The small oligomers formed from Mg-G-actin under favorable conditions were studied by sedimentation velocity ultracentrifugation. The critical concentration of actin at pH 7.8 in the presence of 100 μM MgCl₂ and 200 μM ATP was 12.5 ± 2.8 μM. Under these conditions, about 15% of 7.5 μM Mg-actin was converted to oligomers of subunit size four to eight in 5 h at 20 °C. In 100 μM MgCl₂ and no free ATP, the critical concentration was about 6.5 μM, and about 22% of 7.5 μM Mg-actin was converted to dimers in 80 min. There were no detectable higher oligomers or F-actin present in either case. As determined by the analysis of ATP hydrolysis, most, if not all, of the oligomer subunits contained ATP. When 28.5 μM actin was polymerized to steady state in 100 μM MgCl₂ and 200 μM ATP, about 50% of the actin was present as F-actin, consistent with the critical concentration (~12.5 μM), about 50% as oligomers as large as seven subunits, and only about 5% as monomers. When solutions containing oligomers were diluted the oligomers dissociated. Alternatively, when the MgCl₂ concentration was raised to 1 mM, the solutions containing oligomers polymerized more rapidly than monomeric Mg-G-actin and to the same final steady state. These data are entirely consistent with the condensation-elongation model for helical polymerization proposed by Oosawa and Kasai (Oosawa, F., and Kasai, M. (1962) J. Mol. Biol. 4, 10–21) according to which, under certain conditions, substantial amounts of short linear and helical oligomers should be formed below the critical concentration and linear oligomers should coexist with monomers and F-actin at steady state.

In 1962, Oosawa and Kasai (1) developed the theory for the helical polymerization of macromolecules in contrast to the generally accepted concept of linear polymerization. For simple linear polymers, in which each subunit interacts with no more than two other subunits, monomer and polymer are present at all concentrations of the macromolecule. The relative amounts of each are determined, according to the laws of mass action, by the total subunit concentration and by a simple equilibrium constant. On the other hand, helical polymerization, where each subunit in a helical polymer interacts with at least four other subunits, is similar, but not identical, to the condensation of a gas or to the crystallization of a solute. In the most simplified analysis, only monomer will exist until a certain critical concentration of macromolecules is reached after which all additional subunits will be present as helical polymer, the monomer concentration remaining fixed at the critical concentration. Kinetically, if helical polymerization is initiated with monomers above the critical concentration, the monomers will, relatively slowly, condense to form nuclei which will then rapidly elongate until the monomer concentration falls to the critical concentration and the system reaches a steady state.

In recent years, experiments in a number of laboratories (reviewed in Refs. 2–5) using highly purified protein and sensitive assays for the formation of polymers have confirmed and extended the earlier observations of Oosawa and his colleagues (reviewed in Refs. 1, 6, and 7) that the polymerization of actin fulfills the theoretical criteria for helical polymerization (see "Discussion"). However, the Oosawa-Kasai model is more complex than is implied by this simplified description. For example, it was recognized from the beginning that long filaments could fragment and reanneal (1, 6), and some investigators have found it necessary to introduce terms for at least one of these processes to model their experimental data for actin polymerization (8, 9). More importantly in the present context, Oosawa and Kasai (1, 6, 7) recognized that short actin oligomers will coexist with G-actin monomers and F-actin filaments both during the polymerization process and at steady state. Although the concentration of these small oligomers is too low to measure experimentally under the conditions generally used to study actin polymerization, their formation must be included to fit a theoretical model to the polymerization data. In one study (10), it was calculated that trimer nuclei continue to form until the overall polymerization process is about 80% complete, and, in another study (11), the kinetics of formation of oligomers ranging in size from dimer to hexamer were explicitly included in the model. However, except under certain special circumstances (e.g. the polymerization of cross-linked dimers (12) or when a very high concentration of dimers is formed before the onset of elongation (13)), there is no compelling reason to believe that either fusion of oligomers or the addition of oligomers to elongating filaments has a significant role in the polymerization process as normally studied in vitro.

Our current interest in oligomers derives from the possibility that, although (or perhaps because) the concentration of short actin oligomers in cells may never be very high, small oligomers could provide an excellent site for the biological regulation of the polymerization process. In fact, Lambooy and Korn (14, 15) have recently described a protein, actobinin, that may regulate actin polymerization in vitro by interacting with small oligomers (16). Thus, we decided to investigate the properties of small actin oligomers that might coexist in vivo with G-actin monomers and F-actin polymers.

Oosawa and Kasai (1, 6, 7) pointed out that because actin polymerization is essentially one-dimensional it is not a pure condensation process and, under appropriate experimental
conditions, relatively high concentrations of oligomers can exist with monomers when the total actin concentration is below and even slightly above the critical concentration. Several investigators have taken advantage of this phenomenon to study the formation of actin oligomers under conditions where the critical concentration is high enough to allow analysis of the composition of the unpolymerized fraction (17–19).

In the experiments described in this paper, oligomers formed from Mg-actin below and above its critical concentration in the presence and absence of free ATP were characterized and quantified by analytical ultracentrifugation. This procedure provided information about the sizes of the different macromolecular species that were present. Also, the extent of ATP hydrolysis accompanying actin polymerization was determined, and the polymerization competence of the oligomers and their ability to dissociate when diluted were assessed. We found convincing evidence for the formation of polymerization competent oligomers comprising two to perhaps eight actin-ATP subunits, depending upon the solvent conditions, when the total actin concentration was below or slightly above the critical concentration. Also, short oligomers seemed to coexist with F-actin in a steady state.

**EXPERIMENTAL PROCEDURES**

Muscle G-actin was prepared from a lyophilized powder of rabbit leg and back muscles by the method of Spudich and Watt (20). Pyrenylactin was prepared and the extent of labeling quantified by the procedure of Koyama and Mihashi (21) as modified by Brenner and Korn (22). G-actin and pyrene-G-actin in buffer containing 2 mM Tris (pH 7.8), 20 mM CaCl$_2$, 100 mM dithiothreitol, 200 mM ATP, and 0.01% sodium azide (buffer A) were clarified by centrifugation at 150,000 × $g$ for 2 h before loading onto a Sephacryl 200 column and were eluted with the same buffer at 2°C. Fractions were pooled from the top two-thirds of the elution profiles and were used within 30 h of collection. The concentration of G-actin was determined either by the absorbance at 290 nm, using a molar extinction coefficient of 62,000 M$^{-1}$ cm$^{-1}$ (23), or by the method of Lowry et al. (24). The purity of the G-actin was monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (25). For some experiments, free ATP was removed from the actin solutions by using the anion exchange resin AG-1X8 (26). Just before the start of an experiment, Ca$^{2+}$ in buffer B was converted to Mg-G-actin by simultaneously adding 50 μM EGTA and 50 μM MgCl$_2$ (final concentrations) (27) and incubating at 20°C for 10–20 min. Only actin preparations that showed at least a 25-fold increase in fluorescence (6–10% pyrenylactin) when polymerized by addition of 2 mM MgCl$_2$ were used.

G-actin was labeled with $[^{32}P]$ATP, and the bound nucleotide (0.02–0.06 mol/mol) was determined by the method of Oosawa and Kasai (1) and elaborated with respect to small macromolecular species that were present. Also, the extent of ATP hydrolysis accompanying actin polymerization was determined, and the polymerization competence of the oligomers and their ability to dissociate when diluted were assessed.

The scans were then fit with the function

$$C_0 = \sum_i c_{i,j} \exp(n_i(r^2 - F^2)) / (1 + \exp(n_i(r^2 - F^2))) + b$$

(1)

obtaining the values of the fitting parameters by means of nonlinear least-squares curve-fitting (31). It can be readily demonstrated (see Appendix) that this function, while arbitrary, will give the radial position, $r$, that corresponds to the second moment position of the boundary provided that

$$r_2 = 2r^2 - r_0^2.$$  

(2)

In these equations, $c_{i,j}$ is the plateau concentration of the ith component at time $t$, $n_i$ is a boundary width parameter related to the apparent diffusion coefficient, and $b$ is the base-line value for the absorbance in the depleted region of solution adjacent to the meniscus. The quality of the fit can be assessed by determining whether or not it satisfies Equation 2, by examining the overall appearance of the fit, by examining the distribution of the residuals, and from the root mean square error of the fit. The relationship of the fitting function to second moment analysis is discussed briefly in the Appendix and in greater detail elsewhere.

In all cases the scans were fit for single and multiple boundaries. The smallest number of components possible were used for fitting the data, using improvement of the values of the root mean square error and the coefficient of determination as criteria. In order to visualize the concentration gradients, the equivalents of Schlieren patterns were obtained by plotting the first derivatives of Equation 1 with respect to radius as functions of radial position in the cell for each component using the parameters obtained from fitting the concentration distributions. The sedimentation coefficients were obtained from conventional plots of the natural logarithm of $r$ as a function of time and corrected for buffer viscosity and density to give values of $s_{20,w}$.

All reagents used were of analytical grade. ATP and EGTA were purchased from Sigma; N-(1-pyrene)iodoacetamide was obtained from Molecular Probes; AG-1X8 was from Bio-Rad; $[^{32}P]$ATP (10 Ci/mmol) was from Du Pont-New England Nuclear. All solutions were prepared in deionized water.

**THEORY**

The helical polymerization theory for actin was developed by Osawa and Kassai (1) and elaborated with respect to small oligomers by Newman et al. (17). In the most general case, at equilibrium

$$C_0 = C_i(1 - K_iC_i) + \sigma C_i(1 - K_iC_i)^2 = (c_0 + 2eK_0)$$

(3)

where $C_i$ is the total actin concentration; $C_i$ is the monomer concentration; $K_i$ is the association constant for the linear polymer; $\sigma = \gamma(K_i/K_0)^2$, where $\gamma$ is related to the extra energy
Formation of Actin Oligomers

Theoretical plots of the formation of oligomers according to the Oosawa-Kasai model of polymerization of macromolecules. The percent of total subunits in linear oligomers (panel A) and the average degree of polymerization (panel B) of the linear oligomers are plotted as functions of the ratio of the total subunit concentration, $C_0$, to the association constant for linear polymerization, $K_i$. The curves were calculated using only the first term of Equation 3 (see text). The percent of total subunits in helical oligomers (panel C) and the average degree of polymerization (panel D) are plotted as functions of the ratio of the total subunit concentration, $C_0$, to the association constant for helical polymerization, $K_h$. Curves 1-4 are for $\sigma$ values of 0.0001, 0.001, 0.01, and 0.1, respectively. These curves were calculated using Equation 4.

RESULTS

Formation of Oligomers—To find whether experimental observations would support the Oosawa-Kasai theory, it was first necessary to establish conditions in which the critical concentration was sufficiently high to allow analysis by sedimentation velocity of all of the actin species that might be present. We used conditions similar to those of Newman et al. (17), 0.2 mM ATP and 0.1 mM MgCl$_2$ in low ionic strength (buffer B), and obtained a similar $C_c$ of $12.5 \pm 2.8 \mu M$ (Fig. 2) for five different actin preparations. Sedimentation velocity

$$C_c = C_0 + \sigma C_i/(1 - K_i C_i)^2$$

Solution of this equation ignores the formation of linear oligomers and estimates only the formation of helical polymers. As Newman et al. (17) have shown, when the value for $\sigma$ (which is determined by solution conditions) is very low, a very small amount of helical polymer of high degree of polymerization appears (Fig. 1, C and D, curves 1) as the total actin concentration approaches the critical concentration ($C_c = K_h^{-1}$), and an almost spontaneous transition to large amounts of long polymers occurs above the $C_c$ (not shown). As $\sigma$ increases, however, much more of the total actin will be in the form of much smaller helical oligomers below the $C_c$ (Fig. 1, C and D, curves 2-4) and the transition to larger oligomers above the $C_c$ becomes more gradual (data not shown).
FIG. 3. Sedimentation velocity analysis of the species composition of actin solutions incubated below the critical concentration in the presence of free ATP. Mg-G-actin (7.5 μM) in buffer A (0 min) or after incubation in buffer B at 20°C was analyzed by centrifugation at 52,000 rpm as described under “Experimental Procedures.” The cells were scanned at 280 nm (upper panel of each set). The first scans were taken within 10–18 min of the time the rotor had reached 2/3 of the final speed. The incubation times indicated on the panels are the elapsed times from the increase in MgCl2 concentration to 100 μM MgCl2 to the time of the scans. The time intervals between scans was 8 min for the 0-, 18-, 54-, and 119-min sets, except that the interval between scans 3 and 4 of the 18-min set was 16 min. The scans for the 248- and 320-min sets were 12 min apart. Deviation from linearity in the rate of transport of the boundary position probably reflects polymerization and depolymerization of oligomers. The lower panel of each set shows the concentration gradient distribution of the last four scans obtained by plotting the derivative of the fitted function to the experimental data. The widths of the peaks indicate the heterogeneity of the species present. The data are summarized in Table I. For further details, see “Experimental Procedures.”
transport was faster for the species were calculated from the data in Figs. 3, 5, and 7.5 \mu M Mg-actin in buffer A (Fig. 2). It should be noted, however, that oligomers accounted for 91% of the non-F-actin and 49% of the total actin in this experiment.

**Polymerization Competence of Oligomers**—To obtain independent evidence for the formation of oligomers at actin concentrations below \( C_s \) and to determine whether the oligomers were polymerization competent, separate samples of 7.5 \mu M Mg-actin containing 6% pyrenyl-actin were incubated in buffer B for up to 240 min exactly as in the experiment described in Fig. 3. At different times, the MgCl$_2$ concentration in duplicate samples was increased to 1 mM, and polymerization to F-actin was monitored by the increase in fluorescence. The initial rate of polymerization after increasing the MgCl$_2$ concentration increased with the time of prior incubation in 100 \mu M MgCl$_2$ to a maximum for the samples incubated for 50-65 min (Fig. 5). This was consistent with the formation of oligomers in 0.1 mM MgCl$_2$ that could then act as seeds and accelerate polymerization when the concentration of MgCl$_2$ was raised to 1 mM. Samples incubated for longer than 70 min before increasing the MgCl$_2$ concentration polymerized more slowly than the maximum observed rates but still substantially faster than the Mg-G-actin control, i.e. the 0-min sample (data not shown). Irrespective of their initial rates of polymerization, all of the samples reached the same final steady-state concentration of F-actin as judged by the increase in fluorescence (Table II), demonstrating that the oligomers were fully competent to polymerize. As the oligomer

DISTRIBUTION OF ACTIN SPECIES

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Incubation time</th>
<th>Sedimentation coefficient</th>
<th>Subunit size</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin ( \mu M )</td>
<td>MgCl$_2$ ( \mu M )</td>
<td>ATP ( \mu M )</td>
<td>min</td>
<td>( s_{obs}^0 \pm S.E. )</td>
</tr>
<tr>
<td>7.5</td>
<td>100</td>
<td>200</td>
<td>0</td>
<td>3.2 ( \pm 0.1 )</td>
</tr>
<tr>
<td>18</td>
<td>3.8 ( \pm 0.1 )</td>
<td>&gt;1</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>54</td>
<td>5.0 ( \pm 0.7 )</td>
<td>2</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>119</td>
<td>4.6 ( \pm 0.3 )</td>
<td>2</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>195</td>
<td>4.0 ( \pm 0.8 )</td>
<td>&gt;1</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>248</td>
<td>3.2 ( \pm 0.1 )</td>
<td>1</td>
<td>88</td>
<td></td>
</tr>
<tr>
<td>8.9 ( \pm 0.5 )</td>
<td>4-7</td>
<td>12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>320</td>
<td>3.2 ( \pm 0.2 )</td>
<td>1</td>
<td>83</td>
<td></td>
</tr>
<tr>
<td>9.6 ( \pm 0.9 )</td>
<td>5-8</td>
<td>17</td>
<td></td>
<td></td>
</tr>
<tr>
<td>28.5</td>
<td>100</td>
<td>100</td>
<td>1,440</td>
<td>3.2 ( \pm 0.2 )</td>
</tr>
<tr>
<td>23</td>
<td>3.1 ( \pm 0.2 )</td>
<td>&gt;1</td>
<td>27</td>
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</tr>
<tr>
<td>3.9 ( \pm 0.1 )</td>
<td>&gt;1</td>
<td>32</td>
<td></td>
<td></td>
</tr>
<tr>
<td>83</td>
<td>3.5 ( \pm 0.1 )</td>
<td>1</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>5.4 ( \pm 0.4 )</td>
<td>2</td>
<td>50</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**TABLE I**

where \( R \) is the Stokes' radius of the actin subunit (taken as 2 nm (19)) and \( R_i \) is the distance between subunits \( i \) and \( j \) for all subunits (3.8 nm for linear oligomers and 2.7 nm for helical oligomers (19)).

experiments were then performed on duplicate samples of 7.5 \mu M Mg-G-actin in buffer A (Fig. 3, 0-min samples) and parallel samples of 7.5 \mu M Mg-G-actin left standing for increasing times in buffer B at 20°C (Fig. 3).

For the 0-min sample (i.e. without 100 \mu M MgCl$_2$), transport of the boundary position was linear with time. Boundary transport was faster for the 18-, 54-, and 119-min samples than for the 0-min sample, reflecting conversion of the monomeric actin into species of larger size. Also, for these three samples, the rate of transport was nonlinear with time, indicating that the heavier species were continuing to undergo changes in size during the course of centrifugation. After incubation for 195 min, boundary transport between the respective scans was relatively linear. Moreover, the sixth scan of the 195-min sample clearly indicated the presence of two distinct species, and, after incubation for 248 and 320 min, a second species was definitively resolved. The average values of the sedimentation coefficients determined in these experiments, and the relative amounts of each species when more than one was resolved, are summarized in Table I. The subunit size of each species was estimated from theoretical curves for linear and helical oligomers (Fig. 4). At 248 and 320 min, distinct oligomeric species (tetramer to octamer) comprised 12 and 17%, respectively, of the total actin. Higher concentrations of small oligomers may have been present at earlier times (Table I). As expected, because the actin concentration was well below \( C_s \), no F-actin was detected in these experiments, however, there was a 20% increase in fluorescence in samples incubated for 24 h.

To determine whether oligomers might coexist with F-actin, 28.5 \mu M Mg-actin (i.e. more than twice \( C_s \)) was incubated in buffer B in a centrifuge cell for 20 h at 20°C before centrifugation (Fig. 5). The scan taken at 12 min showed the formation of a clear boundary between a very heavy species (F-actin) and the rest of the actin pool. This heavy species had almost completely sedimented to the bottom of the cell by the time of the 20-min scan. Subsequent scans at 50 min through 86 min were best fit by a three-component model, and three components (i.e. G-actin and two oligomeric species as indicated in Table I) were also required to fit the data for the non-F-actin fractions of the earlier scans. The sedimentation coefficients, relative concentrations and subunit sizes of all these species are summarized in Table I. About 13.2 \mu M F-actin was formed in this experiment, and the sum of the concentrations of the other three species was 15.3 \mu M, not very different from the estimate of the \( C_s \) in buffer B, 12.5 \mu M, determined by the fluorescence assay (Fig. 2). It should be noted, however, that oligomers accounted for 91% of the non-F-actin and 49% of the total actin in this experiment.

**Formation of Actin Oligomers**

The sedimentation coefficients and the relative amounts of each species were calculated from the data in Figs. 3, 5, and 9. The subunit sizes were estimated from the calculated sedimentation coefficients and the theoretical curves in Fig. 4. Where a range of subunit sizes is given, the lower value would be for a helical oligomer and the larger value for a linear oligomer. A subunit size of 1 is a monomer and a dimer can only be a linear oligomer.

**FIG. 4.** The calculated relationship between the sedimentation coefficient and oligomer subunit size. The calculations were made for linear and helical oligomers for identical spherical subunits using the Kirkwood equation (32).

\[
S_n/S_1 = 1 + R/n \sum_{i=1}^{n} \sum_{j=1}^{n} 1/R_{ij}
\]

where \( S_i \) is the sedimentation coefficient for \( n \) associated subunits, \( S_1 \) is the sedimentation coefficient for monomeric Mg-G-actin (component with lowest \( S \) value in Figs. 3, 5, and 9), \( R \) is the Stokes' radius of the actin subunit (taken as 2 nm (19)) and \( R_{ij} \) is the distance between subunits \( i \) and \( j \) for all subunits (3.8 nm for linear oligomers and 2.7 nm for helical oligomers (19)).
Formation of Actin Oligomers

**Fig. 5.** Sedimentation velocity analysis of the species composition of a solution of F-actin at steady state. Mg-G-Actin (28.5 μM) was incubated in buffer B for 20 h at 22 °C in the centrifuge cell before analyzing as described in the legend to Fig. 3. The two upper panels show the scans at 280 nm taken at the times indicated. The two lower panels show the corresponding derivative plots. To fit the data, three components were necessary in addition to F-actin. The data are summarized in Table I. See text for further details.

**Fig. 6.** Polymerization competence of actin oligomers. Mg-G-actin (6% pyrene-actin) was incubated for the indicated times in buffer B, exactly as in the experiment described in Fig. 3. The MgCl₂ concentration was then increased to 1 mM to lower the Cᵢ and the final fluorescence was measured after 20 h at 20 °C to determine the concentration of F-actin. None of the values (not all time points are shown) differed significantly from the mean for the entire site, 6.16 ± 0.08 μM.

**Table II**

<table>
<thead>
<tr>
<th>Time of incubation</th>
<th>F-actin formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>in 0.1 mM MgCl₂</td>
<td>in 1 mM MgCl₂</td>
</tr>
<tr>
<td>min</td>
<td>μM</td>
</tr>
<tr>
<td>0</td>
<td>6.27 ± 0.08</td>
</tr>
<tr>
<td>10</td>
<td>6.18 ± 0.010</td>
</tr>
<tr>
<td>20</td>
<td>6.16 ± 0.006</td>
</tr>
<tr>
<td>40</td>
<td>6.25 ± 0.08</td>
</tr>
<tr>
<td>60</td>
<td>6.29 ± 0.12</td>
</tr>
<tr>
<td>80</td>
<td>6.14 ± 0.15</td>
</tr>
<tr>
<td>100</td>
<td>6.03 ± 0.11</td>
</tr>
<tr>
<td>120</td>
<td>6.15 ± 0.14</td>
</tr>
<tr>
<td>180</td>
<td>6.16 ± 0.18</td>
</tr>
<tr>
<td>240</td>
<td>6.12 ± 0.12</td>
</tr>
</tbody>
</table>

concentration should have been at least 1.1 μM (15% of 7.5 μM, see Table I), nonpolymerizable oligomers would have been readily detected. Also, in all the samples, the electron microscopic images of negatively stained filaments were quantitatively and qualitatively typical of normal preparations of F-actin, and no aggregates were seen (data not shown), eliminating the remote possibility that nonspecific aggregation of denatured oligomers might have resulted in the same increase in fluorescence as polymerization to F-actin. Similar experiments were carried out to assess the polymerization competence of the oligomers that coexisted with F-actin in 0.1 mM MgCl₂ (Fig. 7). When the MgCl₂ concentration was raised to 1 mM, the oligomers polymerized more rapidly and to the same extent as an equal concentration of G-actin (Fig. 7).
Reversibility of Oligomer Formation—If the oligomers formed below the C are to be considered equivalent to normal intermediates in the polymerization process, not only should they be polymerization competent, as just shown, but they should also dissociate upon dilution. To test this, actin oligomers were formed by incubating 7.5 μM Mg-G-actin containing 6% pyrene-actin for 42 min at 20 °C as described in Figs. 3 and 6. MgCl₂ was then added to one sample to a concentration of 1.5 mM, another sample was diluted to 3.75 μM in 1.5 mM MgCl₂, and a third sample was diluted to 3.75 μM in 0.1 mM MgCl₂ and allowed to stand for 10 min before addition of MgCl₂ to 1.5 mM. The initial rates of polymerization of the three samples were compared to appropriate controls of Mg-G-actin (Fig. 8). As previously observed, the undiluted sample polymerized more rapidly than the control (Fig. 8A), indicating the presence of oligomers. The sample diluted immediately into 1.5 mM MgCl₂ polymerized more slowly than the undiluted sample, as expected, but still significantly more rapidly than its control (Fig. 8B). However, the rate of polymerization of the diluted sample that had stood for 10 min in 0.1 mM MgCl₂ before addition of 1.5 mM MgCl₂ was indistinguishable from that of the control, consistent with dissociation of the oligomers originally present.

ATP Hydrolysis—To characterize more completely the oligomers formed below the C, hydrolysis of ATP was measured under conditions identical to those used in Fig. 3 except that the actin was first equilibrated with [γ-32P]ATP. The difference in the rate of ATP hydrolysis in the presence and absence of 100 μM MgCl₂ was linear and amounted to about 0.26 mol/mol of total actin by 4.5 h (Fig. 9A). This is equivalent to about 1.7 mol of ATP hydrolyzed/mol of oligomer subunit, however, because of the possibility of exchange of free and actin-bound ATP it was not possible to associate the hydrolysis of ATP with the oligomerization reaction in these experiments.

In order to be able to correlate ATP hydrolysis more specifically with oligomer formation, the experiments described in Figs. 3 and 9A were repeated in the absence of free ATP, i.e. 7.5 μM Mg-G-actin was incubated at 20 °C in buffer B minus ATP. Under these solvent conditions, the sample concentrations were slightly above C, which was determined to be 6.7 μM (Fig. 10). After incubation for 23 and 83 min, analytical ultracentrifugation provided clear evidence for two species (Fig. 11) whose sizes and relative amounts are given in Table I, as much as 50% of the total actin was present as oligomers (dimers). Under these conditions, the difference in the rates of ATP hydrolysis in the presence and absence of 100 μM MgCl₂ was very small (Fig. 9B), about 0.046 mol/mol of total actin after 4.5 h, or about 0.03 mol/mol of oligomer subunit formed in 83 min. As were the oligomers formed in the presence of ATP, the oligomers formed in the absence of free ATP were fully competent to polymerize when the concentration of MgCl₂ was increased (Table III).

Before discussing the implications of the results reported in this paper, it is worth summarizing some of the reasons why the formation of oligomers is highly unlikely to have been an artifact of the analytical ultracentrifugation used to detect them. Had that been the case, oligomer formation from G-actin near the critical concentration would not have been a function of the time of incubation before centrifugation, and the samples would not have polymerized more rapidly.
than G-actin when the concentration of MgCl₂ was increased prior to ultracentrifugation. Furthermore, the oligomers co-existing with F-actin at steady state, as well as those formed from G-actin below the critical concentration, were formed at the expense of the monomer pool, i.e. the concentration of F-actin determined by analytical ultracentrifugation was as predicted by conventional assays. In as much as the subunit volume of F-actin is larger than that of G-actin, the pressure effects of ultracentrifugation would be expected to depolymerize F-actin and oligomers to monomers (33) rather than induce formation of oligomers from monomers. Finally, evidence for oligomer formation was obtained in samples that had not been subjected to ultracentrifugation (Figs. 6–8).

**DISCUSSION**

The results presented in this paper document the formation of small actin oligomers below (in the presence of 200 μM ATP) and just above (in the absence of free ATP) the critical concentration in 100 μM MgCl₂, pH 7.8. The most prominent species formed in the initial stages was dimer but, with time, these grew to the size of about tetramers or octamers, the predicted subunit size depending on whether they were linear or helical oligomers. These observations are in general agreement with most, but not all, of the observations reported by others using different assays.

Newman et al. (17) reported that a mixture of Ca-actin and Mg-actin below its critical concentration in 100 μM MgCl₂, 200 μM ATP underwent a 25% decrease in its translational diffusion coefficient over a 1–2-h period consistent with the formation of small oligomers of unidentified size. In similar experiments, Mozoso-Villarias and Ware (18) measured a decrease in the diffusion of Mg-actin below its critical concentration in 67 μM MgCl₂, 200 μM ATP, to an extent said to be consistent with the conversion of about 40% of the actin to trimers or tetramers. Godette et al. (19) estimated that about 25% of actin in 50 μM MgCl₂, 100 μM ATP was converted to dimers in 4 h. Grazi and co-workers (reviewed in Ref. 34) have inferred the formation of actin oligomers under several different ionic conditions, and similar results were obtained in pioneering studies by Ooi (35).

The oligomers formed in the first two studies (17, 18) were at least partially competent to polymerize when the divalent cation concentration was increased, but, in contrast, the dimers formed in the experiments reported by Godette et al. (19) were apparently incapable of polymerization to F-actin. In agreement with the implications of the first two studies, we found that the oligomers formed in the presence and absence of free ATP were fully competent to polymerize to F-actin when the critical concentration was lowered by addition of more MgCl₂ and, as would be expected, at a more rapid rate than monomeric G-actin. This result also proves that formation of oligomers was independent of the conditions of the analytical ultracentrifugation that was used to characterize and quantify them. These data, together with the data for oligomer dissociation upon dilution, provide strong evidence that the oligomers were not non-specific, denatured aggregates.

In addition, we found a very low level of ATP hydrolysis associated with the formation of the oligomers, indicating that they comprised predominantly, if not exclusively, ATP-actin subunits. This is consistent with previous evidence that ATP hydrolysis occurs on F-actin and the suggestion that actin nuclei may contain bound ATP (reviewed in Ref. 5).

We also found that actin oligomers coexist with F-actin and monomeric actin at steady state in 100 μM MgCl₂ and 200 μM ATP. Indeed, and surprisingly, about 90% of the non-F-actin appeared to be in the form of small oligomers and only about 10% was monomeric under these conditions. Qualitatively similar results have been reported by Lanni and Ware (36) who determined the diffusion coefficients of the actin species remaining in the supernatant after centrifugal removal of F-actin. In our experiments, the sum of the concentrations of monomers (1.5 μM) plus small oligomers (13.5 μM) as determined by analytical ultracentrifugation, was similar to, but slightly greater than, the critical concentration (12.5 μM) determined by the fluorescence assay. These results are compatible if it is assumed that pyrenyl-actin subunits in the small oligomers had only about 18% of the fluorescence.
Fig. 11. Sedimentation velocity analysis of the species composition of Mg-actin solutions incubated slightly above the critical concentration in the absence of free ATP. Mg-G-actin (7.5 μM) was incubated in buffer B minus ATP, and data were collected and analyzed exactly as described in the legend of Fig. 3. The 0-min sample was taken before addition of 100 μM MgCl₂. The first scans (upper panels) were taken within 10–18 min of the rotor reaching 2/3 of the final speed (52,000 rpm). Intervals between scans were 8 min for the 0-min set (except for 9.5 min between scans 3 and 4) and 12 min for the other two sets. The lower panels show the concentration gradient distribution of the last four scans in each case. The results of this analysis are summarized in Table I. See text for further details.

<table>
<thead>
<tr>
<th>TABLE III</th>
<th>Polymerization competence of oligomers formed below the critical concentration in the absence of free ATP</th>
</tr>
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<tbody>
<tr>
<td>Mg-G-actin (7.5 μM, 6% pyrene-actin) was incubated in buffer B (0.1 mM MgCl₂) in the absence of free ATP at 20 °C as described in the legend to Fig. 10 to allow oligomers to form. Samples were removed at the indicated times, and the MgCl₂ concentration was increased to 1 mM to lower the C. The samples were sonicated for 2 s about 10 min after addition of MgCl₂. The maximum fluorescence intensity reached after sonication was used to calculate the concentration of F-actin. Mean value for the entire set: 6.24 ± 0.08 μM.</td>
<td></td>
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<table>
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<tr>
<th>Time of incubation in 0.1 mM MgCl₂</th>
<th>F-actin formed in 1 mM MgCl₂</th>
<th>μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>6.27 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>6.40 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>6.23 ± 0.09</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>6.23 ± 0.08</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>6.29 ± 0.06</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>6.18 ± 0.10</td>
<td></td>
</tr>
<tr>
<td>80</td>
<td>6.22 ± 0.10</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>6.10 ± 0.08</td>
<td></td>
</tr>
</tbody>
</table>

Quantum yield of the pyrenyl-actin in F-actin.

Contrary to our observation, one might have expected approximately the same concentration and size distribution of oligomers in the presence and absence of F-actin inasmuch as the solution conditions were the same. The difference may have been due to the fact that steady state between monomers and oligomers was probably not reached in the experiments using 7.5 μM actin, as evidenced by the continual change in composition even during the sedimentation velocity analysis (Fig. 3 and Table I). It is also possible that the nucleotide bound to the actin oligomers was ADP in the experiments in which F-actin was present at a steady state, whereas it was shown to be ATP in the absence of F-actin.

Both in the presence and absence of F-actin, theory suggests that linear and helical oligomers should be present. In addition, more than one kind of dimer conformation is possible. For example, covalent cross-linking has been found to produce at least two different cross-linked dimers early in the polymerization process (albeit under different ionic conditions than those used in the present study), one of which becomes dominant as a steady state is reached (37). Possibly the formation of two different cross-linked products is related to the different types of oligomers (linear and helical) discussed in this paper.

It is worth re-emphasizing that the accumulating evidence for substantial oligomer formation under certain solvent conditions is entirely consistent with the conventional view of actin polymerization in which oligomers are not generally considered, other than in the nucleation event. The conventional interpretation is supported by numerous observations in many laboratories that the rate of elongation of F-actin filaments added to solutions of G-actin is directly proportional to the monomer concentration and that the critical concentration determined in this way is the same as the critical concentration determined by measuring the concentration of un polymerized actin at steady state conditions (reviewed in Refs. 2–5). This model is further supported by the fact that the effects of the monomer-binding protein, profilin, on the concentration of F-actin at steady state (38), on the rate of elongation of filaments added to solutions of monomeric actin (39), and on the kinetics of actin polymerization de novo (40) are all consistent with the formation of a 1:1 actin-profilin complex with the same Kₐ.

The apparent contradictions between the formation of relatively large amounts of small actin oligomers in the present experiments (and in earlier experiments by others) and their absence in studies of the kinetics of polymerization is readily rationalized. Actin polymerization is usually studied under approximately physiological ionic conditions where the critical concentration and the value of σ are both expected to be very low and, therefore, as illustrated in Fig. 1, the oligomer concentration will be very small and the transition from monomer to long polymers will be very sharp, i.e. the conventional, simplified version of condensation-elongation model as formulated in Equation 4 will apply. In contrast, oligomers are formed in relatively large amounts, and the transition from monomer to polymer is very gradual when the solution conditions are chosen to give a high critical concentration and a high σ value (Fig. 1). In this case, for a complete analysis, all of the terms in Equation 3 must be considered, i.e. linear oligomers, helical oligomers, and monomers can coexist below the C, and monomers, linear oligomers and helical oligomers, or F-actin can simultaneously be present above the C. Even
under physiological conditions, however, both linear and helical oligomers will occur to a finite, albeit small, extent, and their potential interaction with regulatory proteins could be important. The observations described in this paper should provide an experimental basis for studying such interactions in vitro.

**APPENDIX**

The radial position $\bar{r}$ obtained by second moment analysis is defined by

$$\bar{r} = r^2 - \frac{1}{C_2} \int_0^\infty C_2 \, dr^2$$

Using the definition of $c_2$ from Equation 1 in the integral above then gives, for each component that

$$\int_0^\infty C_2 \, dr^2 = \int_0^\infty c_2 \left( \exp(n(r^2 - \bar{r}^2)) / (1 + \exp(n(r^2 - \bar{r}^2))) \right) \, dr^2$$

performing the appropriate algebra then gives the following:

$$c_2(r^2 - \bar{r}^2) =$$

$$c_2 \left[ \ln(1 + \exp(n(r^2 - \bar{r}^2))) - \ln(1 + \exp(n(r^2 - \bar{r}^2))) \right] / n$$

$$\exp(n(r^2 - \bar{r}^2)) =$$

$$(1 + \exp(n(r^2 - \bar{r}^2)) / (1 + \exp(n(r^2 - \bar{r}^2))$$

$$1 + \exp(n(r^2 - \bar{r}^2)) =$$

$$\exp(n(r^2 - \bar{r}^2)) + \exp(n(r^2 - \bar{r}^2)) \exp(n(r^2 - \bar{r}^2))$$

$$1 = \exp(n(r^2 - \bar{r}^2)) \exp(n(r^2 - \bar{r}^2))$$

$$0 = n(r^2 - \bar{r}^2) + n(r^2 - \bar{r}^2)$$

Since the concentration distribution of the boundary must be of finite width, a zero value for $n$ is not possible. Thus, Equation 2 is the consequence of Equation vii. As long as the conditions of Equation 2 are met and Equation 1 fits the concentration distribution well, the value of $\bar{r}$ obtained in the fitting procedure represents the second moment position of the boundary.

**REFERENCES**