Growth conditions control the size and order of actin bundles in vitro

David L. Stokes and David J. DeRosier
Rosenstiel Basic Medical Sciences Research Center, Brandeis University, Waltham, Massachusetts USA 02254

ABSTRACT The bonding rules for actin filament bundles do not lead to a particular packing symmetry, but allow for either regular or disordered filament packing. Indeed, both hexagonal and disordered types of packing are observed in vivo. To investigate factors which control bundle order, as well as size, we examined the effect of protein concentration on the growth of actin-fascin bundles in vitro. We found that bundles require 4–8 d to achieve both maximum size and order. The largest and best ordered bundles were grown at low fascin and high actin concentrations (an initial fascin/actin ratio of 1:200). In contrast, a much larger number of poorly ordered bundles were formed at ratios of 1:25 and 1:50, and most surprisingly, no bundles were formed at 1:300 or 1:400.

Based on these observations we propose a two-stage mechanism for bundle growth. The first stage is dominated by nucleation, which requires relatively high concentrations of fascin and which is therefore accompanied by rapid growth. Below some concentration threshold, nucleation ceases and bundles enter the second stage of slow growth, which continues until the supply of fascin is exhausted. By analogy with crystallization, we hypothesize that slower growth produces better order. We are able to use this mechanism to explain our observations as well as previous observations of bundle growth both in vitro and in vivo.

INTRODUCTION

How do actin bundles assemble and why is it that some bundles contain hexagonally packed actin filaments, while others contain filaments with liquid-like packing (Fig. 1)? Such bundles occur in a wide range of biological contexts and although the filaments are always actin, the identity of the bundling protein(s) depends both on the species and on the type of cell. Nevertheless, the observation of different filament packing within these bundles need not be due to different bonding geometries between the constituent proteins. Quite the contrary, the geometry of the interfilament bond, and even the actual binding site on actin, could be the same for both hexagonal and liquid-like filament packing (DeRosier and Tilney, 1982).

If the difference between hexagonal and liquid-like bundles does not arise from a difference in the binding geometry of the cross-bridge, then how might the cell control the packing of filaments within its bundles? Tilney and DeRosier (1986), in their study of embryogenesis of stereocilia in the inner ear, suggest that well-ordered bundles within these stereocilia are formed in two stages. First, a small hexagonal seed is produced and second, filaments are gradually added to the seed to produce a large well-ordered bundle. They argue that if one cross-bridges many filaments together simultaneously, disordered filament packing will be locked into the structure and a disordered bundle will result. Thus, they predict that a limiting supply of cross-bridging protein is present, which reduces the rate of cross-bridging and thereby produces well-ordered seeds; then, these seeds grow into well-ordered bundles.

The dependence of bundle formation on the concentration of cross-bridging protein has been studied in vitro by Bryan and Kane (1978), using fascin isolated from sea urchin oocytes. They measured the amount of protein incorporated into bundles, the actin-to-fascin ratio in these bundles, and the actin-to-fascin ratio left behind in the unbundled material. They found that the amount of protein in bundles increased linearly with the fascin concentration, and that at low fascin concentrations, not only were fewer filaments bundled but fewer cross-bridges were formed per filament (i.e., the fascin-to-actin ratio was lower). From a Scatchard plot, they measured a binding constant of $\sim 2\ \mu M^{-1}$ and a fascin-to- actin ratio at saturation of 1:4.6, which corresponds to three cross-bridges per actin cross-over. In the electron microscope, Bryan and Kane observed that bundles formed at low fascin concentrations appeared smaller and more disordered than those at higher fascin concentrations, in apparent contradiction with the predictions of Tilney and DeRosier (1986). R. E. Kane (personal communication) finds, however, that large, extremely

Dr. Stokes' current address is Department of Physiology, Jordan Hall, University of Virginia Health Sciences Center, Charlottesville, VA 22908.

0006-3495/91/02/456/10 $2.00

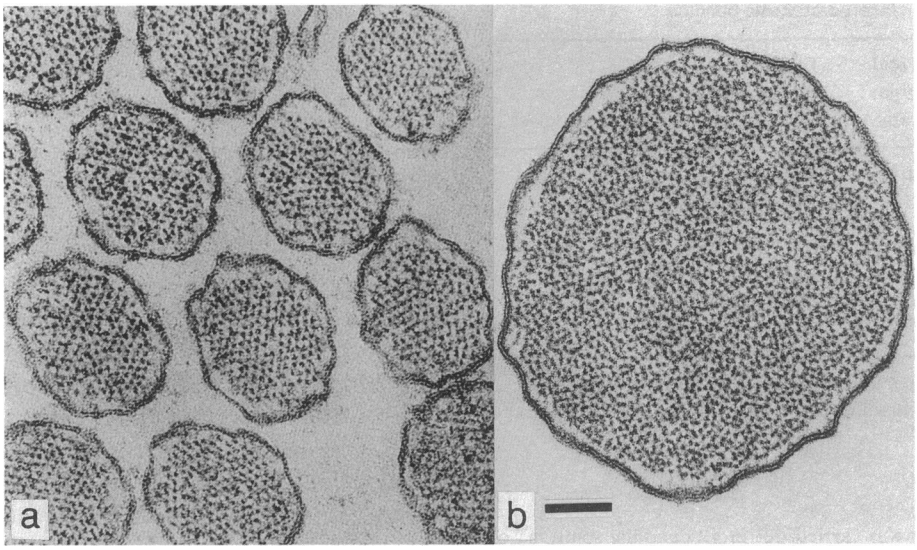


FIGURE 1 Different filament packing in actin bundles in vivo. (*a*) Transverse thin section through hexagonally packed bundles. These bundles form the core of the stereocilium, which is a microvillus-like structure found on the surface of hair cells in the inner ear. These bundles of hexagonally packed filaments are from the chicken. Although the packing is not perfect, the filaments lie roughly along lines that intersect at 60 degree angles. Thus, most filaments have six nearest neighbors. (*b*) Transverse section through a bundle with liquid-like packing. This section is from a stereocilium from the alligator lizard. In contrast to those in *a*, the filaments do not lie along regular lines but are randomly packed. On average, filaments have five nearest neighbors instead of six. Reprinted from the work of Tilney et al. (1983*b*) in the *Journal of Cell Biology* with permission. The bar represents 80 nm.

well-ordered bundles form in actin preparations that are slightly contaminated by fascin. Besides corroborating the predictions of Tilney and DeRosier, this observation suggested that it would be important to look at bundle formation at lower fascin-to-actin ratios than had previously been studied.

We therefore measured the size and order of bundles grown in vitro at various actin-to-fascin ratios. Unlike Bryan and Kane (1978), we increased the actin concentration proportionate to a decrease in fascin concentration to force bundle formation even at very low fascin concentration. Also, unlike Bryan and Kane, who were interested in the average protein composition of bundles and in the binding constant of fascin for actin, we focused on the size and order within individual bundles as a function of time and of initial fascin-to-actin ratio. We found that bundles grew bigger and better ordered over a 4–8 d period. Moreover, the largest, best-ordered bundles grew at the lower fascin-to-actin ratios, with the exception that at the lowest concentration ratios (below 1:300) no bundles were formed. We will elaborate on the mechanism of bundle formation presented by Tilney and DeRosier (1986) and will use this mechanism to explain observations in vitro by ourselves and by Bryan and Kane as well as those in vivo by Tilney and DeRosier.

METHODS

Preparation and microscopy of actin bundles

Actin bundles were grown in vitro by adding an extract of sea urchin eggs, which contained fascin, to F-actin. F-actin was prepared by the method of Spudich and Watt (1971) at a final concentration of ~15 mg/ml. The extraction of eggs from the sea urchin *Tripneustes gratilla* followed the method of Kane (1975). Briefly, a crude extract was first made by removing the coat of jelly from the eggs, then washing and homogenizing them in 0.9 M glycerol, 5 mM EGTA, and 0.1 M PIPES. After centrifuging at 100,000 × *g* for 1 h, the clear supernatant was removed and dialyzed overnight against 10 mM PIPES, 0.1 mM ATP, and 0.1 mM EGTA, pH 7.0. The preparation was then centrifuged at 25,000 × *g* for 15 min and 1 mM EGTA and 1 mM NaN_3 were added. The resulting egg extract was frozen in liquid nitrogen and stored at −70°C. After up to 8 mo of storage, the egg extract was thawed slowly, in an ice bath, and centrifuged at 25,000 × *g* for 20 min. The viability of the extract was tested by its ability to form a gel; 20 mM KCl and 1 mM ATP were added to a small aliquot of extract, which was then incubated at 40°C for 30–60 min. If an opalescent gel formed, the protein in the extract was deemed viable, and was ready to be used for bundle formation (Bryan and Kane, 1978).

Bundles were grown over a range of fascin-to-actin ratios. To obtain ratios of 1:25, 1:50, 1:100, 1:200, and 1:400 (fascin/actin), we mixed F-actin with egg extract which has a fascin concentration of ~0.17 mg/ml (Otto et al., 1980). At each of these ratios, we constrained the product of actin and fascin concentrations to be 0.25 $(mg/ml)^2$, thereby ensuring complete incorporation of fascin into actin bundles (see

TABLE 1 **Amount of fascin incorporated into bundles**

Concentration ratio*	Total molar ratio	Total actin conc.	Fascin conc.	Fascin in bundles‡
	μM	μM	μM	
1:25	1:33	60	1.8	1.8
1:50	1:66	83	1.3	1.3
1:100	1:132	120	0.9	0.9
1:200	1:264	167	0.64	0.64
1:300	1:391	206	0.53	0.53
1:400	1:530	238	0.45	0.44

*The weight ratio of fascin to actin in milligrams per milliliter.
‡Calculations based on binding constant K_b = 1.9 μM^{-1} (Bryan and Kane, 1978).

Table 1). The bundling was carried out in a "bundling solution" of 30 mM KCl, 5 mM K_2HPO_4, and 1 mM NaN_3 at pH 7.0 at 0°C. Approximately 3 ml of solution was made at each concentration ratio.

Aliquots of each concentration ratio were prepared for electron microscopy after periods of 3 h, 1 d, 2 d, 4 d, 8 d, and 16 d. Each aliquot, 0.5 ml in volume, was twice centrifuged at 12,000 × *g* for 15 min, and was resuspended each time with an equal volume of bundling solution. Also, a control sample, consisting only of egg extract, was tested for spontaneous bundle formation at each time point.

Solutions containing actin bundles were negatively stained with 1% uranyl acetate on carbon-coated grids. Micrographs were recorded with an electron microscope (Philips EM420; Philips Electronic Instruments Co., Mahwah, NJ) at 10500× such that a randomly chosen group of 50–100 bundles was photographed at each concentration ratio and time point. The 395 Å repeat of tropomyosin paracrystals was used to calibrate the magnification of the microscope.

Measurement of bundle size and order

The lengths and widths of all bundles that were entirely within the field of the micrographs were measured with a digitizing pad (MM1200; Summagraphics Corp., Fairfield, CT), which was connected to a computer (VAX 11/780; Digital Equipment Corp., Marlboro, MA). The order within each group of bundles was assessed with a qualitative scoring scheme. Scores ranged from 0 to 1 and corresponded to the fraction of the bundle length that possessed hexagonally packed and axially aligned filaments. Thus, a score of 0.15 (shown in Fig. 2 *a*) corresponds to a largely disordered bundle, a score of 0.5 to a bundle that was ordered along half its length, and a score of 1 to a bundle that was hexagonally packed along its entire length (Fig. 2 *b* or *c*). To assign a score to a particular bundle, its micrograph was examined at a ten-fold magnification in an optical projector (Shadowgraph; Nikon Inc., Garden City, NY). At a given place along the bundle, hexagonal packing was considered to be present if the filaments in the bundle were straight, parallel to one another, evenly spaced and appeared to be superimposed along one of the hexagonal lattice lines. This superposition gives rise to a distinct zigzag pattern of actin subunits from which the axial alignment of neighboring filaments could be verified; if filaments were hexagonally packed then they inevitably were axially aligned. Smaller bundles were difficult to score because they contained a small number of filaments (usually <10) and therefore contained a minimal amount of filament superposition; such superposition was relied on for judging both hexagonal packing and axial alignment. Thus, for a given time and concentration ratio, bundles that were smaller than average were ignored.

RESULTS

Bundle formation

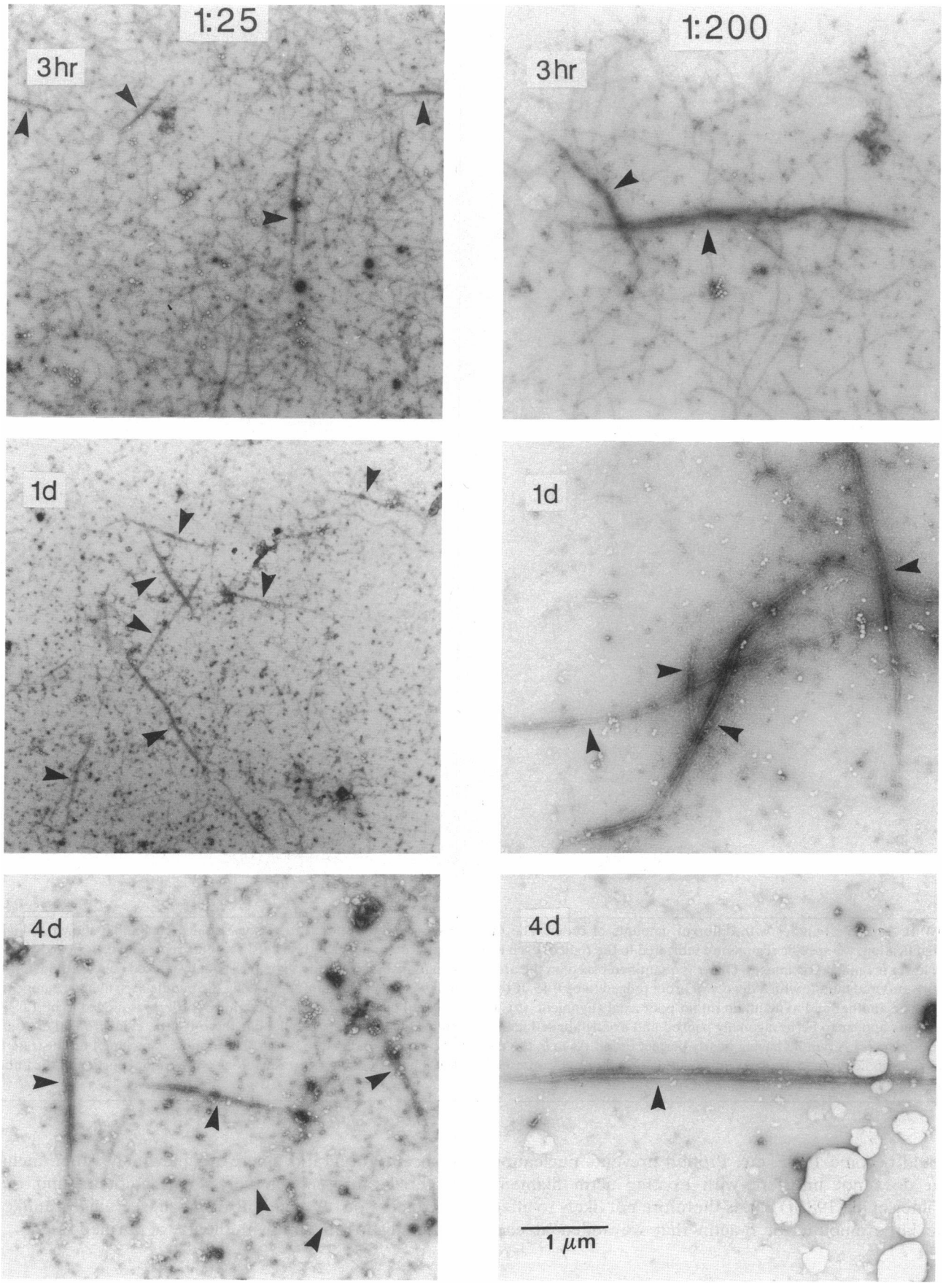
As expected from the work of Bryan and Kane (1978), the size of the pellets was roughly proportional to the concentration of fascin in the initial mixture; that is, the ratio of 1:25 (fascin to actin) always produced the largest pellet, while pellets from lower ratios (i.e., 1:50 to 1:200) were progressively smaller and the ratios of 1:300 and 1:400 yielded no pellet at all even after longer, higher speed centrifugation. At a given ratio, pellet size increased over the first 2 d but changed little after that.

Electron microscopic observations of resuspended pellets confirmed the correspondence between pellet size and number of bundles. Resuspensions of large pellets (e.g., from the 1:25 ratio) contained so many bundles that further dilution was necessary for electron microscopy, while resuspensions of smaller pellets contained many fewer bundles (see Fig. 3). Solutions from ratios of 1:300 and 1:400 contained no bundles at all, nor did control solutions, consisting only of egg extract.

Because we used an egg extract as our source of fascin, there was potential for other proteins to interfere with bundle formation. However, after adding extract to actin, SDS gels indicate that pellets contain only actin, fascin, and sometimes 220 kD protein, an actin gelation factor (Bryan and Kane, 1978, and our unpublished results). It is possible that other minor proteins in the extract could influence bundling without being incorporated into the bundles. However, most of the proteins in the extract have been characterized and considering their normal functions, one would not expect them to interfere under the conditions we used for bundling. For example, under the proper conditions bundles can be cross-linked into a structural gel by the 220-kD protein and, under different conditions, including the addition of ATP, myosin can combine with actin and sea urchin filamin to produce a contractile gel (Kane, 1980; Bryan and Kane, 1982). However, our conditions were different and most importantly, none of our bundling solutions produced either structural or contractile gels, but remained fluid. Endogenous actin, which may be complexed to profilin, is also present in egg extract at a concentration of ~0.8 mg/ml (Otto et al., 1980); if this actin formed filaments it would only change the concentration ratios slightly: e.g., the concentration ratio of 1:25 would become 1:30, 1:50 would become 1:55, 1:100

FIGURE 2 Three bundles with different amounts of order. The three images at the bottom are regions from each bundle shown at higher magnification; the specific regions are indicated to the right of each lower magnification image by brackets. The bars represent 0.1 μm and apply to the lower magnification images. Order is quantified based on the area of the bundle that is judged to be hexagonally packed (see Methods). (*a*) A poorly ordered bundle with a degree of order judged to be 0.15. If the image is viewed at glancing angle along the bundle axis, filaments appear to cross one another and to maintain rather poor axial alignment. (*b*) A well-ordered bundle in which the degree of order was judged to be 1.0. The filaments appear to be hexagonally packed and axially aligned along the entire length of this bundle, but there is little evidence of 125 Å axial periodicity. (*c*) A bundle having nearly perfect order. As in *b*, the degree of order was judged to be 1.0. This bundle is included to illustrate the 125-Å periodicity characteristic of cross-bridging in perfectly ordered bundles. This periodicity is present along a substantial portion of the bundle and is most clearly seen in the enlarged region at the lower right.

would become 1:105, etc. Profilin prevents nucleation, but does not interfere with existing actin filaments (Tilney et al., 1983*a*) and is therefore not likely to affect the large amounts of F-actin that we added at each concentration ratio. There are, inevitably, other uncharacterized proteins in very small amounts and their participation in bundling, though seemingly unlikely, can only be ruled out with the use of purified fascin.


1:25
3hr
1:200
3hr
1d
1d
4d
4d
1 μm

Size and order of individual bundles

We measured bundle size and order from low magnification micrographs (10,500×) taken at each concentration ratio and at a number of time points (e.g., see Fig. 3). Because order varies along an individual bundle, there is no way to quantitate bundle order from its diffraction pattern. We therefore characterized bundle order directly from the images according to the fractional area of each bundle that showed regularly spaced and axially aligned filaments characteristic of hexagonal packing (Fig. 2). The characteristic 125-Å striations produced by fascin cross-bridges (DeRosier et al., 1977; DeRosier and Censullo, 1981) were visible in some bundles but generally were not strong features. This is similar to previous observations of actin bundles in the stereocilia of the chicken, where mosaicity in the hexagonal packing reduces the magnitude of the lateral striations (see, for example, Figs. 1, *d* and *e*, and 2, *e* and *f*, from Tilney and DeRosier, 1986).

Bundle size

The plots in Fig. 4 represent the average length (*a*) and width (*b*) of the bundles from various concentration ratios as a function of time. The error bars represent the standard error of the mean, but the variability of the data, judged from the scatter of the means about a smooth curve, is greater than these standard errors would predict. This may reflect sources of error not represented by these standard errors, such as unavoidable differences in handling individual aliquots, and we have not attempted tests of statistical significance.

Curves for bundle length generally increase over the first 4 or 8 d; this is especially clear at ratios of 1:25 and 1:200. At both these ratios, length appears to reach a maximum at 4–8 d after which the average length becomes shorter, possibly due to degradation of protein. Data from 1:50 is similar to that from 1:25 and therefore has been omitted for clarity. The bundles from the ratios of 1:25 and 1:50 are consistently shorter than those from 1:100 and 1:200 throughout the 16-d period, and in general, ratios of 1:25 produce the shortest bundles while ratios of 1:200 produce the longest. The width of bundles increases fairly steadily with time, and reaches a maximum between 4 and 8 d at ratios of 1:25 and 1:200. Also, the bundles from 1:200 are wider than those from 1:25 at all time points.

Bundle order

Bundle order within the various samples is plotted in Fig. 4 *c*; these data suggest that low concentrations of fascin produce the best order and that this order increases with time. In general, maximal order was achieved between 4 and 8 d, though this increase was most dramatic at a ratio of 1:200. Thus, the bundles grown at 1:200 attained the greatest order, while those grown at 1:25 were consistently less ordered. The presence of 125 Å transverse striations across the width of the bundles (see Fig. 2 *c*) confirmed that bundles grown at 1:200 were the best ordered. 10 bundles grown at 1:200 exhibited these striations at 16 d, three bundles at 8 d, and two bundles at 4 d. In contrast, only one bundle with striations was seen at 8 d from both the 1:100 and the 1:50 ratios. No bundles with striations were observed at the other time points or concentration ratios.

All of these various types of observations are summarized in Table 2.

DISCUSSION

Our motivation for the current work was to see if and how the degree of order in a bundle can be controlled. We found that at fascin-to-actin ratios of 1:25, many small and poorly ordered bundles formed; as the ratio dropped, fewer bundles formed but they ultimately grew to be bigger and better ordered. In other words, as the bundles grow larger they become better ordered, as is shown by Fig. 4 *d* where length, width, and order have been replotted on a normalized scale for a ratio of 1:200. Notably, at very low ratios of either 1:300 or 1:400, no bundles were observed at all.

Based on our observations we suggest the following two-stage mechanism for bundle formation, consisting of nucleation and growth. According to our mechanism, these two processes have a different dependence on the concentrations of the constituent proteins; nucleation requires relatively high concentrations and only occurs above some threshold, while growth occurs at both high and low concentrations. This concentration dependence gives rise to a time course consisting of an initial period (at high concentrations of free material) dominated by bundle nucleation followed by a longer period (at lower concentrations of free material) dominated by growth. The order within the resulting bundles depends on the speed with which they are assembled, which in turn

FIGURE 3 Representative fields of actin bundles taken at different times and at different fascin-to-actin ratios. The concentration ratios and times are indicated on each panel. All bundles are indicated by arrowheads; other structures are either single or paired actin filaments. Note that bundles are generally larger at later times and that those at 1:200 are generally larger than those at 1:25. At this magnification, it is not possible to judge the degree of order; however, by examining the images at ~10 × magnification, filament packing can be observed and the order judged (see Fig. 2).

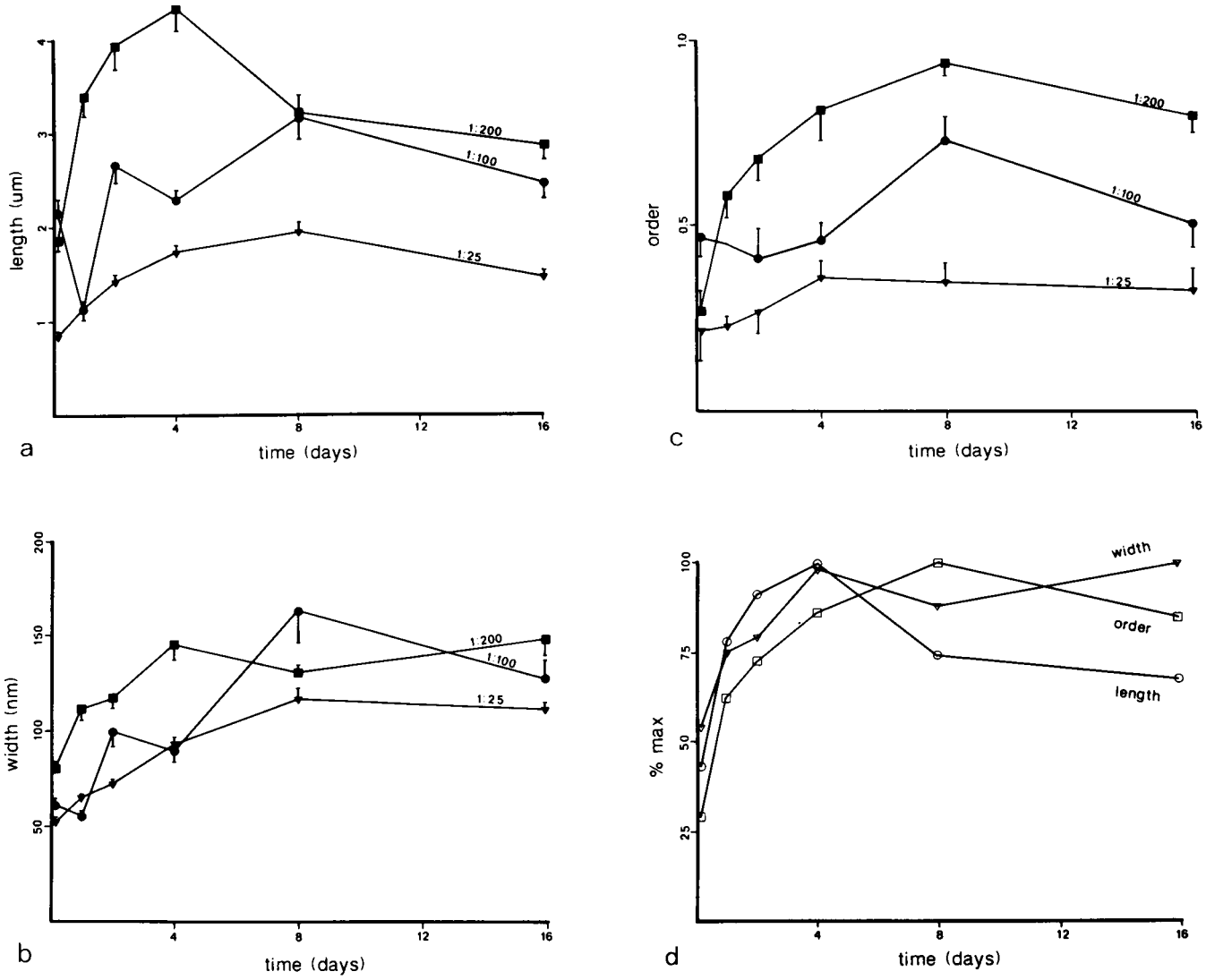


FIGURE 4 Bundle growth over time. Data obtained from different fascin-to-actin ratios are plotted in *a* (length), *b* (width), and *c* (order). The concentration ratios are indicated on the appropriate curves, and the error bars represent the standard error of the mean. Data for the ratio of 1:50 is similar to that at 1:25 and has been omitted for clarity. Data from the ratio of 1:200 are replotted in *d*; each parameter is plotted as the percent of its maximal value. Note that length, width, and order all follow a similar hyperbolic curve.

depends on the concentration of free material. Thus nuclei, which are formed rapidly at high concentrations, are disordered, while subsequent growth occurs more slowly and therefore results in progressively better ordered bundles.

Threshold for nucleation

According to the first-order binding constant calculated by Bryan and Kane (1978), the total amount of bundled material at a ratio of 1:400 should be about two-thirds that at 1:200 (Table 1). Naturally, this binding constant was derived from concentrations at which bundles are nucleated and therefore describes the extent of growth once nuclei are present. The absence of bundles at ratios below 1:300, however, indicates that there is a threshold below which bundle nucleation does not spontaneously occur.

Bundle size

According to our proposed mechanism, this nucleation threshold produces the observed correlation between protein concentrations and bundle size. At concentration ratios that initially nucleate bundles, the concentra-

TABLE 2 **Summary of observations on bundle formation**

Concentration ratio	Number of bundles	Ultimate size	Ultimate order	Time dependence	
				Size	Order
1:25	***‡	*	*	*	*
1:50	***	*	**	*	**
1:100	**	**	**	**	**
1:200	*	***	***	***	***
1:300	0	—	—	—	—
1:400	0	—	—	—	—

‡Number of *'s represents the observed magnitude for each parameter.

tion of free fascin (because it is in least supply) will eventually fall below the threshold as material is bundled; thereafter, nucleation will stop. Growth, however, will continue until equilibrium is reached, which in our experiments corresponds to the incorporation of all the fascin into bundles (see Table 1). The number of nuclei produced will depend on the initial concentrations relative to the threshold; for example, if the initial concentration of fascin is close to the threshold, few nuclei will be produced whereas if it is far from the threshold many nuclei will be produced. Thus, the number of nuclei will not be proportional to the total amount of bundled material. It is this lack of proportionality that we propose gives rise to differences in the average size of bundles; the average size in a given sample depends on the amount of bundled material relative to the number of nuclei. Thus, a ratio of 1:200, which is close to the nucleation threshold and therefore produces few nuclei, generates the largest bundles and a ratio of 1:25, which is far from the threshold and produces many nuclei, generates the smallest bundles.

The foregoing discussion assumes that bundles grow by recruiting free fascin and actin rather than by aggregation of small bundles into larger ones. Also, we assume that small bundles do not dissolve, thereby contributing their components to the growth of larger ones. These assumptions are consistent with our finding that solutions with the lowest concentration of bundles and with the highest concentration of free material (e.g., 1:200) produce the largest bundles. In addition, aggregation and selective dissolution are inconsistent with our observation that small bundles at 1:25 never become large, despite their presence in high concentrations at which both mechanisms should be favored.

Rate of growth

Given that bundles recruit free material and that the concentrations of free materials decrease over time (due to their incorporation into bundles), the rate of growth must also decrease over time. In particular, growth during early times, concurrent with nucleation, must proceed more rapidly than growth during later times, after nucleation has stopped. Indeed, the hyperbolic aspect of our plots of bundle length and width vs. time supports this reasoning (see Fig. 4).

Bundle order

Overall order in a population of bundles can change by either of two mechanisms: by rearrangement of disordered bundles to a more ordered state or by the ordered addition of material onto a disordered core. We can rule out the first mechanism because the bundles at 1:25 never become well ordered even after 8 or 16 d. At this ratio there were no well-ordered bundles whereas at 1:200 the majority of bundles were well ordered. Because at 1:25 well-ordered bundles did not appear with time, it appears that once formed, poorly ordered bundles remain poorly ordered. As for the alternative mechanism, we observed that as bundles grow larger they become better ordered and we therefore propose that this is achieved by the orderly addition of material onto poorly ordered "seeds". According to this mechanism, the rate at which order increases should parallel the rate of growth and indeed our data on large bundles confirm this correlation (see Fig. 4 *d*).

These ideas about bundle order are supported by general experience with protein crystallization. First, the more quickly crystals grow, the less well ordered they are. Second, a poorly ordered crystal never becomes ordered with time. Finally, one can use small poorly ordered crystals to seed solutions that do not otherwise produce crystals; under the appropriate conditions, slow growth onto the seed produces large well-ordered crystals. It is presumed that the small disordered seed remains intact at the center of the larger crystal.

By applying these ideas to bundles, we can explain the time course and concentration dependence of order. At first, when concentrations are relatively high, growth is faster than at later times, when the concentrations are lower; hence the small bundles formed initially are poorly ordered and their order does not improve with time. In contrast, when growth continues at lower concentrations, material is added more slowly and with better order. Thus, as a bundle grows larger, filaments are added with increasing order and its overall order becomes progressively better. Bundles formed at ratios of 1:25 are never well ordered because after nucleation, there is less growth of individual bundles at 1:25 than there is at 1:200.

Relationship to earlier studies

Earlier observations of bundle order by Bryan and Kane (1978) are also consistent with our proposed mechanism. While they did not report a wide variety of concentrations, they did report well-ordered bundles at very high ratios (1:3) and poorly ordered bundles at intermediate ratios (1:10). A ratio of 1:10 is not dissimilar from our ratio of 1:25 and therefore, the observation of disordered bundles is not surprising. However, a ratio of 1:3 is quite different because actin now represents the limiting component for bundle formation (the molar ratio of fascin/actin at saturation is 1:4.5 or 1:3.4 by weight). Our mechanism can nevertheless explain the observation of large, well-ordered bundles by assuming limited nucleation, due in this case to a decrease in actin concentration below the nucleation threshold. This is different from our ratio of 1:200 where fascin concentration is limiting and where a decrease in fascin concentration below threshold limits nucleation. However, in both cases (i.e., at 1:200 or at 1:3) the limited number of nuclei formed go on to incorporate the relatively large amount of remaining free material into large, well-ordered bundles.

Bundle growth in vivo

How does our mechanism fit the observations of Tilney and DeRosier (1986) in vivo? As was the case with our observations in vitro, there seems to be a correlation between bundle growth and increased order in vivo. At 11 d the stereocilia possess small, rather poorly ordered bundles. Bundle size and order increase significantly during the next 6 d (see Fig. 1 of Tilney and DeRosier, 1986). During this time the bundles double in width by the lateral addition of filaments. However, neither these older stereocilia nor mature ones possess perfectly ordered bundles. Rather, the bundles seen in cross-section have regions of disordered packing that are comparable in size and disorder to 10-d old bundles (i.e., the "seeds"). Although there is no direct evidence on this point, it is possible that the initial bundle seeds remain intact at the heart of the mature bundle. In light of these observations, we suggest that the seed bundle in stereocilia needn't be and probably isn't well ordered, but that order increases by the orderly addition of filaments to this seed. Slow, orderly growth can be achieved by keeping the cross-bridging protein in short supply.

In contrast to the hexagonally packed actin bundles from these chicken stereocilia, the bundles from the alligator lizard have only liquid-like filament packing even though they grow to be much larger (DeRosier et al., 1980). No observations have been made of lizard stereocilia during embryogenesis; however, the liquid packing in the bundles suggests that they are formed much more quickly than in chickens. The cell could maintain a high rate of growth by providing a relatively high concentration of proteins; under these conditions, the components would be rapidly bundled without time to take up hexagonal packing.

This two-stage mechanism of nucleation followed by growth accounts for observations in vitro and provides plausible explanations for observations in vivo. Further testing of this mechanism in vitro will require use of purified fascin, measurement of actin filament length, of pellet size, of the number of bundles, thorough characterization of the hypothesized nucleus, in addition to the measurements we have reported. Nevertheless, our observations confirm earlier predictions that different assembly conditions, rather than different bonding rules between constituent proteins, can be responsible for differences in the order of actin bundles from different sources.

We thank Bob Kane for kindly giving us the extracts from sea urchin eggs, Lew Tilney for his inexhaustible enthusiasm and helpful comments about the manuscript, Noreen Francis for helping with the biochemistry, and Claudio Villa for patiently printing our figures.

This work was supported by National Institutes of Health grant GM26357.

Received for publication 25 May 1990 and in final form 17 September 1990.

REFERENCES

Bryan, J., and R. E. Kane. 1978. Separation and interaction of the major components of sea urchin actin gel. *J. Mol. Biol.* 125:207–224.

Bryan, J., and R. E. Kane. 1982. Actin gelation in sea urchin egg extract. *In* Methods in Cell Biology. Leslie Wilson, editor. Academic Press, New York. 25:175–199.

DeRosier, D. J., and R. Censullo. 1981. Structure of F-actin needles from extracts of sea urchin oocytes. *J. Mol. Biol.* 146:77–99.

DeRosier, D. J., and L. G. Tilney. 1982. How actin filaments pack into bundles. *Cold Spring Harbor Symp. Quant. Biol.* 46:525–540.

DeRosier, D., E. Mandelkow, A. Sillman, L. Tilney, and R. E. Kane. 1977. The structure of actin-containing filaments from two types of non-muscle cells. *J. Mol. Biol.* 113:679–695.

DeRosier, D. J., L. G. Tilney, and E. Egelman. 1980. Actin in the inner ear: the remarkable structure of the stereocilium. *Nature (Lond.).* 287:291–296.

Kane, R. E. 1975. Preparation and purification of polymerized actin from sea urchin egg extracts. *J. Cell Biol.* 66:305–315.

Kane, R. E. 1980. Induction of either contractile or structural actin-based gels in sea urchin egg cytoplasmic extract. *J. Cell Biol.* 86:803–809.

Otto, J. J., R. E. Kane, and J. Bryan. 1980. Redistribution of actin and fascin in sea urchin eggs after fertilization. *Cell Motil.* 1:31–40.

Spudich, J. A., and S. Watt. 1971. The regulation of rabbit skeletal muscle contraction. *J. Biol. Chem.* 246:4866–4871.

Tilney, L. G., and D. J. DeRosier. 1986. Actin filaments, stereocilia, and hair cells of the bird cochlea IV. How the actin filaments become organized in developing stereocilia and in the cuticular plate. *Dev. Biol.* 116:119–129.

Tilney, L. G., E. M. Bonder, L. M. Coluccio, and M. S. Mooseker. 1983*a*. Actin from Thyone sperm assembles on only one end of an actin filament: a behavior regulated by profilin. *J. Cell Biol.* 97:112–124.

Tilney, L. G., E. H. Egelman, D. J. DeRosier, and J. C. Saunders. 1983*b*. Actin filaments, stereocilia, and hair cells of the bird cochlea: II. Packing of actin filaments in the stereocilia and in the cuticular plate and what happens to the organization when stereocilia are bent. *J. Cell Biol.* 96:822–834.