rod minifilaments is detected even prior to any substantial dissociation of these structures by pyrophosphate. The decrease, amounting to 5 and 8% for myosin and rod minifilaments, respectively, is saturated at 5 mM pyrophosphate. At this substrate concentration, 90% of the protein remains in the minifillament form. Numerically, the observed spectral changes closely correspond to the predicted decreases in the α-helical content of myosin and rod, should those arise from the melting of S-2 alone [at room temperature, about 15% of the S-2 structure exists in the unfolded coiled state (Tsong et al., 1979)]. This is consistent with our observation that the secondary structure of S-1 is not perturbed by pyrophosphate binding. Pyrophosphate (5 mM), although fully dissociating the LMM polymers, does not induce any detectable change in the far-UV spectrum of this protein. This further supports our suggestion that the structural perturbation of myosin induced by pyrophosphate binding is localized in the S-2 fragment. Results of direct CD titrations of S-2 with pyrophosphate are consistent with this conclusion. The observed effect upon binding of pyrophosphate to S-2 corresponds to a 10% decrease in the α-helical content. Since the estimate of the original α-helical content of S-2 in the absence of pyrophosphate is the least precise among all determinations (because of indirect protein concentration determinations), it is doubtful whether quantitive comparisons of the observed structural changes should be presently carried to any further extent.

The detection of conformational changes in the myosin and rod minifilaments and their localization in the S-2 segment are consistent with the basic postulate of the Tsong et al. (1979) model for contraction. According to this model, the contractile force is generated through a α-helix–coil transition within the S-2 element during each cycle of the cross bridge. Tsong et al. (1979) and Sutoh et al. (1978) suggest that the α-helical conformation of the S-2 fragment is stabilized in the resting state by the adjacent myosin molecules within the thick filament. Upon activation, the S-2 segment is believed to be released from the stabilizing environment of the thick filament surface and to undergo a rapid melting process. Under physiological conditions, 150–200 residues of S-2 located next to the LMM–HMM junction could undergo a α-helix–coil transition on a time scale close to the cycle time of a cross bridge (Tsong et al., 1979). Our study suggests that an overall change occurs within the original myosin structure without substantial, or possibly any (under physiological conditions), disruption of the filament backbone. The mechanism of the pyrophosphate- (or ATP-) induced structural change is presently unknown. An obvious question which awaits resolution is whether the above changes are triggered, as suggested by Harrington and his collaborators, by the release of S-2 from the filament surface. Preliminary support for such an idea has been obtained by following the rate of cross-linking of myosin heads and the S-2 fragment to the thick filament surface (S. Oda and E. Reisler, unpublished experiments). In these experiments carried out with regular synthetic filaments in 0.1 M KCl, at pH 7.0, as described by Sutoh et al. (1978a), we have noticed that substituting 10 mM phosphate for 40 mM imidazole buffer results in much reduced rates of cross-linking of S-1 and S-2 fragments to the filament backbone. Thus, it appears that in the presence of phosphate ions the cross bridges may be released from the filament surface. Similar investigation of the minifilaments should provide the desired information on the radial disposition of cross bridges under conditions conducive to melting of the S-2 fragment.

It seems now very likely that myosin filaments can undergo structural changes which originate in the S-2 region and are pertinent to contraction. The striking effects of pyrophosphate and probably ATP, the largely diminished effects of their metal complexes, and the almost complete lack of structural transitions in high-salt solutions (monomeric rod and myosin) suggest an important role for charge interactions in the observed processess. Future work should delineate the physiological substrates involved in the possible structural modulation of the thick filament and couple such modulation to contractile events.

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References

Godfrey, J. E., & Harrington, W. F. (1970) *Biochemistry 9*, 886-893.
Gratzer, W., & Lowey, S. (1969) *J. Biol. Chem. 244*, 22-24.
Greenfield, N., & Fasman, G. D. (1969) *Biochemistry 8*, 4108-4117.
Harrington, W. F., & Burke, M. (1972) *Biochemistry 11*, 1448-1455.
Harrington, W. F., & Himmelfarb, S. (1972) *Biochemistry 11*, 2945-2952.
Josephs, R., & Harrington, W. F. (1966) *Biochemistry 5*, 3474-3487.
Lake, J. A. (1979) *Methods Enzymol. 61*, 250-257.
Mendelson, R. A., & Cheung, P. (1976) *Science (Washington, D.C.) 194*, 190-192.
Morales, M. F. (1955) *Physiol. Rev. 35*, 475-505.
Morales, M. F., & Boots, J. (1953) *Discuss. Faraday Soc. 13*, 125-130.
Reisler, E., Smith, C. A., & Seegan, G. (1980) *J. Mol. Biol. 143*, 129-145.
Rome, E. (1967) *J. Mol. Biol. 27*, 591-602.
Seidel, J. C., & Gergely, J. (1971) *Biochem. Biophys. Res. Commun. 44*, 826-831.
Sutoh, K., Chiao, Y. C., & Harrington, W. F. (1978a) *Biochemistry 17*, 1234-1239.
Sutoh, K., Sutoh, K., Karr, T., & Harrington, W. F. (1978b) *J. Mol. Biol. 126*, 1-22.
Thomas, D. D., Seidel, J-C. Gergely, J., & Hyde, J. S. (1975) *J. Supramol. Struct. 3*, 376-390.
Tsong, T. Y., Karr, T., & Harrington, W. F. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 1109-1113.
Weeds, A. G., & Taylor, R. S. (1975) *Nature (London) 257*, 54-57.
Weeds, A. G., & Pope, B. (1977) *J. Mol. Biol. 111*, 129-157.
Young, D. M., Himmelfarb, S., & Harrington, W. F. (1964) *J. Biol. Chem. 239*, 2822-2829.

Assay of Total Estradiol Receptor in Tissue Homogenate and Tissue Fractions by Exchange with Sodium Thiocyanate at Low Temperature†

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ABSTRACT: After injection of radioactive estradiol to ovariectomized rats, the [^{3}H]estradiol–receptor complex transferred to the nuclei can be solubilized by low concentrations of NaSCN. The extraction by NaSCN is significantly more efficient than that obtained by KCl and is, in fact, complete; i.e., no radioactivity can be found in the nuclei after extraction. Since NaSCN also induces the exchange of receptor-bound estradiol with free hormone [Sica, V., Puca, G. A., Molinari, A. M., Buonaguro, F. M., & Bresciani, F. (1980) *Biochemistry 19*, 83], a simple assay method has been set up which measures receptor in tissue and tissue fractions, including nuclei and whole homogenate, at 0–4 °C, irrespective of whether the receptor is or is not interacting with endogenous hormone. The procedure consists of a simple incubation step at 0–4 °C overnight (16 h) of the nuclear fraction, cytosol, and a total homogenate in the presence of excess radioactive estradiol and 0.5 M NaSCN. This method is very easy to carry out, accurate, and precise and avoids the loss of binding sites which results from the heating procedures utilized in other methods. The ability to measure the binding in both the soluble and the particulate fractions of rat uterus permits the determination of the rate of the cytoplasmic to nuclear transfer of estrogen after injection of various hormone concentrations. No nuclear transfer was observed after administration of other nonestrogen hormones such as progesterone, testosterone, or hydrocortisone while a nonsteroid antiestrogen, tamoxifen, was able to translocate the receptor. It was found that 2 h after injection of estradiol into ovariectomized rats total receptor content of uterus shows a decrease which is proportional to the amount of hormone injected. After injection of a hyperphysiological dose of 17β-estradiol, a certain amount of the receptor–hormone complex remains in the cytosol for at least 4 h. The nuclear turnover of estradiol receptor related to the progesterone receptor induction has been studied. Actinomycin D and cycloheximide prevent nuclear processing.

When estradiol receptor is assayed in normal or neoplastic tissues of postpuberal animals, [^{3}H]estradiol[1] binding assays conducted at 0 °C do not measure preformed receptor–estrogen endogenous complexes because at temperatures close to 0 °C the dissociation rate of the estrogen–receptor complex is so low that only minimal exchange occurs during incubation time (Truong & Baulieu, 1971; Sanborn et al., 1971; Katzenellenbogen et al., 1973).

Because of the obvious importance of being able to assess total receptor content of the cell, and specifically the receptor complexed to endogenous hormone in the nucleus, there have been numerous attempts to optimize conditions for increasing

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[1] Abbreviations used: [^{3}H]estradiol, 17β-estradiol-6,7-t_2; DTT, dithiothreitol; DCC, dextran-coated charcoal; TED buffer, 10^{-2} M Tris-HCl, 10^{-3} M EDTA, and 10^{-3} M dithiothreitol, pH 7.4.

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