for nucleation, consistent with the theoretical prediction that the number of polymers at equilibrium is determined by the excess free energy of nucleation (Oosawa, 1983).

In summary, the decrease in $D_{20}$ on treatment of actin with 0.1 mM $MgCl_2$ represents the physical aggregation of monomeric units. The observation that this decrease in $D_{20}$ is in large part reversible suggests that this aggregation is not associated with denaturation. The fluorescence intensity experiments (Figure 6) demonstrate the presence of functional nuclei in actin solutions resulting from incubation with 0.1 mM $MgCl_2$. We thus conclude that functional small polymers (oligomers) of actin do exist at actin concentrations lower than the critical actin concentration.

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# Actin Oligomers below the Critical Concentration Detected by Fluorescence Photobleaching Recovery†


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ABSTRACT: Measurement of the diffusion coefficient of G-actin using fluorescence photobleaching recovery reveals that a hydrodynamically larger species is formed in $Mg^{2+}$-containing buffers but not in $Ca^{2+}$-containing buffers. In buffer A (2 mM Tris-HCl, 0.2 mM $CaCl_2$, 0.2 mM ATP, and 0.5 mM 2-mercaptoethanol, pH 8.0) and other $Ca^{2+}$-containing buffers, the data indicate a single species with a diffusion coefficient of (7.98 ± 0.17) × $10^{-7}$ $cm^2/s$. In $Mg^{2+}$-containing buffers the average diffusion coefficient was (5.52 ± 0.06) × $10^{-7}$ $cm^2/s$, and there was evidence of more than one component. The distinction between these buffer systems was reversible by dialysis. The conjecture that the putative oligomeric species may be active in promoting actin assembly was confirmed in two independent experiments. Assembly of actin in the presence of these species produced shorter filaments, as would be expected if they act to increase the number of nucleation sites. In addition, trace quantities of these species added to buffer A actin had the ability to accelerate the kinetics of actin assembly. It is concluded that G-actin below the critical concentration forms oligomeric species in the presence of 50-200 μM $Mg^{2+}$ and that these species either are assembly nuclei or at least are more readily converted to assembly nuclei than is monomeric actin.


The capacity of actin for reversible self-assembly is believed to be an indispensable element of its role in cytoplasmic motility (Pollard & Weihing, 1974; Taylor & Condeelis, 1979; Stossel et al., 1982; Korn, 1982). Extensive studies of the kinetics of actin assembly in vitro have led to an established model in which, for any given set of solution conditions, there is a critical concentration of actin that remains unassembled

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and thus below which no filaments are formed. There is further evidence that the steady state achieved above the critical concentration involves a dynamic exchange between G-actin monomers and F-actin filaments with essentially no oligomeric species present. It is clear, however, that the initital event in the nucleation of actin assembly must be the dimerization and/or oligomerization of G-actin.

In previous studies of the actin assembly process, we have utilized the technique of fluorescence photobleaching recovery (FPR), which permits the simultaneous characterization of assembled and unassembled species (Lanni et al., 1981; Lanni & Ware, 1984; Mozo-Villarias & Ware, 1984). These experiments have led us to believe that under certain conditions the presence of small oligomers of actin in solution is a stable condition. In this report we provide direct evidence of the reversible oligomerization of G-actin below the critical concentration, and we examine the possible role of these oligomers in actin assembly.

## Materials and Methods

Actin was obtained by the method of Pardee & Spudich (1982) from acetone powder of rabbit muscle and was purified by successive cycles of polymerization and depolymerization as described elsewhere (Lanni et al., 1981). Actin was labeled by reaction of actin filaments with 5-(iodoacetamido)-fluorescein (Molecular Probes) according to the method of Wang & Taylor (1980). This procedure attaches a single fluorescein residue at Cys-374. Separation of unreacted label from protein was accomplished by passage through a Sephadex G-25–150 column; separation of labeled actin from unlabeled actin was accomplished by using a DE-52 column. Purified actins (labeled and unlabeled) were polymerized and stored as pellets at 0 °C for no longer than 1 month. In preparation for experiments, the homogenized actin pellet was depolymerized by homogenization in and dialysis against the appropriate buffer. The resulting solution was clarified by centrifugation. The final G-actin concentration (always around 9 $\mu$M) was measured by the Lowry method (Lowry et al., 1951) using bovine serum albumin as the standard and applying the correction factor given by Houk & Ue (1974). The proportion of labeled actin in these experiments was always between 10% and 15%. G-Actin was never left in solution for more than 3 days. Stability of G-actin for this period of time was verified by independent demonstrations of its competency for reversible assembly.

The buffers used in these experiments were variations of the standard buffer A (2 mM Tris-HCl, 0.2 mM $CaCl_2$, 0.2 mM ATP, and 0.5 mM 2-mercaptoethanol, pH 8.0) and buffer B (2 mM Tris-HCl, 50 $\mu$M $MgCl_2$, 0.2 mM ATP, and 0.5 mM 2-mercaptoethanol, pH 8.0). All reagent-grade chemicals were purchased from Sigma Chemical Co. and used without further purification. We have varied the concentrations of $Ca^{2+}$, $Mg^{2+}$, and ATP in a number of our experiments. In such cases the concentrations of free $Mg^{2+}$, free $Ca^{2+}$, and free ATP were calculated by using the best literature values for dissociation constants available for our conditions (pH 8, low salt). The values of the dissociation constants used were 100 $\mu$M for CaATP (Heyde & Rimai, 1971) and 20 $\mu$M for MgATP (Laget et al., 1973).

Translational diffusion coefficients of labeled actin were determined by the technique of fluorescence photobleaching recovery (FPR) [for a recent review, see Ware (1984)]. In this approach the motion of labeled species is detected by photobleaching a portion of the sample and measuring the characteristic time for the bleached region to fade away as bleached and unbleached species randomize their positions by translational diffusion. The theory and methodology of our approach to the FPR measurement have been described elsewhere (Lanni & Ware, 1982). To summarize briefly, a periodic photobleaching pattern is produced in the sample by brief, intense illumination through a grating. The grating is then translated at constant speed through the reference beam to produce a moving pattern on the specimen. A modulation of fluorescence emission is produced as the bleached pattern and the moving illumination fall into and out of phase. The resulting photocurrent contains an ac component whose frequency is determined by the spacing and velocity of the grating and whose amplitude relative to the dc component is determined by the extent of photobleaching. In effect, the modulation ac envelope $E(t)$ is a measure of the spatial Fourier component of the fluorescence intensity with wave vector $K'$ given by

$$K' = 2\pi/L \tag{1}$$

where $L$ is the spacing of the projection of the grating lines in the specimen. If the labeled species have a single diffusion coefficient, then the ac component decays by diffusion in exponential form:

$$E(t) = E(0)e^{-DK'^2t} \tag{2}$$

where $D$ is the translational diffusion coefficient. If there are several hydrodynamically distinct labeled species, then the envelope decay will be a sum of exponentials, with each exponential weighted by the fraction of total label incorporated into that species. Previous studies of actin assembly using FPR have made use of the fact that F-actin filaments have a very much lower diffusion coefficient and therefore can be characterized by this technique, since the photobleaching recovery due to filaments occurs on a longer time scale than that due to monomers (Lanni et al., 1981; Tait & Frieden, 1982a,b; Tellam & Frieden, 1982; Mozo-Villarias & Ware, 1984). In the present study we are also concerned with the high-mobility, shortest time scale recoveries characterizing monomers and low oligomers. These data were fitted to functions with either one or two exponential components. The single exponential was preferred whenever an attempt at a two-component fit resulted in no clear distinction between the two time constants. In all cases the criterion of best fit was a minimum in the relative standard deviation function between the experimental and analytical values. The search for this minimum follows the numerical derivative of the relative standard deviation function (Bevington, 1969).

FPR specimens were prepared by mixing labeled G-actin and native G-actin solutions in the proper proportions and loading 5 $\mu$L of the solution into washed flat glass microcapillary tubes having a depth of 100 $\mu$m (Vitro Dynamics, Rockaway, NJ). The total actin concentration was 9 $\mu$M in all experiments. The filled capillaries were placed on a flat black, anodized aluminum plate that was maintained at a constant temperature of 20 °C. Each end of the capillary was sealed and bonded to the plate with a drop of cover slip mounting cement (Permount, Fisher Scientific Co., Pittsburgh, PA). Convection or lateral flow was never detected in this type of specimen.

## Results

Representative FPR data traces are shown in Figure 1. For each trace the solid line is the experimental modulation envelope $E(t)$ and the dotted line is a computer-generated functional fit. Trace a is the data from a solution of G-actin in buffer A, and the fit is to a single exponential. From the

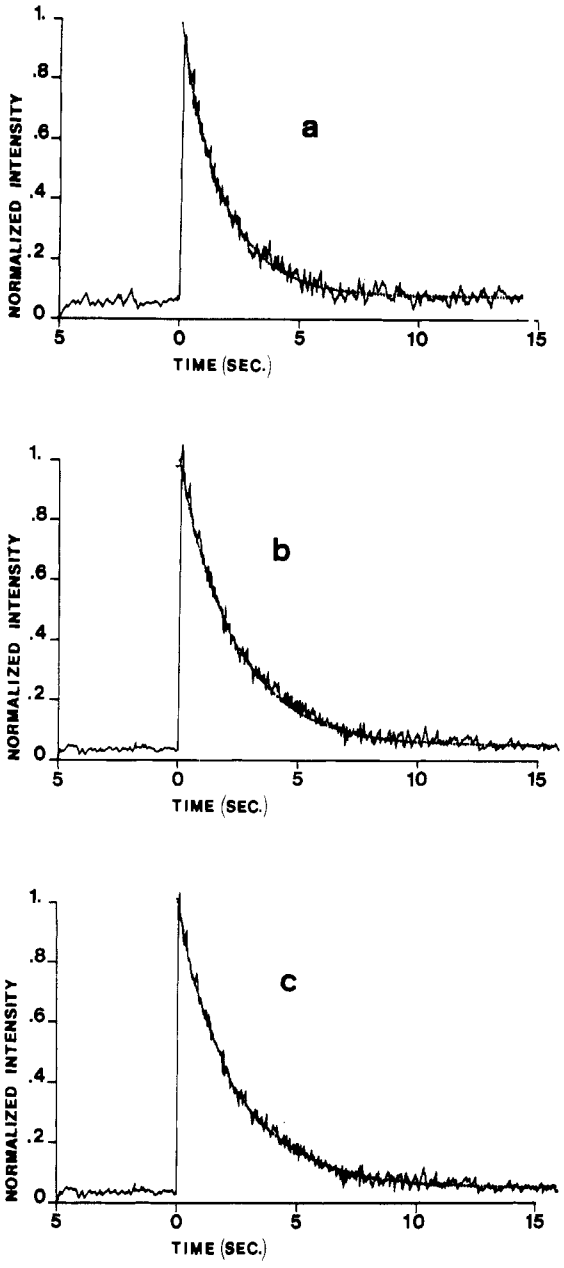


FIGURE 1: FPR data traces for G-actin samples. In each trace the normalized amplitude of the modulation envelope is plotted as function of time, where zero time is the instant of photobleaching. The dotted lines are computer-generated functional fits. In each case the actin concentration was 9 $\mu$M, the temperature was 20 °C, and the wave vector $K'$ was 841 $cm^{-1}$. Trace a was taken from a solution of G-actin in buffer A and is fitted to a single exponential. Traces b and c were taken from a solution of G-actin in buffer for which $Ca^{2+}$ was replaced by 67 $\mu$M $Mg^{2+}$. Trace b is fit to a single exponential, and trace c is fit to two exponentials.

decay time of 1.80 s and the experimental wave vector $K'$ of 841 $cm^{-1}$, we obtain a diffusion coefficient of $7.85 \times 10^{-7}$ $cm^2/s$. Trace b was collected from a sample of G-actin in a buffer that differs only in the replacement of $Ca^{2+}$ by 67 $\mu$M $Mg^{2+}$. The time constant of 2.34 s and $K'$ of 841 $cm^{-1}$ give $D = 6.05 \times 10^{-7}$ $cm^2/s$. Even though the noise in this case was lower, the quality of the single-exponential fit was visibly poorer. Trace c shows the result of a two-exponential fit to the same data as in trace b. The two exponentials had time constants of 1.78 and 3.57 s and coefficients of 0.62 and 0.38, respectively. The corresponding diffusion coefficients are $7.96 \times 10^{-7}$ $cm^2/s$ and $3.96 \times 10^{-7}$ $cm^2/s$. The two-exponential fit had a 25% lower standard deviation than the single-exponential fit. No significant improvement of fit could be achieved by two-exponential fits to the data for samples in $Ca^{2+}$-containing buffers.

Measurements such as those in Figure 1 were repeated for a range of $[Ca^{2+}]$ from 20 to 200 $\mu$M, with [CaATP] held constant at 50 $\mu$M by adjusting the total ATP concentration. The diffusion coefficient was invariant with $[Ca^{2+}]$, and the average value among 20 samples was $(7.98 \pm 0.17) \times 10^{-7}$ $cm^2/s$. In a similar experiment the free $[Ca^{2+}]$ was held constant at 50 $\mu$M while [CaATP] was varied between 17 and 150 $\mu$M; results were essentially identical with an average diffusion coefficient of $(8.05 \pm 0.19) \times 10^{-7}$ $cm^2/s$. The diffusion coefficients quoted are the averages among different samples, where the sample value itself is the mean of several independent measurements. Uncertainties quoted are standard deviations among the mean values for different samples.

FPR data on G-actin in buffer B and other $Mg^{2+}$-containing buffers were of similar form, but the measured diffusion coefficient was invariably lower. When [MgATP] was held constant at 50 $\mu$M and $[Mg^{2+}]$ was varied between 20 and 175 $\mu$M, the results were invariant with concentration, and the average diffusion coefficient among 10 samples using single-exponential fits was $(5.53 \pm 0.06) \times 10^{-7}$ $cm^2/s$. A parallel experiment in which $[Mg^{2+}]$ was held constant at 50 $\mu$M and [MgATP] was varied between 30 and 150 $\mu$M showed no variation with concentration and produced an average diffusion coefficient of $(5.65 \pm 0.04) \times 10^{-7}$ $cm^2/s$.

As illustrated in Figure 1, attempts were made to fit the FPR data with a sum of two exponentials. In buffers containing $Ca^{2+}$, these efforts did not produce a significant improvement in the quality of fit, and the two terms generally converged to the average value around $8 \times 10^{-7}$ $cm^2/s$. In buffers containing $Mg^{2+}$, attempts to fit the data to a sum of two exponentials produced a somewhat better fit (standard deviations decreased an average of 10–15%) and reproducibly led to a result of two components in about a 3:2 ratio with diffusion coefficients of $(8.06 \pm 0.21) \times 10^{-7}$ $cm^2/s$ and $(4.02 \pm 0.41) \times 10^{-7}$ $cm^2/s$. These results were obtained independent of the initial estimated parameters of the fit, over a range of about a factor of 3. The average weighting coefficient selected for the higher mobility term was $0.61 \pm 0.05$.

The foregoing measurements are a clear demonstration that G-actin samples in the presence of $Mg^{2+}$ are hydrodynamically distinct from G-actin samples in the presence of $Ca^{2+}$. The next distinction that we sought to establish was the assembly competence of the two preparations. Assembly was induced by addition of either KCl (to 100 mM) or $MgCl_2$ (to 2 mM), and successive FPR traces were measured to follow the course of assembly as described previously (Lanni et al., 1981; Lanni & Ware, 1984; Mozo-Villarias & Ware, 1984). The fraction of immobilized actin, $f_{LM}$, was determined by subtracting the fraction of high-mobility recovery from the FPR trace of a fully assembled preparation. For the sample prepared in buffer B, the $f_{LM}$ for assembly by 100 mM KCl was 0.91, and the $f_{LM}$ for assembly by 2 mM $MgCl_2$ was 0.94. These values correspond to critical concentrations of 0.8 and 0.5 $\mu$M, respectively, which agree within experimental error (~30%) with the values of $f_{LM}$ and literature values of critical concentration determined by us and others for actin assembled from buffer A. Thus, we see no difference in the assembly competence of the two preparations. An important difference is perceived, however, if we use the slopes of the plateaus of the FPR traces to measure the diffusion coefficients of the assembled filaments, from which we can calculate their lengths. In parallel experiments, the average diffusion coefficient of filaments

assembled from buffer A was $6.4 \times 10^{-9}$ cm$^2$/s, which corresponds to a length of about 4 $\mu$m (Lanni & Ware, 1984); the average diffusion coefficient of filaments assembled from buffer B was $20 \times 10^{-9}$ cm$^2$/s, which corresponds to a filament length of about 0.7 $\mu$m. The simplest interpretation of these data is that the buffer B sample contains aggregates that either are nuclei for assembly or are readily converted to nuclei, so that a larger number of nuclei initiate assembly when the assembling medium (100 mM KCl or 2 mM $MgCl_2$) is added. This larger number of filaments then exhausts the pool of monomer before attaining the length that would have been achieved if no nuclei or nucleus precursors were present in the initial medium. To test this hypothesis, we assembled buffer A actin in the presence of trace quantities of buffer B actin. The expected decrease in filament length was observed, in rough proportion to the amount of buffer B actin added. For example, a solution with 2% buffer B actin produced filaments of about 2 $\mu$m, roughly half the length of filaments assembled from buffer A alone. The lengths of filaments obtained are not simple weighted averages of the values for buffer A alone and buffer B alone, but rather are weighted more toward the lengths for buffer B. This probably indicates that when buffer B actin is assembled, the nuclei and growing short filaments undergo some combination, so that the eventual number of filaments is substantially less than the initial number of nuclei.

The hypothesis that buffer B actin contains assembly nuclei or nucleus precursors can also be tested by observing the effect of addition of trace quantities of buffer B actin on the kinetics of assembly of buffer A actin. We conducted such experiments, but due to the limited time resolution of the FPR method, we were constrained to suboptimal assembly media in order to retard the time scale of assembly. Figure 2a shows the time course for assembly of actin filaments after addition of KCl up to 25 mM to a sample of buffer A actin (9 $\mu$M). In the experiment represented by solid circles, the added solution contained trace (1%) buffer A actin; in the experiment represented by the open circles, the added solution contained the same trace level of buffer B actin. Figure 2b shows a similar experiment using the same protocol, except that the polymerizing medium was 0.3 mM $MgCl_2$. In both experiments the presence of trace levels of buffer B actin measurably accelerated the rate of actin assembly.

In order to test the reversibility of the hydrodynamic transition brought about by the substitution of $Mg^{2+}$ for $Ca^{2+}$, we performed an experiment of successive dialyses. A sample of G-actin in buffer A with $D = 8.0 \times 10^{-7}$ cm$^2$/s was dialyzed for 24 h against buffer B. An aliquot of this sample was taken, and the diffusion coefficient was measured by FPR to be $5.6 \times 10^{-7}$ cm$^2$/s. The remaining sample was redialyzed against buffer A, and the measured diffusion coefficient was $7.5 \times 10^{-7}$ cm$^2$/s. Finally, this sample was polymerized with 2 mM $MgCl_2$, and from the steady-state $f_{LM}$ the critical concentration was found to be about 0.9 $\mu$M, within the range for normal buffer A actin. We conclude that, despite the long time required for this experiment, no significant amount of the actin was denatured, and the hydrodynamic transition was essentially reversible.

## Discussion

The primary result of this paper is that solutions of G-actin below the critical concentration at 20 °C form hydrodynamically larger species in $Mg^{2+}$-containing buffers but not in $Ca^{2+}$-containing buffers such as the standard buffer A. A very similar result has recently been reported by Newman et al. (1985). Using the technique of quasi-elastic light scattering, they have found that G-actin in buffer A has a diffusion

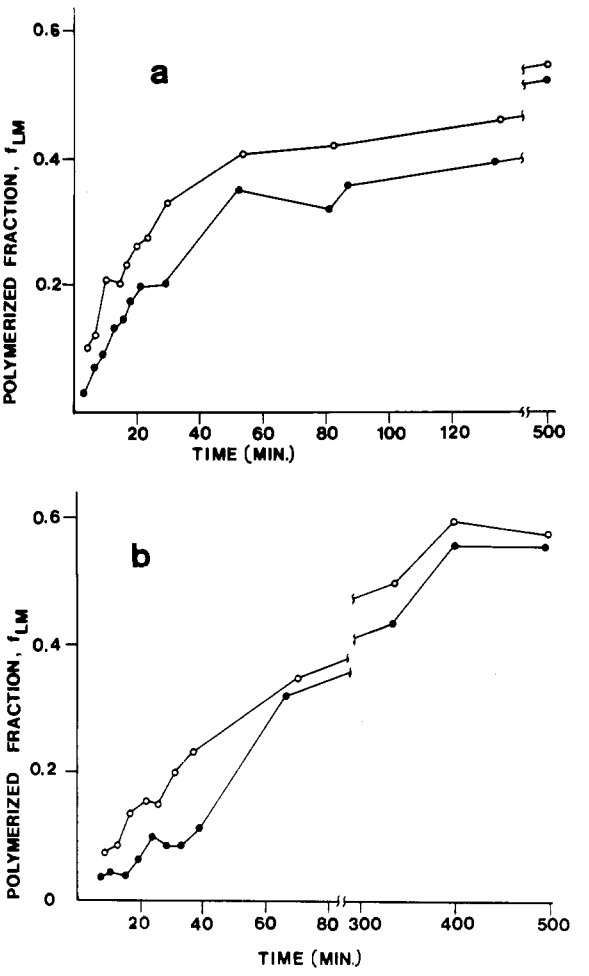


FIGURE 2: Variation of the polymerized fraction of actin, $f_{LM}$, as a function of time after addition of KCl up to 25 mM (trace a) or $MgCl_2$ up to 0.3 mM (trace b). The initial solution contained 9 $\mu$M buffer A actin. Closed circles correspond to addition of trace (1%) buffer A actin with the added salt. Open circles correspond to separate experiments in which trace (1%) buffer B actin was added with the salt. Acceleration of kinetics in the latter case indicates more rapid nucleation of assembly.

coefficient of $(7.88 \pm 0.11) \times 10^{-7}$ cm$^2$/s at infinite dilution; addition of 0.1 mM $MgCl_2$ caused the diffusion coefficient of 20 $\mu$M G-actin to decrease from $(8.2 \pm 0.12) \times 10^{-7}$ cm$^2$/s to $(6.2 \pm 0.7) \times 10^{-7}$ cm$^2$/s. They also observed that this decrease in the diffusion coefficient could be partially reversed upon addition of lower concentrations of $CaCl_2$. Our respective values for the diffusion coefficients in the presence of $Ca^{2+}$ and $Mg^{2+}$ are in agreement with their values, and the agreement between these independent measurements using different techniques permits a high degree of certainty that the results are not artifactual.

We also agree with the suggestion of Newman et al. (1985) that these results probably indicate that $Mg^{2+}$-actin is undergoing oligomerization. Although a $Mg^{2+}$-initiated change in the molecular conformation of G-actin has been suggested as an important step in actin assembly (Frieden, 1982), the hydrodynamic transition that we observe is probably of too great a magnitude to be accounted for by an intramolecular rearrangement. Double-exponential fits to our data led to a consistent result that $Mg^{2+}$-G-actin consisted of about 60% monomer and about 40% of a species with a diffusion coefficient half that of monomer. Depending upon the packing configuration, such a species could be a trimer or, more probably, a tetramer. However, we must emphasize that the statistical reliability of the resolution of two exponential terms

differing only by a factor of 2 is severely limited for signal-to-noise ratios that are typical for these data. We presented the results of this attempt because the determinations were remarkably consistent over many fitting attempts on 10 independent data sets, but strict interpretation is not warranted.

Accurate values of critical concentrations for the conditions of our experiments are not known. We have observed no evidence of filament formation in any of our samples, each of which was observed for longer than 1 day. Indeed, it is not known whether normal actin filaments can be grown at such low concentrations of salt and divalent cations. Newman et al. (1985) attempted to measure the critical concentration for actin under conditions in this range (20 $\mu$M $Ca^{2+}$ and 100 $\mu$M $Mg^{2+}$) and found only that it was greater than 50 $\mu$M and thus more than 5 times the actin concentration we have used. They also found no evidence of filaments in their light scattering measurements, which were performed in similar buffers at 20 $\mu$M actin. At 9 $\mu$M actin we have observed filament formation only when the free $Mg^{2+}$ concentration was about 200 $\mu$M or greater. The fact that the diffusion coefficients we measure are independent of [$Mg^{2+}$] over more than an order of magnitude is evidence that the species detected is a stable particle and not a progressive aggregate induced by electrostatic effects of increasing numbers of bound cations.

Although our value for the G-actin monomer diffusion coefficient agrees with the value reported by Newman et al. (1985) and with the values obtained by Montague et al. (1983), also using quasi-elastic light scattering, we must observe that previous reports from our group (Lanni et al., 1981; Lanni & Ware, 1984) and from another group using FPR (Tait & Frieden, 1982) have been somewhat lower. We attribute these discrepancies to the fact that FPR is still a developing technology. After several improvements in our experimental apparatus, we find now no significant difference between our values and those obtained by quasi-elastic light scattering.

We believe that our initial studies on the assembly properties of buffer B actin indicate that the oligomers present are species of relevance to the elucidation of the mechanism of actin assembly. We have demonstrated that the formation of these oligomers is reversible by dialysis against buffer A, and we have shown that our buffer B actin has the same critical concentration as buffer A actin. Both these findings rule out the possibility that denaturation can account for the observed hydrodynamic transition. This is a particularly important issue because we have necessarily worked at low levels of free divalent cation and free ATP, both of which are important for the stabilization of the G-actin native conformation (Waechter & Engel, 1977; Engel et al., 1977; Frieden, 1982, 1983). However, our conditions always maintained a total divalent cation concentration (including actin-bound, ATP-bound, and free cation) and a total ATP concentration of at least 75 $\mu$M each. In our experience this is a sufficient condition for the stabilization of 9 $\mu$M G-actin, even when the free $Mg^{2+}$, $Ca^{2+}$, or ATP concentration may be as low as 20 $\mu$M. From the known stability constants, this observation implies that MgATP and CaATP are able to interact directly with actin in their undissociated form. By contrast we observe that assembly ensues only when the free divalent cation concentration is raised above 200 $\mu$M.

We have shown that buffer B actin, and buffer A actin to which traces of buffer B actin have been added, assembles to a shorter steady-state filament length, as would be expected if the oligomers present are nuclei for assembly. We have also shown that traces of buffer B actin accelerate the kinetics of assembly of buffer A actin. We conclude that G-actin solutions in buffer B and other $Mg^{2+}$-containing buffers form substantial portions of oligomers, at least some of which are assembly nuclei or at least are more readily converted to assembly nuclei than is monomeric G-actin.

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