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Liquid crystal domains and thixotropy of filamentous actin suspensions

(cytoplasm/rheology/cell motility)

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ABSTRACT **The thixotropic properties of filamentous actin suspensions were examined by a step-function shearing protocol. Samples of purified filamentous actin were sheared at 0.2 sec^{-1} in a cone and plate rheometer. We noted a sharp stress overshoot upon the initiation of shear, indicative of a gel state, and a nearly instantaneous drop to zero stress upon cessation of shear. Stress-overshoot recovery was almost complete after 5 min of "rest" before samples were again sheared at 0.2 sec^{-1}. Overshoot recovery increased linearly with the square root of rest time, suggesting that gel-state recovery is diffusion limited. Actin suspensions subjected to oscillatory shearing at frequencies from 0.003 to 30 radians/sec confirmed the existence of a 5-min time scale in the gel, similar to that for stress-overshoot recovery. Flow of filamentous actin was visualized by polarized light observations. Actin from 6 mg/ml to 20 mg/ml showed the "polycrystalline" texture of birefringence typical for liquid crystal structure. At shear rates <1 sec^{-1}, flow occurred by the relative movement of irregular, roughly ellipsoidal actin domains 40–140 μm long; the appearance was similar to moving ice floes. At shear rates >1 sec^{-1}, domains decreased in size, possibly by frictional interactions among domains. Eventually domains flow in a "river" of actin aligned by the flow. Our observations confirm our previous domain-friction model for actin rheology. The similarities between the unusual flow properties of actin and cytoplasm argue that cytoplasm also may flow as domains.**

Cytoplasm and suspensions of actin have unusual and complex fluid behaviors that must play a role in such phenomena as cytoplasmic streaming, amoeboid movement, movement of vesicles through the cytoplasm, and bulk flow of polymer. Both cytoplasm and actin solutions are shear thinning; their viscosity decreases with increasing shear rate (1–8). Shear thinning of actin differs from most polymer solutions in the absence of a Newtonian viscosity plateau at very low shear rates (1–4, 7). Further, cytoplasm and cytoskeletal suspensions show a constant shear stress (force) for shear rates between 0.001 and 1 sec^{-1} (1, 5). Flow rate, therefore, is not fixed by the force; no flow occurs at forces less than the constant and fluid velocity is limited by inertia alone at forces higher than the constant. We (1) called this "flow indeterminacy," which, for example, helps explain the capacity of *Physarum* to support very rapid cytoplasmic streaming within "walls" of cytoplasm that do not shear away: High flow rates do not necessarily require large forces and a small difference in filament concentration could account for the difference between flowing and nonflowing cytoplasm. Cytoplasm and actin suspensions are also thixotropic (3, 4, 8–10)—i.e., show time-dependent decreases in viscosity at steady shear rate and subsequent recovery when the flow is discontinued (11). Thixotropy is frequently discussed in terms of gel → sol transitions familiar to biologists and has been among the most durable concepts in our understanding of the mechanisms of amoeboid movement (8, 12–14).

We (1) have proposed that the flow indeterminacy of actin and microtubule suspensions results from solid-like domains of liquid crystalline polymer sliding past one another, rather than from movement of individual filaments as for flow of most polymer fluids. Solid friction-like interactions among domains would explain indeterminate flow because sliding friction is independent of sliding velocity. Also, static friction is greater than sliding friction, possibly explaining thixotropy. In support of this model, we found that actin and microtubule solutions formed birefringent domains when viewed in polarized light (1), indicative of a domained liquid crystal structure (15, 16). As the name implies, liquid crystals show both fluid properties, such as flow, and crystal properties, such as elasticity and birefringence of polarized light. Liquid crystals are frequently regarded as a distinct phase of matter, commonly seen in suspensions/solutions of long polymers (16, 17), including biological molecules (1, 18–20). Indeed, it has long been appreciated that liquid crystal properties may be responsible, in part, for the mechanical properties of cytoplasm, the polarity of cells, and their unusual shape (21, 22).

We report here a quantitative rheological study of the thixotropy of filamentous actin (F-actin) suspensions. We also visualized the flow of these suspensions by polarization microscopy.

METHODS

Actin was prepared from acetone powder of rabbit skeletal muscle (23). Some actin preparations were purified further by chromatography over Sephadex G-150 or by ion exchange on DEAE-cellulose (23). Protein concentrations were determined by the method of Bradford (24).

Rheological measurements were made on a Rheometrics fluid spectrometer model 8400 (Rheometrics, Piscataway, NJ) configured with an environmental chamber and with a 0.02-radian angle cone and plate of 50 mm diameter. Newtonian viscosity standards of 9.95 poise and 307.2 poise (Cannon Instrument) were used to calibrate the instrument and to confirm that stress overshoots were not artifacts. Globular actin was polymerized and loaded onto the instrument as described (1).

For thixotropic measurements, an experimental procedure was adopted as outlined by Mewis (11). To avoid fluid structures unique to freshly polymerized actin, polymerized samples were initially sheared at 0.2 sec^{-1} for 20 min; this shear rate is thought to be within the physiological range. Samples were then rested for 5 min (preliminary experiments indicated most of the recovery occurs within this period) and

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Abbreviation: F-actin, filamentous actin.

sheared again at 0.2 sec^{-1}. The stress overshoot (σ^*) was measured at the beginning of this shearing as was the steady-state shear stress after 5 min (σ_{const}). Shearing was then stopped for a period between 5 sec and 3 hr and the stress overshoot (σ_{run}) and steady-state stress (σ_{const}) were again measured during another 5-min shear. Recovery of the peak overshoot was expressed as a fraction of the initial peak:

$$\% \text{ recovery} = [\sigma^* - \sigma_{const}]/[\sigma_{run} - \sigma_{const}] \times 100\%.$$

For viscoelastic measurements, actin samples at 6 mg/ml were subjected to oscillatory shear at frequencies from 0.003 to 30 radians/sec at 10% strain at 22°C. Preliminary strain-sweep experiments indicated that the gel state of actin was undisrupted at this strain. Relaxation spectra were calculated by the method of Tschoegl (25) from loss modulus data (G'') from the rheometer.

Actin flow was visualized for samples at various concentrations between 6 mg/ml and 20 mg/ml by placing the protein between a glass microscope slide and a coverslip separated by a spacer, nominally 0.15 mm thick. In some experiments actin was polymerized *in situ* between the slide and coverslip; in other experiments the actin was polymerized then placed between the slide and coverslip. These specimens were viewed through crossed polarizers in a Leitz Ortholux Pol I microscope (15). Flow was induced by slowly poking (depressing) the coverslip to achieve shear. Records of flow were made using a silicon-intensified video camera (Dage-MTI). Shear rate was estimated according to the formula

$$\bar{\gamma} = v(\mu m/sec)/(d/2) = 2v/150\ \mu m,$$

where $\bar{\gamma}$ is average shear rate, v is average velocity measured as described below, and d is the distance between the two plates. This analysis assumes that the velocity profile between the plates is approximately parabolic and that the microscope is focused centrally between the plates. Average velocity of domains was determined by marking the positions of easily recognized domains in videotape frames "frozen" at 1- to 3-sec intervals, measuring the distance moved, and dividing by the elapsed time.

RESULTS

Actin thixotropy was studied by applying 5-min periods of constant shear rate to F-actin at 6 and 10 mg/ml interrupted by periods of "rest." As shown in Fig. 1, we measured the magnitude and duration of the stress (force) overshoot after the onset of shear (indicative of the breakdown of some structured "gel" state), its decay to a steady-state stress (indicative of a fluid), and the subsequent recovery of the stress-overshoot/gel state after the "rest" periods (11). The stress overshoot was very steep, leading to a peak in less than 2 radians. Upon cessation of shear, shear stress dropped to zero nearly instantaneously. As shown in Fig. 1, subsequent shearing caused stress overshoots that increased with increasing recovery period; the overshoot after a recovery of 5 min was 85–95% of the value of the peak from the first 5-min shearing. These recovery time data are summarized in Fig. 2 for various actin preparations at 6 mg/ml. The recovery for actin at 10 mg/ml was nearly identical (data not shown). A striking observation here was that stress overshoot recovery appeared to be unaffected by the actin preparation or purity.

We were interested in determining whether overshoot recovery depended on diffusion or on some reaction-limited process such as polymer annealing. A simple method for distinguishing these alternatives involves plotting the recovery data against the square root of time (Fig. 3). The straight line shown there at the short recovery time limit indicates a

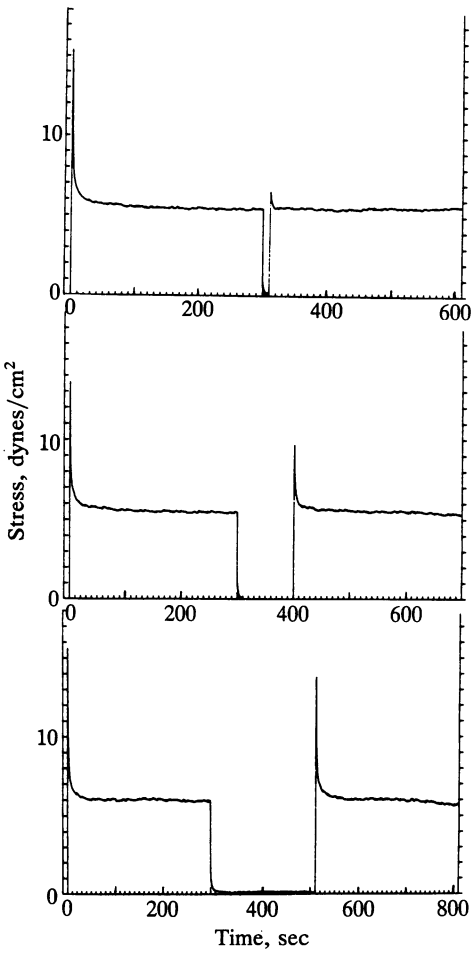

FIG. 1. Shear stress as a function of time (each time division is 10 sec) for actin subjected to a step-function shearing protocol with various periods of recovery between 5-min bouts of steady shearing at 0.2 sec^{-1}. After a period of steady shearing to homogeneity, actin purified by chromatography on DEAE-cellulose (23) at 6 mg/ml was subjected to a step-function protocol of steady shearing at 0.2 sec^{-1} in 5-min bouts, followed by various periods of recovery, followed by steady shearing at 0.2 sec^{-1} for 5 min. Shown here are rheograms for recovery times of 10 sec (*Top*), 100 sec (*Middle*), and 240 sec (*Bottom*). Note that peak shear stresses (overshoots) increase with increasing time of recovery.

diffusion-limited process (27). Since reaction-limited models are linear with time in the short time limit, reaction-limited models should not produce a reasonable fit to the stress-overshoot data.

Viscoelastic Measurements. The experiments above indicate a diffusion process with a time scale of 5 min for the disrupted gel state to recover. We wished to determine whether a similar time scale was apparent in the gel state itself. If so, viscoelastic measurements (i.e., assessing fluid and solid properties) on the undisrupted gel should show a similar 5-min time as the longest over which the gel stores energy (solid behavior). We subjected F-actin suspensions at 6 mg/ml to sinusoidally oscillating nondisruptive shear at frequencies from 0.003 to 30 radians/sec. Because frequency is 1/time, varying the frequency of the sinusoid at a sufficiently small strain (10%) to ensure nondisruption of the gel state assesses the viscoelastic behavior of this actin state at several time scales.

Fig. 4 is a relaxation spectrum obtained from loss modulus data for two experiments at 22°C. In one sample (data set 1) dynamic measurements were initiated immediately after the 1-hr polymerization period with no prior shearing. The other sample (data set 2) was sheared at 0.2 sec^{-1} for 20 min prior

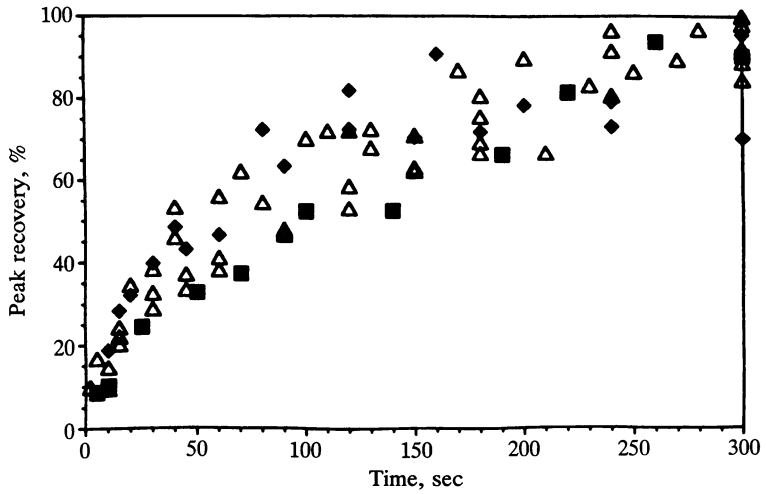

FIG. 2. Proportional recovery of peak stress (overshoot) relative to peak stress at the beginning of the first 5-min shearing (= 100%) as a function of recovery time for actin at 6 mg/ml, purified by various standard methods (23). △, Purified by extraction essentially as described by Spudich and Watt (26); ◆, purified by extraction followed by G-150 Sephadex chromatography; ■, purified by extraction followed by DEAE-cellulose chromatography.

to beginning dynamic measurements, as for the thixotropic recovery experiments above. As shown in Fig. 4, both experimental samples show a peak in the relaxation spectrum at 300 sec. We do not see evidence of the essentially instantaneous time scale for stress relaxation, presumably because it is too fast (i.e., the maximum experimental frequency was too short to observe it). The relaxation spectra differed between sheared and unsheared samples in absolute value but not in their qualitative change with a characteristic time, τ. We found equally large differences in absolute values between different preparations of actin treated identically. The relatively constant H for τ of less than 300 sec indicates substantial energy storage (elasticity) at these time scales. The peak in H at 300 sec and the sharp decline thereafter indicate that the maximum time scale for elastic storage is 5 min, at longer times the material flows (dissipates energy/force). Fig. 4 also shows the relaxation spectrum predicted for actin at 6 mg/ml and 22°C by assuming the length distribution of filaments of Kawamura and Maruyama (29) and that it behaves according to a rigid-rod model for isotropic (nonliquid crystal, nondomain) polymers (28). As shown, the viscoelastic behavior of actin at 6 mg/ml is significantly different than that predicted by this rigid-rod model.

Flow Visualization of F-Actin Suspensions. We wished to confirm our previous proposal (1) that actin at these concentrations is flowing as domains of liquid crystals. Liquid crystals are typically visualized by the form birefringence (i.e., optical anisotropy) of the material when viewed in the polarizing microscope (15).

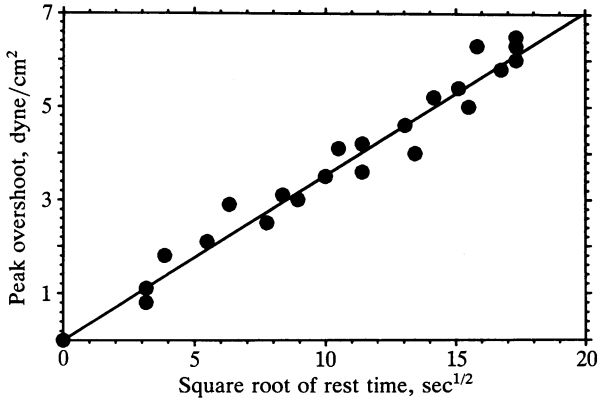

FIG. 3. Peak overshoot stress (stress maximum minus steady-state stress) as a function of the square root of recovery time for three experiments (over 3 days) of actin from the same preparation. The close fit to a straight line strongly suggests that recovery is a diffusion-limited process. (1 dyne = 100 mN.)

F-actin at various concentrations between 6 mg/ml and 20 mg/ml was viewed through crossed polarizers. For all samples, the entire field appeared birefringent as samples were rotated indicating that the suspension was not a collection of ordered regions embedded in an isotropic surrounding. Actin polymerized in the observation chamber showed uniform birefringence or broad stripes, similar to the observations of Hitt *et al.* (20) for microtubules, suggesting that the liquid crystallinity was not due to flow during polymerization. "Polycrystalline" texture characteristic of various liquid crystal systems (16) was observed after very light "poking" on the coverslip with forceps or by placing previously polymerized actin into the observation chamber.

Flow of these samples was initiated by slowly depressing the coverslip. The initial response was elastic; the entire field moved and returned as a unit when the coverslip was depressed. Additional, more forceful poking, presumably exceeding the stress overshoot documented above, broke the actin into irregular domains which then moved past one another. The appearance was similar to moving ice floes. Actin domains were often roughly ellipsoidal with long dimensions of 40–140 μm. Each domain contained an irregular pattern of positive and negative birefringence. We observed no alteration in the alignment of filaments within the domains after several cycles of flow. Fig. 5 shows frames from the video record of the flow of an unusual round domain over 20 sec. The average estimated shear rate for this sequence is 0.45 sec^{-1}. This sequence was chosen for presentation because the more typical irregular domains change their birefringent appearance too significantly to recognize in still photography from one frame to another. Flow is difficult to demonstrate clearly except on videotape; the authors have a modest number of videotapes that interested readers may borrow.

Our observations of actin flow included shear rates from 0.06 to 5.5 sec^{-1}. At shear rates less than about 1 sec^{-1}, domains maintained their integrity throughout the observations, 20–30 min. Flow indeterminacy, postulated in Buxbaum *et al.* (1), is obvious in these records; domains in the same vicinity move at different rates when subjected to the same shear stress. However, shearing at 1–2 sec^{-1} or greater appeared to wear down domain sizes after several minutes. These smaller domains then move in a "river" of uniformly flowing actin. The filaments in this uniform flow clearly show alignment as observed by Maruyama (30) at shear rates of 2 and 27 sec^{-1}. To follow up the thixotropic measurements above, we observed the effect of 5-min rest periods between shearing bouts at approximately 1 sec^{-1}. It clearly required more forceful poking to achieve flow after a 5-min rest. Visually, however, the domains did not change in shape, size,

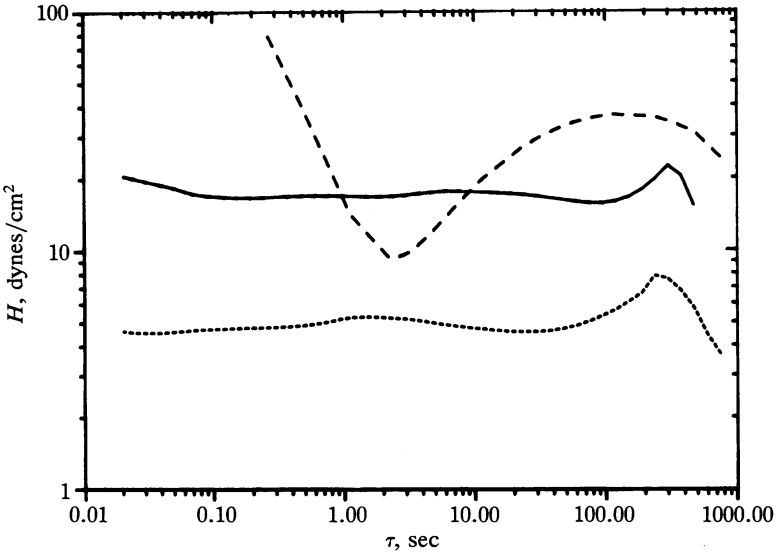

FIG. 4. Relaxation spectra for actin at 6 mg/ml and 22°C. Data set 1 (solid line) is from a sample unsheared prior to the beginning of measurements. Data set 2 (dotted line) is from a sample sheared for 20 min at 0.2 sec^{-1}, as in thixotropic experiments above, prior to dynamic measurements. The dashed line shows the prediction of an isotropic rigid-rod model (28). This analysis considers the material as an infinite array of Maxwell elements (linear viscoelastic elements modeled by a spring in series with a dashpot). Each Maxwell element is associated with a characteristic time (τ) over which force relaxes to $1/e$ of an initial value (i.e., τ is essentially a half-life for force decay). This time is derived from the ratio of the stiffness of the spring and the viscosity of the dashpot. A parameter, H, equivalent to a relaxation modulus, is plotted as a function of these characteristic times. This analysis provides an assessment of the important time scales for a material and provides a qualitative assessment for classifying the response of various materials based on similarities of relaxation spectra.

or relative positions during this period. After several pokes the flow became indistinguishable from that before the rest period.

DISCUSSION

Our rheological and optical observations indicate that actin suspensions at concentrations of 6 mg/ml or greater are liquid crystalline. Shear converts actin, apparently irreversibly, into a polycrystalline domain structure typical of many liquid crystal phases (16). We postulate that the flow of this material as domains (i.e., without movement of filaments within domains) is responsible for both the steady-shear behavior of actin and the thixotropic properties for the following reasons. First, we observed that flow was discrete; a uniform stress did not result in a uniform velocity gradient. Instead, at shear rates between 0.06 and 1 sec^{-1} actin flows by the movement of discrete domains. These observations are similar to reports of flow of "domained" liquid crystals (31) and flow of non-liquid-crystalline domained fluids (submicrometer parti-

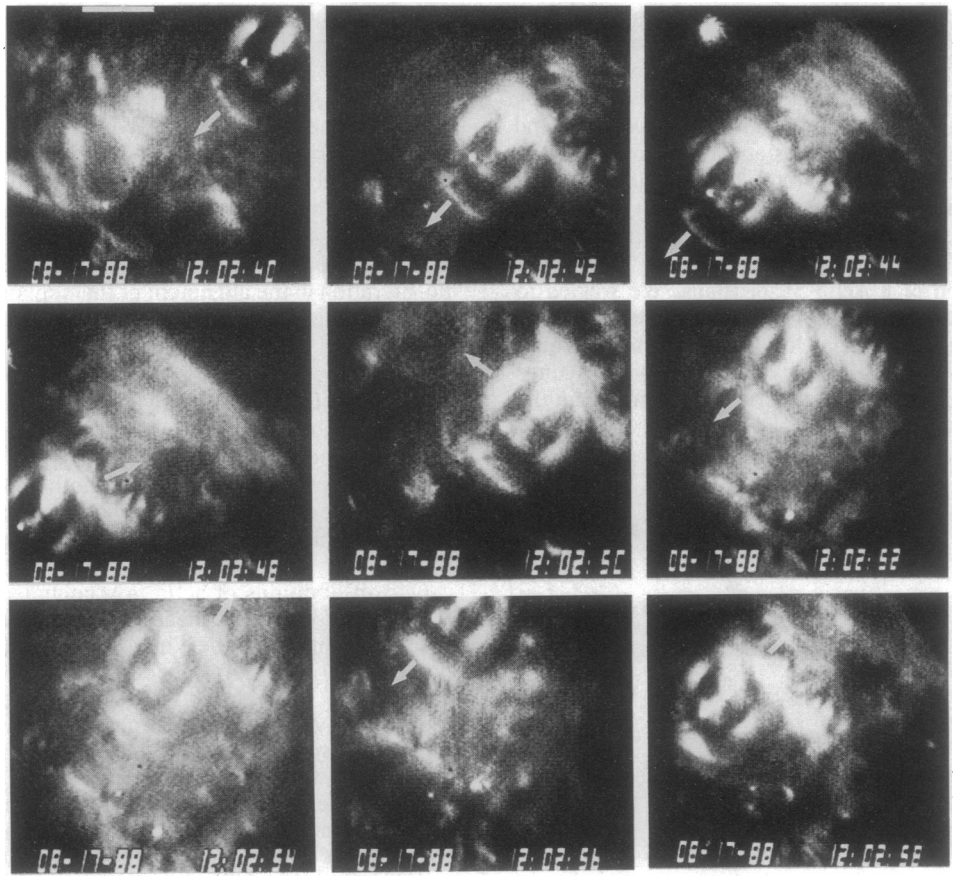

FIG. 5. Flow of liquid crystal actin domains. A sequence of still photographs of an unusual round actin domain subjected to shear stress. The tail of each arrow is near the round domain and the arrows point in the direction of domain movement. Photographs were taken every 2 sec from a videotape of polarized light images recorded by a silicon-intensified television camera. The image at 12:02:46 is omitted because the domain was in the same position as at 12:02:44. (Bar = 40 μm.)

cles) (32). An interesting similarity of the particle fluid (32) and actin (1, 2, 4, 6, 7) is the absence of a Newtonian viscosity plateau at very low rates of constant shear. The filament alignment and domains we observed at shear rates greater than 1 sec^{-1} [where shear-thinning behavior of actin begins to level off (4)] is seen in many liquid crystal systems (16, 31). Several prominent rigid-rod models, however, do not correspond with this observation of rod alignment at higher shear rates (28, 33, 34).

An attractive theory has been proposed (35), involving self-similar defects within domains, that can explain how such domain flow might underlie shear-thinning, thixotropic, and indeterminate-fluid behaviors. Alternatively, frictional interactions and domain breakdown may play major roles in the flow and alignment of actin suspensions at moderate to high concentration. Shear stress develops and decays virtually instantaneously (Fig. 1), as with solid friction. Possibly a structured, "gel" state forms that recovers (thixotropy) by way of a diffusive interaction of about 5 min. One particularly simple model is the diffusion of rods from adjoining domains interdigitating with each other within 5 min, thus "knitting together" the domains into a gel state. Shear stress applied over a period longer than 5 min would allow flow to occur (Fig. 4) because, on average, the rods have sufficient time to diffuse into one or another domain. The stress overshoot observed for the gel → sol transition would then be the stress required to break apart the interdigitated polymers, creating the irregular domains. Shear stress is a constant in steady flow (1) because flow requires only the force necessary to break or bend those rods that extend from one domain to another; this force is independent of shear rate as for solid friction.

A significant aspect of this work is that the available evidence is consistent with domain flow, as distinct from liquid crystallinity, underlying the flow of cytoplasm and actin generally. As outlined above, the phenomenology of cytoplasmic flow is similar to the fluid behaviors of actin, properties that can be accounted for by domain flow as explained above. The behavior of actin at concentrations as low as 1 mg/ml resembles that for the concentrations used here. At high and low concentrations, F-actin shows stress overshoots (3, 4) and indeterminate-flow (1, 2), shear-thinning (1–4, 7), and similar viscoelastic behaviors (36). Also, diffusion data on actin at 1 mg/ml from Frieden and coworkers (37, 38) indicates filament diffusion is significantly slower than expected for movement of individual rods but consistent with filament immobilization within domains. These actin samples also show evidence of "microheterogeneity" upon shearing (38), implying a fluid structure larger in scale than individual molecules (i.e., domains). In addition to explaining flow properties, the possibility of cytoplasmic domains is of interest given recent work invoking microcompartmentation of cellular metabolites (39) [e.g., energy metabolites within smooth muscle (40)]. Such compartmentation and indications of a length-scale dependence of molecular movement in cytoplasm (5, 41) may reflect molecules moving quickly between filaments while moving slowly between domains.

Finally, we note that experiments investigating amoeboid movement, cytoplasmic streaming, etc., *in vivo* have confirmed the intimate link between contraction (shearing) of cytoplasm and its gel → sol transition (12, 13). Our data suggest that any contractile mechanism generating sufficient shearing forces would cause, by itself, a gel → sol transition. This is the simplest explanation we have seen for the close link between cytoplasmic contraction and the gel → sol transition.

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