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Optical Rotatory Dispersion of G-Actin

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Optical rotatory dispersion data for G-actin prepared by several different methods are presented. Reproducible values of $[\alpha]_D = -44°$, $\lambda_c = 252$ mμ, and $b_0 = -184$ were obtained for purified preparations obtained by cold-extraction or by repeated polymerization and depolymerization, including a Mg^{++}-induced polymerization step. Actin which had been extracted at room temperature or subjected to only a single Mg^{++}-induced polymerization step gave rotatory dispersion values suggesting the presence of a protein other than native G-actin-ATP. The presence of a protein with the properties of tropomyosin in preparations which had been extracted at room temperature was confirmed. From the change in optical rotatory properties and the increased susceptibility to proteolytic digestion by a bacterial protease, by chymotrypsin, and by trypsin after the loss of bound nucleotide, it is concluded that the loss of the bound nucleotide causes a change in the protein conformation. Optical rotatory dispersion reveals three structural states: the most ordered state in the presence of the bound nucleotide ($b_0 = -184$), a less ordered state after the bound nucleotide is removed ($b_0 = -74$), and a random coil found in concentrated urea or guanidine-HCl solution ($b_0 = 0$).

The muscle protein, actin, has a tightly bound adenine nucleotide (Straub and Feuer, 1950; Laki *et al.*, 1950; Mommaerts, 1952b), which undergoes dephosphorylation during polymerization and a rephosphorylation (Strohman, 1959), or, more likely an exchange reaction (Martonosi *et al.*, 1960) upon depolymerization *in vitro*. The removal of the bound nucleotide from actin is an irreversible process under most conditions, although under certain conditions a time-dependent partial reversibility has been observed (Bárány *et al.*, 1961a,b; Grubhofer and Weber, 1961; Maruyama and Gergely, 1961; Strohman and Samorodin, 1962). It has been suggested that the dissociation of G-actin and ATP is accompanied by a conformational change of the protein (Asakura, 1961; Strohman and Samorodin, 1962).

This study concerns the optical rotatory properties of actin with and without its bound nucleotide. Some optical rotatory dispersion data on actin have been published by Kay (1960) and some unpublished observations are mentioned elsewhere (Asakura, 1961; Laki and Kenton, 1961). Data will be presented which indicate that Kay's observation represents the special case of a nucleotide-free actin. Data will also be given showing that changes in optical rotatory parameters and in the susceptibility to proteolytic digestion indicate changes in the conformation of actin when the bound nucleotide is lost.

MATERIALS AND METHODS

Three different methods of actin preparation were used. Crude actin was prepared according to Bárány and Bárány (1959). The acetone-dried powder was extracted with 25 volumes of H_2O for 20 minutes at room temperature (warm extract) or at 4° (cold extract, Drabikowski and Gergely, 1962) and purified by ultracentrifugation according to the method of Mommaerts (1951).

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The F-actin pellet was homogenized in a Potter-Elvehjem type homogenizer and diluted to a protein concentration of about 6–8 mg/ml with 4×10^{-4} M ATP, 10^{-3} M tris(hydroxymethyl)-aminomethane (Tris) buffer, *p*H 7.6, and was depolymerized by dialysis against 50 volumes of the same ATP solution with inside-outside stirring for 24 hours. The small amount of polymer actin at the end of dialysis was removed by centrifugation at 105,000 × *g* for 3 hours and the remaining G-actin was used in further experiments, since the results with the second and third sedimentation by the polymerization-depolymerization cycle did not change the measured parameters.

The third actin preparation was made from the purified G-actin. Martonosi (1961) reported that G-actin equilibrated with a $MgCl_2$ concentration ranging from 4×10^{-4} to 8×10^{-4} M in 10^{-3} M Tris buffer, *p*H 7.6. in the presence of about 4×10^{-4} M ATP showed a fast-moving broad main peak which comprises the polymerized actin, followed by a smaller peak and a slow-moving sharp peak in ultracentrifugal analysis. The slow-moving component can be separated from the polymerized actin by a 3-hour centrifugation in the Spinco preparative ultracentrifuge, No. 40 head, at 40,000 rpm. The upper layer is removed with a syringe and a long needle. It is called the "γ-component" (Martonosi, 1962). The very viscous bottom part is polymerized actin and is diluted to a protein concentration of about 6 mg/ml with 4×10^{-4} ATP solution, *p*H 7.6, and dialyzed against 4×10^{-4} ATP, 10^{-3} M Tris, *p*H 7.6, to decrease the Mg^{++} concentration to $< 10^{-6}$ M for depolymerization. The G-actin thus formed was freed from any polymers by centrifugation as described above and was used in further experiments.

It is usually considered that a preparation of G-actin-ATP is pure if it gives a single peak on ultracentrifugal sedimentation, or if, after polymerization, all of the protein can be sedimented in the ultracentrifuge. Neither of these criteria can be regarded as an absolute indication of purity. We estimated the per cent purity of the native G-actin from the number of moles of inorganic phosphate liberated for each 60,000 g of protein during polymerization of G-actin preparations (3–5 mg protein/ml) in solutions containing 0.1 M KCl, 4×10^{-4} M ATP, 10^{-4} M $MgCl_2$, 10^{-3} M Tris buffer, *p*H 7.6. The molecular weight of the monomer actin was taken as 60,000, based on recently published molecular weight determinations in the range of 57,000 to 66,000 (Mommaerts, 1952a; Kay, 1960; Laki and Standaert, 1960; Ulbrecht *et al.*, 1960).

Dowex 1 (Cl^-) (200–400 mesh) suspension in an equal volume of 10^{-3} M Tris buffer, *p*H 7.6, was used for removal of the free nucleotide from the actin solutions (Hayashi and Tsuboi, 1960; Asakura, 1961). G-actin containing 4×10^{-4} M free ATP and 10^{-3} M Tris buffer, *p*H 7.6, was mixed with 0.1 volume of the Dowex 1 suspension at room temperature for 5 minutes with frequent stirring.

Protein was determined by the biuret method (Gornall *et al.*, 1949; Bárány and Bárány, 1959) calibrated by the Kjeldahl method. ATP was purchased from the Sigma Chemical Company. Inorganic phosphate was determined by the method of Rockstein and Herron (1951).

The viscosity of actin was measured at 25° in an Ostwald-type viscometer with an outflow time of 40 seconds at 25°, in a total volume of 4 ml.

Sedimentation analysis was carried out with a Spinco model E analytical ultracentrifuge.

Optical rotation was measured with a Rudolph model 80 photoelectric spectropolarimeter with an oscillating polarizer prism. The light source was a General Electric A-H6 water-cooled high-pressure arc or a Hanovia 510B mercury-xenon compact-arc light. The symmetrical angle was set at 2°. All measurements were performed in 10-cm polarimeter tubes with quartz end-plates. The wave length range used was 310-600 mμ. Protein concentration varied from 1–6 mg/ml. Measurements were made at room temperature. When measurements under 310 mμ were attempted the symmetrical angle was set at 15° and a fused quartz cell of 1 cm length was used. The actin concentration was 1–2 mg/ml, which corresponds to absorbancy values of over 1.0 at low wave lengths.

With the exception of purified "γ-component," all of the data for actin preparations from 330–600 mμ fitted the one-term Drude equation, $[\alpha]_\lambda = \frac{K'}{\lambda^2 - \lambda_c^2}$. The refractive index correction term $\left(\frac{n^2+2}{3}\right)$ is incorporated into the rotation constant K'. The constant, λ_c, was calculated graphically with the modified plot of Yang and Doty (1957). The constant, b_0, of Moffitt's (1956) equation,

$$[m']_\lambda = \frac{a_0\lambda_0^2}{\lambda^2 - \lambda_0^2} + \frac{b_0\lambda_0^4}{(\lambda^2 - \lambda_0^2)^2}$$

was obtained graphically (Moffitt and Yang, 1956) from the slope of plots of $[m']\ (\lambda^2 - \lambda_0^2)$ against $(\lambda^2 - \lambda_0^2)^{-1}$. Tentatively, per cent "helix content" is given assuming that $b_0 = -630$ for a 100% helical protein (Cohen and Szent-Györgyi, 1957). "Helix content," calculated from λ_c as proposed by Yang and Doty (1957) or by an alternate relationship (Urnes and Doty, 1961), gives an excellent agreement with the calculation based on b_0 value. λ_0 was taken as 212 mμ. In Moffitt's equation the data are expressed in terms of $[m']_\lambda$, defined as

$$[m']_\lambda = [\alpha]_\lambda \times \frac{\text{MRW}}{100} \times \frac{3}{n^2 + 2}$$

where $[\alpha]_\lambda = (100\ \alpha)/(lc)$, and α is the observed rotation in degrees, l is the length in decimeters, c is the concentration of the protein solution in

g/100 ml, MRW is the mean amino acid residue weight, n is the refractive index of the solvent, and λ is the wave length at which the observation is made. The MRW was taken as 115. Refractive index values were taken at the sodium D line for water and guanidine HCl and at the measured wave lengths for urea solutions[1]; sample calculations demonstrated that for the wave lengths and rotations measured in this study there is no significant difference in λ_c or b_0 values introduced by corrections for the dispersion of refractive index in water or urea solutions.

For proteolytic digestion three enzymes were used. A bacterial protease, nagarse, was obtained from the Teikoku Chem. Industry Co., Ltd., Osaka, Japan. Crystallized, salt-free trypsin was obtained from Armour Laboratories, England. β-Chymotrypsin, three - times crystallized, was a Worthington Biochemical Co. product. The nucleotide-free actin for digestion was prepared by two methods: (1) Denaturation of the actin by 8 M urea, followed by removal of urea by dialysis; and (2) the addition of EDTA at room temperature, which completely removes the bound nucleotide from actin in 60 minutes (Bárány *et al.*, 1961b; Grubhofer and Weber, 1961; Maruyama and Martonosi, 1961; Strohman and Samorodin, 1962), followed by dialysis or Dowex 1 treatment. The rate of proteolytic digestion was followed by use of the ninhydrin reagent according to Moore and Stein (1954).

Polymerizability of EDTA-treated actin was followed with time as the change of relative viscosity after addition of 3×10^{-4} M ATP, 0.1 M KCl, and 10^{-4} M $MgCl_2$, to Dowex 1-treated samples. The viscosity was measured 60 minutes after addition of salt at a protein concentration of 2 mg/ml.

The nucleotide content of actin solutions was determined spectrophotometrically from the filtrate at 260 mμ after precipitation of the Dowex 1–treated actin with perchloric acid. The molar extinction coefficient was taken as 14,200 for the adenine nucleotide (Cohn and Carter, 1950). The effect of 10^{-3} M EDTA on the bound nucleotide content was measured as follows: EDTA was added to Dowex-treated G-actin–ATP solution. At time intervals samples were removed, the dissociated ATP was removed by Dowex 1 treatment, and the bound nucleotide was measured spectrophotometrically in the filtrate after denaturation of actin by 3% perchloric acid.

Results

Optical Rotatory Properties of Actin Extracted at Room Temperature.—The room temperature–extracted actin was prepared as described in the Materials and Methods section. The G-actin–ATP content of these preparations, as determined by the phosphate-liberation method, varied from 75 to 86%. The optical rotatory dispersion data on several of these preparations are given in Table I. V[b] shows that removal of the *free* nucleotide from the solution has little or no effect on the rotatory dispersion properties. The lower part of Table I gives the data on room temperature–extracted actin subjected to various treatments. If the G-actin–ATP is polymerized to F-actin, and then depolymerized with 0.6 M KI in the presence of free ATP, the rotatory dispersion values are the same as for G-actin–ATP which is treated with 0.6 M KI without previous polymerization. Both of these values are practically the same as the values of untreated G-actin–ATP. These data suggest that the conformation of G-actin–ATP is the same whether it is prepared in the conventional way or prepared from F-actin by KI depolymerization in the presence of free ATP. The data also show that KI does not affect the conformation of G-actin–ATP in the presence of free ATP. However, if G-actin–ATP is dialyzed against repeated changes of 0.6 M KI in the absence of free nucleotide, the bound nucleotide is removed and an irreversible change in the conformation of the protein occurs, as reflected by the change in rotatory properties. This state, however, still differs from the completely random coil structure. The characteristic dispersion constants for a completely random coil protein structure can be brought about only

TABLE I

OPTICAL ROTATORY DISPERSION OF SEVERAL ACTIN PREPARATIONS EXTRACTED AT ROOM TEMPERATURE AND PURIFIED ACCORDING TO MOMMAERTS

G-Actin Preparation[a]	$-[\alpha]_D$, deg.	λ_c, mμ	$-b_0$	"% Helix"
I	41	262	248	39
II	43	261	243	39
III	43	260	225	36
IV	40	261	243	39
V	42	260	225	36
V[b]	45	259	219	35
F-Actin (KI, ATP)	40	260	248	39
G Actin (KI, ATP)	41	260	248	39
G Actin (KI)[c]	60	227	70	11
G Actin, 8 M urea	110	213	0	0
G-Actin, 8 M urea	112	213	0	0
G-Actin, 8 M urea	108	215	0	0
G-Actin, 3 M guanidine-HCl	104	217	0	0
F-Actin, 8 M urea	105	216	0	0
F-Actin, 3 M guanidine-HCl	108	214	0	0

[a] Protein concentration = 1–6 mg/ml, in 4×10^{-4} M ATP, 10^{-3} M Tris buffer, pH 7.6. KI solutions were 0.6 M, containing 0.006 M $Na_2S_2O_3$. Free ATP was removed from the G-actin solutions before treatment with urea or guanidine-HCl by Dowex 1 treatment. [b] After removal of the free nucleotide by Dowex 1 treatment. [c] Dialyzed against repeated changes of 0.6 M KI containing 0.006 M $Na_2S_2O_3$ and 10^{-3} M Tris buffer, pH 7.6.

[1] Values for the dispersion of refractive index were taken from unpublished data of Foss, Kang, and Schellman, kindly provided by Dr. G. D. Fasman.

TABLE

OPTICAL ROTATORY DISPERSION OF ACTIN PREPARATIONS PURIFIED BY PARTIAL POLYMERIZATION WITH Mg^{++}

Actin Preparations[a]	$-[\alpha]_D$, deg.	λ_c, mμ	$-b_0$	"α Helix"
I[b]	51	244	153	24
II[b]	49	240	132	21
III[b]	48	242	146	23
I[c]	51	244	152	24
IV[d]	44	255	185	29
γ-Component[e]	59	241	151	24
45–75% $(NH_4)_2SO_4$ fraction[f]	28	nonlinear	490	78

[a] Protein concentration = 1–6 mg/ml in 4×10^{-4} M ATP, 10^{-3} M Tris buffer, *p*H 7.6. [b] Mg^{++}-purified actin preparations. [c] Free ATP removed by Dowex 1 treatment. [d] Additional KCl polymerization and depolymerization of Mg^{++} purified actin. [e] Average value of four preparations. [f] After fractionation of γ-component at 0°, the $(NH_4)_2SO_4$ was removed by dialysis against 10^{-3} M Tris buffer, *p*H 7.6.

by treatment with a high concentration of urea or guanidine-HCl. Dowex treated G- or F-actin in a high concentration of urea or guanidine-HCl give the same rotatory dispersion parameters. Values obtained after such treatment are given in Table I.

The Purity of Actin Extracted at Room Temperature.—During an attempt to determine the rotatory properties of partially polymerized actin, we used Martonosi's method (1961) to prepare the "γ-component." The preparation, however, does not have the properties of actin. The optical rotatory properties of such preparations are given in Table II. In the ultracentrifuge, the preparation sedimented as a sharp single peak at a protein concentration of 5 mg/ml and two very close distinct peaks at a concentration of 1 mg/ml protein in the presence of 4×10^{-4} M $MgCl_2$, 10^{-3} M Tris, *p*H 7.6, 4×10^{-4} M ATP. Its intrinsic viscosity in the same solution is 1.8, a relatively high value. Addition of KCl to a final concentration of 0.1 M did not cause polymerization; instead the relative viscosity of the solution dropped significantly (in a 2 mg/ml solution from 2.0 to 1.2). This behavior is similar to that of tropomyosin. Tropomyosin has previously been reported to be a persistent impurity in actin preparations (Laki and Cairns, 1959; Martonosi, 1962).

On ammonium sulfate fractionation, at least two proteins differing in salting-out properties can be obtained from the γ-component: one which is precipitated at an ammonium sulfate saturation of 45%, and a second which is precipitated from 45–75% ammonium sulfate saturation. After removal of salt by dialysis, the main part of the fraction precipitated at 45% ammonium sulfate saturation remains insoluble. The second fraction is soluble and its rotatory dispersion parameters are: $[\alpha]_D = -28°$, $b_0 = -490$ (Table II). The rotatory properties of tropomyosin crystallized according to Bailey (1948) are: $[\alpha]_{578} = -16.0°$, $b_0 = -620$ (Cohen and Szent-Györgyi, 1957). Myosin (Cohen and Szent-Györgyi, 1957) and γ-myosin (Dubuisson, 1948; Kay and Pabst, 1962) are the only other known proteins from rabbit muscle with high b_0 values, but these proteins are denatured under the conditions for the preparation of the tropomyosin-like fraction. The low dispersion values observed with the crude γ-component may have two explanations: (1) The high rotatory dispersion of tropomyosin is counteracted by the low dispersion values of other protein impurities, such as denatured actin. (2) There is an interaction between the tropomyosin and another protein, perhaps actin, resulting in an altered conformation and lower dispersion.

There is a possibility that one or more of the components of the γ-component is a degradation or association product of the actin caused by treatment with Mg^{++}. However, a second treatment of the purified G-actin with Mg^{++} does not give a γ-component after ultracentrifugal separation. A salt-free solution of the ammonium sulfate–fractionated γ-component with high rotatory dispersion values has a relative viscosity of 6 in a concentration of 2.5 mg/ml in 10^{-3} M Tris buffer, *p*H 7.6, which drops to a relative viscosity of 1.5 on the addition of 0.1 M KCl. The γ-component amounts to about 10–20% of the total G-actin extracted at room temperature.

Properties of Actin Purified by Polymerization with Dilute $MgCl_2$.—Actin may be separated from the γ-component by ultracentrifugation after polymerization with dilute Mg^{++}. After depolymerization and another ultracentrifugation step, to remove any polymers, the preparation gives a 100% polymerizable actin. The inorganic phosphate liberated during the complete polymerization of this preparation is only 40–70% of the expected value (Table IV). The optical rotatory properties, despite the large variation in phosphate liberation, do not vary significantly in several preparations (Table II). After a further KCl-induced polymerization step, followed by depolymerization, a purified preparation is obtained which exhibits rotatory dispersion values identical to those of cold-extracted actin (Table II and see below). This preparation contains 1.01 mole of nucleotide and liberates 0.99 ± 0.02 moles of inorganic phosphate/60,000 g on polymerization (Table IV).

Optical Rotatory Properties of Cold-Extracted Actin Preparations.—Table III gives the values obtained with these preparations. Preparation III in this table was the purest G-actin–ATP obtained by this method. It yielded 96% inorganic phosphate during polymerization and its nucleotide content was 98%. This preparation was used for determining rotatory data under different conditions. As was observed with the actin extracted at room temperature, the removal of the free nucleotide does not affect the rotatory

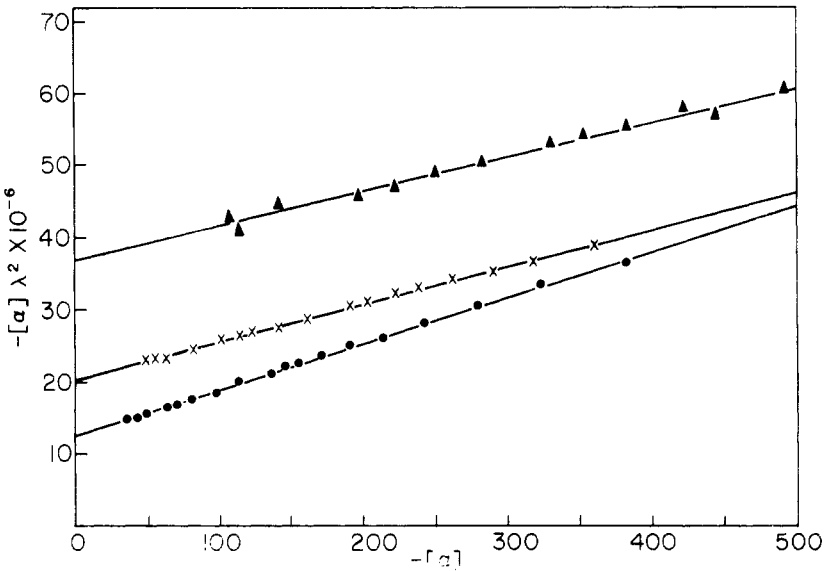

FIG. 1.—Representative examples of the Yang and Doty modified plots of the Drude equation used for calculation of λ_c values (Table III). Native cold-extracted G-actin–ATP, ●; same, after irreversible dissociation from the bound nucleotide with EDTA and Dowex 1 treatment, x; same, in 8 M urea, ▲.

TABLE III

OPTICAL ROTATORY DISPERSION OF SEVERAL ACTIN PREPARATIONS EXTRACTED IN THE COLD FOR 20 MINUTES AND PURIFIED ACCORDING TO MOMMAERTS

G-Actin Preparation[a]	$-[\alpha]_D$, deg.	λ_c, mμ	$-b_0$	"% Helix"
I	43	254	185	29
II	44	252	181	29
III	44	252	183	29
III[b]	44	252	184	29
III + 10^{-3} M EDTA[b,c]	51	243	140	22
III + 10^{-3} M EDTA, 24 hr.[b]	69	228	74	12
III + 8 M urea[b]	108	214	0	0
III removal of urea by dialysis[b]	68	226	63	10
III extensive dialysis, bound nucleotide lost	69	226	63	10

[a] Protein concentration = 2–5 mg/ml in 4 × 10^{-4} M ATP, 10^{-3} M Tris buffer, *p*H 7.6. [b] Free ATP removed by Dowex 1 treatment. [c] Reading made within 30 minutes.

TABLE IV

THE INTRINSIC VISCOSITY, $s_{20,w}$, AND PHOSPHATE LIBERATION ON POLYMERIZATION OF THREE PREPARATIONS OF G-ACTIN[a]

Actin Preparation	$[\eta]$	$s_{20,w}$	Phosphate Liberated[b]		
Room-temperature extraction	0.4–0.8[c]	3.6	81	86	75
Cold extraction	0.12	3.2	92	96	94
Mg^{++} purification	0.1	3.2		40–70	
Pure G-actin–ATP[d]	0.1	3.2		99 ± 2	

[a] The three preparations are as described in Materials and Methods. [b] $\frac{\text{moles inorganic phosphate}}{60{,}000 \text{ g protein}} \times 100$. Values obtained from different preparations. [c] Extrapolations of a plot of η_{sp}/c versus c values from a protein concentration range of 2.0–6.0 mg/ml. The plots showed no concentration dependence for the pure actin preparations, but a considerable concentration dependence for the room-temperature–extracted preparations, requiring a large extrapolation for the estimation of $[\eta]$. [d] Additional KCl polymerization and depolymerization of Mg^{++} purified actin.

properties of G-actin–ATP. Addition of 10^{-3} M EDTA partially removes the bound nucleotide within 30 minutes (Bárány *et al.*, 1961b; Grubhofer and Weber, 1961; Maruyama and Martonosi, 1961; Strohman and Samorodin, 1962) and the rotatory properties begin to change. After 24 hours the bound nucleotide removal by EDTA is complete and the rotatory values are lowered, but still not to the level of a completely random coil structure. The same rotatory values were found after removal of the bound nucleotide by extensive dialysis. The G-actin in 8 M urea solution gives rotatory values characteristic of the completely random coil state of globular proteins (Jirgenson, 1961; Urnes and Doty, 1961). When the urea is removed by dialysis, the rotatory properties approach those found after removal of the bound nucleotide by EDTA or by dialysis.

Two representative plots of optical rotatory data for cold-extracted preparations are given in Figures 1 and 2.

Other physicochemical parameters of the three different actin preparations are given in Table IV. Under the conditions used, the cold-ex-

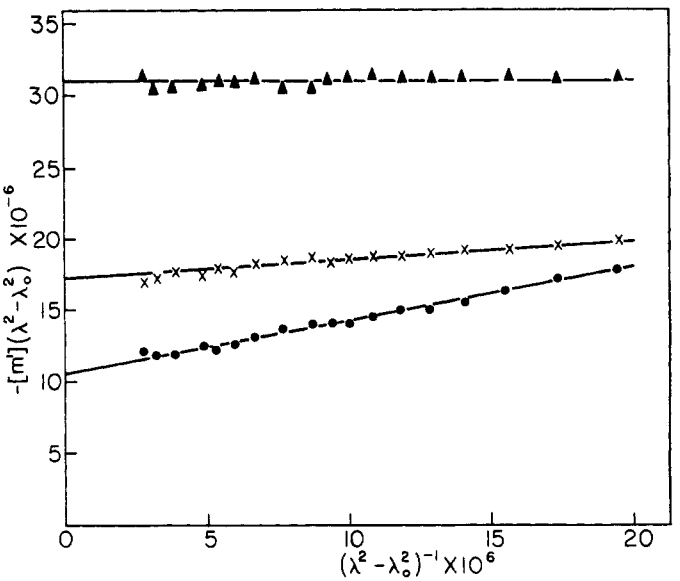

FIG. 2.—An example of Moffitt plots used for calculation of b_0 values. Conditions and symbols as in Figure 1.

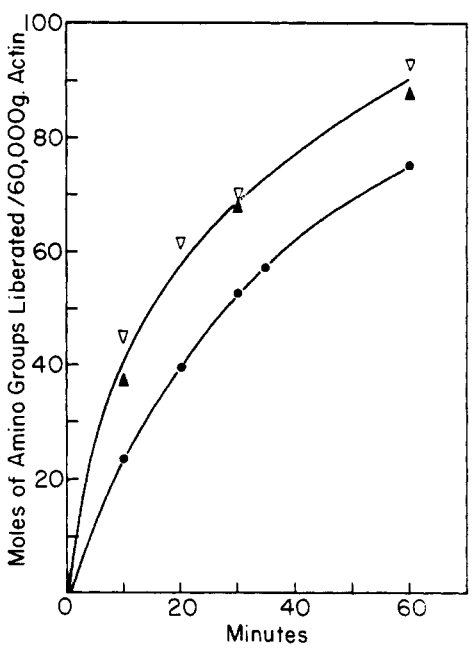

FIG. 3.—The rates of proteolytic digestion by nagarse of native G-actin–ATP and nucleotide-free G-actin in the presence and absence of excess ATP at 25° in 0.1 M phosphate buffer, *p*H 7.8. Weight ratio of actin/nagarse = 20. When free ATP was present, its concentration was 3.3×10^{-4} M. Native G-actin-ATP, ●; G-actin without bound nucleotide, ▲; G-actin without bound nucleotide, free ATP added, ▽.

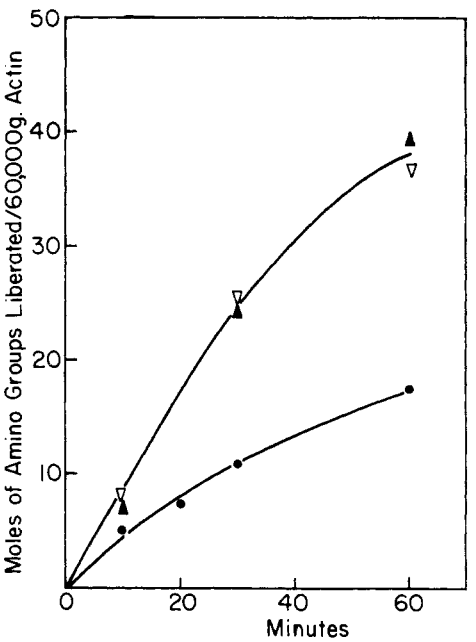

FIG. 4.—The velocity of proteolysis by chymotrypsin of native G-actin–ATP and nucleotide-free G-actin in the presence and absence of excess ATP. Weight ratio of actin/chymotrypsin = 10. Conditions and symbols as in Figure 3.

tracted and the Mg^{++}-purified actin preparations do not show significant differences in $[\eta]$ or in $s_{20,w}$, although the Mg^{++}-purified preparation has somewhat lower $[\eta]$. The reduced viscosity of both the cold - extracted and Mg^{++} - purified preparations showed only a slight concentration dependence. The room temperature–extracted preparations has higher $[\eta]$ and $s_{20,w}$ values than either of the other preparations.

Proteolysis of ATP-Actin and ATP-Free Actin by Nagarse, Chymotrypsin, and Trypsin.—The structural change of the actin molecule when it loses its bound nucleotide is reflected also by the

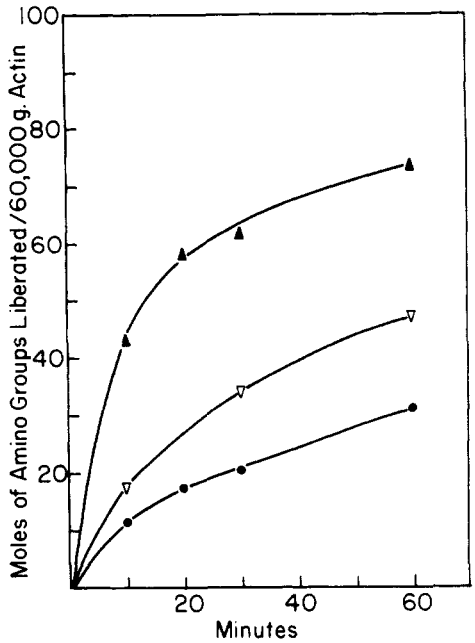

FIG. 5.—The velocity of proteolysis by trypsin of native G-actin–ATP and nucleotide-free G-actin in the presence and absence of excess ATP. Weight ratio of actin/trypsin = 20. Conditions and symbols as in Figure 3.

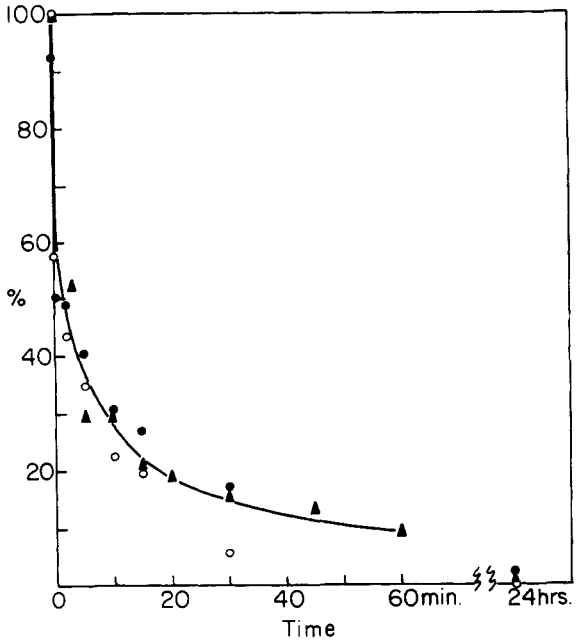

FIG. 6.—The effect of 1×10^{-3} M EDTA on the nucleotide content, polymerizability, and optical rotation at 500 mμ of cold-extracted actin, 5 mg/ml protein, in 1×10^{-3} M Tris buffer, pH 7.6, at room temperature. Per cent bound nucleotide content, ●; polymerizability, ○; [α] at 500 mμ, ▲.

relative change of the rate of proteolysis by proteolytic enzymes. In Figure 3 is shown the proteolysis by nagarse, a protease from *B. subtilis*. Figures 4 and 5 show the proteolysis by chymotrypsin and trypsin, respectively, of actin solutions similar to those shown in Figure 3. The native actin was digested in the presence of 3.3 $\times 10^{-4}$ M free ATP. After removal of the bound nucleotide the protein was digested by the proteolytic enzymes in the absence of ATP and, as a control, in the presence of 3.3×10^{-4} M ATP. The rate of digestion of nucleotide-free actin by nagarse and chymotrypsin is not influenced by added ATP. In the case of trypsin, the rate of digestion in the presence of ATP was not as high as in ATP-free medium. Nevertheless, the rate of proteolysis of G-actin without bound nucleotide in each case is significantly higher than that of the corresponding native G-actin–ATP. Similar changes in susceptibility to proteolytic attack by nagarse, trypsin, and chymotrypsin were observed on the addition of EDTA to G-actin–ATP in nucleotide-free solution, but are not shown in the figures. The enzymes used are not affected by EDTA.

Rate of Bound Nucleotide Removal, Change of Relative Viscosity, and Change of Specific Rotation at 500 mμ by EDTA Treatment at Room Temperature.—As shown in Figure 6, the removal of the bound nucleotide parallels the loss of polymerizability and the negative change in specific rotation. The rotatory dispersion parameters of EDTA-treated G-actin–ATP, 30 minutes after EDTA addition, given in Table III, are between those of native G-actin–ATP and those of ATP-free actin after 24 hours.

DISCUSSION

Numerous studies on actin have shown that the bound adenine nucleotide is necessary for stability in crude or purified actin preparations (Straub and Feuer, 1950; Laki *et al.*, 1950; Mommaerts, 1952b; Ulbrecht *et al.*, 1960; Bárány *et al.*, 1961a,b). The loss of bound nucleotide is irreversible after a few minutes, and free nucleotide (ATP, ADP, ITP) in sufficient concentration has a protective effect (Szent-Györgyi, 1951; Szent-Györgyi and Joseph, 1951; Grubhofer and Weber, 1961; Maruyama and Gergely, 1961; Strohman and Samorodin, 1962). There is a large body of evidence which suggests that the attachment of ATP to G-actin is through a two-point binding involving Ca^{++} and an —SH group (Straub and Feuer, 1950; Bárány, 1956; Bárány *et al.*, 1956; Martonosi and Gouvea, 1961; Strohman, 1961; Strohman and Samorodin, 1962.) Asakura (1961) and Strohman and Samorodni (1962) suggested that a configurational change would account for the irreversibility of nucleotide dissociation. The irreversibility is not prevented by —SH containing compounds, and it was concluded that it cannot be due to oxidation of the —SH group.

The method of optical rotatory dispersion is well established (Djerassi, 1960; Urnes and Doty, 1961) and, despite its uncertain theoretical justification, it is a useful empirical tool for determining changes in protein conformation. If it is assumed that only α-helical and random coil conformations contribute to the observed rotation of a protein, the fraction of the residues in a helical conformation may be estimated from the rotatory dispersion parameters λ_c and b_0 (Schellman and Schellman, 1958, 1961; Urnes and Doty, 1961); the results of such calculations are given in the tables as "% helix."

Preparations of G-actin–ATP which have been prepared by extraction in the cold or purified by repeated polymerization with dilute Mg^{++} and KCl exhibit optical rotatory values of $[\alpha]_D = -43°$, $\lambda_c = 254$ mμ, and $b_0 = -185$.[2] These preparations appear to be relatively free of impurities, and their rotatory parameters may be taken to be those of native G-actin–ATP. By the methods of calculation referred to above, it may be estimated that these preparations have approximately 29% α-helical content.

Our conclusion that the "γ-component," an impurity in room temperature–extracted actin, is tropomyosin, at least in part, confirms the similar conclusion reached by Martonosi by different methods (1962; in press, 1962). This conclusion is based on: (1) precipitation by >45% saturation with ammonium sulfate, (2) a high relative viscosity in salt-free solution, which drops on the addition of 0.1 M KCl, and (3) a b_0 value of -490 for the partially purified "γ-component."

Actin which has been purified by a single polymerization with dilute Mg^{++} (Martonosi, 1962) is completely polymerizable, but has slightly different rotatory properties from more highly purified actin and liberates only 40 to 70% of the expected amount of inorganic phosphate on polymerization. Further investigation is required to define the properties of this material, but it is of interest in this connection that actin may, under certain conditions, undergo polymerization without ATP splitting (Prágay, 1957; Hayashi and Rosenbluth, 1960; Grubhofer and Weber, 1961; Hayashi and Rosenbluth, 1962).

Our results confirm the conclusion of Drabikowski and Gergely (1962) that extraction in the cold greatly reduces contamination of actin by tropomyosin. Cold extraction was suggested previously (Tsao and Bailey, 1953; Mommaerts, 1958; Ulbrecht *et al.*, 1960), but its effect on actin purity was not reported.

The loss of bound nucleotide is accompanied by changes in all of the rotatory parameters. The dispersion constants are $\lambda_c = 226$–228 mμ and $b_0 = -63$ to -74 for nucleotide-free actin (Table III). These dispersion constants do not depend on the method used for nucleotide removal (Table III and also in Table I). The values obtained are very similar to the value given by Kay (1960), of $\lambda_c = 223$, for a preparation which had been dialyzed against repeated changes of 0.5 M KI, 0.05 M K_2HPO_4, 0.025 M KH_2PO_4, at *p*H 7. Such treatment causes loss of the bound nucleotide and irreversible denaturation of the actin.

Actin in concentrated urea or guanidine solutions exhibits an $[\alpha]_D$ value of $-110°$ and the dispersion constants $\lambda_c = 214$ and $b_0 = 0$ (Tables I and III). These values are characteristic of a completely random coil state. Removal of urea by dialysis causes the protein to take up a more ordered conformation, which gives the same rotatory values as are found after removal of the bound nucleotide by dialysis, EDTA treatment, or prolonged 0.6 M KI treatment in the absence of free ATP.

The changes in the rotatory parameters upon removal of the bound nucleotide probably reflect a change in the conformation of the protein, in the same direction as is generally found upon protein denaturation (Schellman and Schellman, 1958; Jirgenson, 1961; Schellman and Schellman, 1961). The change in the value of b_0, from -185 to -70, is the most reliable indicator of such a change (Cohen and Szent-Györgyi, 1957; Simmons *et al.*, 1961; Beychok and Blout, 1961). However, the binding of chromophores to asymmetric macromolecules may induce a Cotton effect in the region of light absorption by the chromophore (Beychok and Blout, 1961; Stryer and Blout, 1961; Li *et al.*, 1962). The possibility must, therefore, be considered that the observed changes in the optical rotatory parameters might reflect change in rotation due to loss of asymmetrically bound nucleotide, rather than a change in the conformation of the protein on removal of the bound nucleotide. Attempts to measure such a Cotton effect near the absorption maximum of ATP at 260 mμ were unsuccessful, because of the high absorbancy of the solutions in this region. Preliminary measurements at still lower wave lengths also did not provide satisfactory quantitative data, because of high absorbancy, but did reveal a decrease in levorotation followed by a small positive rotation at wave lengths below 240 mμ, which is similar to that found for other globular proteins and which is presumably associated with the helical content of these proteins (Jirgenson, 1962; Zimmerman and Schellman, 1962).

To obtain evidence from an independent method, the change in the susceptibility to proteolytic digestion upon removal of the bound nucleotide was determined with three different enzymes (Fig. 3–5). The susceptibility of proteins to proteolytic digestion is increased by structural changes in the direction of denaturation (Putnam, 1953), and similar effects are found

[2] Tonomura *et al.* (1962) have recently reported values of b_0, obtained in very dilute solutions, ranging from -174 to -249 for F-actin in 0.6 M KCl and 0 for F-actin in 9.15 M urea. We have not observed the variability of rotatory properties with different G-actin preparations which was recently reported by Standaert and Laki (1962) and is attributed by these authors to varying amounts of F-actin in their preparations.

with enzymes when bound coenzymes are removed. Shifrin and Kaplan (1960) concluded that the latter effect could be accounted for if the coenzyme holds the protein structure together by bridging through helices or folds of the protein, as has been suggested by Dixon (1955) and London *et al.* (1958). The G-actin–ATP binding may be analogous to such enzyme-coenzyme binding. The change in optical rotatory properties and the increased susceptibility to proteolytic enzymes on loss of the bound nucleotide, as well as the irreversibility of this loss, all strongly suggest a conformational change of actin.

The following scheme would account for the data presented. Steps I and II are in agreement with Grubhofer and Weber (1961).

$$\underset{\text{I}}{\text{G-actin ATP} \rightleftharpoons \text{ATP} + \text{G-actin}} \longrightarrow \underset{\text{II}}{\text{G}'\text{-actin}} \underset{\text{III}}{\rightleftharpoons \text{G}''\text{-actin}}$$

Step I is reversible. The rate in both directions must be sufficiently fast to permit a complete exchange of bound ATP with labeled C^{14}-ATP within a few minutes at 0–2° (Martonosi *et al.*, 1960; Strohman and Samorodin, 1962). Step II is irreversible, and the formation of G′-actin is accelerated if the free nucleotide is continuously removed by Dowex 1 treatment, or if G-actin–ATP is treated with EDTA in the absence of free nucleotide. Nucleotide-free G-actin has a half-life of only a few minutes, which is decreased at room temperature compared to 0°, and by the presence of EDTA (Fig. 6., Grubhofer and Weber, 1961; Maruyama and Gergely, 1961; Maruyama and Martonosi, 1961; Strohman and Samorodin, 1962). Step III is a reversible change of conformation caused by concentrated urea or guanidine-HCl. However, prolonged treatment (10–20 hours) with concentrated urea or guanidine-HCl causes slow aggregation and precipitation of G″-actin.

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Charge Distribution of Fibrinogen as Determined by Transient Electric Birefringence Studies*

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This paper reports the results of a study of the transient electric birefringence of bovine fibrinogen in the *p*H regions of 4.0 to 5.0 and 7.0 to 9.0. A transverse permanent dipole moment leading to negative birefringence in the low *p*H region has been found; anomalous titration behavior associated with it suggests an interpretation which involves an all-or-none titration of several dissociable groups. The presence of more than one fibrinogen species in solution at the same time has been established from analysis of the birefringence transients. The presence of a longitudinal permanent dipole moment in the high *p*H region, as found by previous workers, has been verified. The magnitude and *p*H dependence are consistent with the titration of two α-amino groups, one at each end of the molecule, a distance of about 250 A from the center. From the results and interpretations given the conclusion is reached that the distribution of groups titrating in the *p*H regions studied is symmetrical about the center of the protein molecule.

A recent study by Hartley and Waugh (1960) of the solubility behavior of bovine fibrinogen has indicated that native clottable fibrinogen may exist in a variety of isomeric structures. Because of differences in folding of the polypeptide chains such isomers may differ in number and location of available dissociable groups. A valuable technique for the study of charge distribution in an anisometric molecule like fibrinogen is the method of transient electric birefringence, which is highly sensitive to changes in electrical symmetry. This technique has the additional advantage of providing a continuous check on the gross structure of the molecule through measurement of the rotational diffusion coefficient.

Although previous investigations of the transient electric birefringence of bovine fibrinogen have been reported (Tinoco, 1955a; Billick and Ferry, 1956) use of the more sensitive high-speed apparatus developed by O'Konski and Haltner (1956, 1957) has permitted a more critical study

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