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Morphological Bifurcations Involving Reaction-Diffusion Processes During Microtubule Formation

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Nonlinear chemically dissipative mechanisms have been proposed as providing a possible underlying process for some aspects of biological self-organization, pattern formation, and morphogenesis. Nonlinearities during the formation of microtubular solutions result in a chemical instability and bifurcation between pathways leading to macroscopically self-organized states of different morphology. The self-organizing process, which contains reactive and diffusive contributions, involves chemical waves and differences in microtubule concentration in the sample. Patterns of similar appearance are observed at different distance scales. This behavior is in agreement with theories of chemically dissipative systems.

Spontaneous macroscopic self-organization can occur when a system is moved from a linear equilibrium state to a nonlinear out-of-equilibrium state. After an instability in the initial state, the system bifurcates into one of two possible new configurations (*1–4*). Fields, which are normally too weak to effect equilibrium structures, can play a decisive role in determining the morphology of the final state. Turing (*5*), and later Prigogine and co-workers (*3, 4, 6*), proposed and developed the idea that chemical and biochemical reactions might behave this way via mechanisms involving reactive and diffusive contributions (*7–9*). This type of process, often termed chemically dissipative, is distinguished from other similar nonlinear mechanisms (of, for example, physical origin) by the chemical nature of the processes involved. Turing proposed his theory in 1952 as a physico-chemical explanation for biological pattern formation and morphogenesis. The potential existence of dissipative mechanisms and Turing patterns (*10*) in biology has been the subject of debate and uncertainty. In view of the possible biological importance of mechanisms of this type, I report that a simple biochemical system of microtubule assembly behaves in the manner outlined above.

Microtubules (*11*) play a controlling role in organizing the cytoskeleton. These are tubular molecular assemblies, 280 Å in diameter and several micrometers in length, comprised of the protein tubulin and that can be formed by warming (4° to 35°C) a solution containing purified tubulin and the nucleotide, guanosine triphosphate (GTP). The tubulin spontaneously assembles into microtubules, chemical reactions occur, and GTP is hydrolyzed to guanosine diphosphate (GDP). This reaction continues after microtubule formation is initially completed, by the addition and loss of tubulin at the opposing ends of the microtubules. Frequently, a GTP regenerating system (*12*) is included in which GDP, as produced, is rephosphorylated to GTP by a kinase enzyme in the presence of the appropriate organic phosphate. The concentration of GTP remains constant, and the net reaction is a consumption of the organic phosphate. The assembly process can show nonlinear kinetics (*13*), and the system is hence capable of manifesting various complex nonlinear phenomena. Some microtubular solutions show spontaneous macroscopic space ordering (*14–18*), a phenomenon that has been attributed to chemically dissipative mechanisms (*17, 18*). After assembly in optical cells, stationary periodic horizontal stripes of about 0.5 to 1 mm separation progressively develop in the sample. In each band the microtubules are highly oriented at either an acute or obtuse angle, but adjacent stripes differ in having opposing orientations (*17*). Striped morphologies occur when the microtubules are prepared in upright sample containers, but a different pattern arises when they are prepared in horizontal cells (*19*). This behavior is attributed to the determining role of the direction of the gravitational field (*19*).

According to theories of chemically dissipative systems, the presence of a weak field at the moment of a chemical instability determines whether a system bifurcates along one morphological pathway or another. To establish at what moment the sample morphology depends on the orientation of the cell with respect to gravity, I performed the following experiment. Twenty samples of phosphocellulose-purified tubulin (10 mg/ml) (*20*), together with GTP and a GTP regenerating system (*17*), at 4°C, were placed in optical cells 4 cm by 1 cm by 0.1 cm. Microtubule formation was initiated by placement of the cells upright in a thermostatted room at 35°C. At 1-min intervals consecutive cells were layed flat, and the samples were examined about 12 hours later through cross polars with a wavelength compensater (Fig. 1A). When observed in this way (*21*), acute and obtuse microtubule orientations produce blue and yellow interference colors, respectively. After

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about 6 hours, the structures that have formed are stationary and independent of cell orientation (*17–19*). Twenty minutes after initiation of microtubule formation, there are no obvious signs of stripes (*17–19*), and one might expect all the samples to show the horizontal pattern. This is the case for samples inverted during the first few minutes. However, samples that were upright for 6 min or longer showed striped morphologies similar to preparations that remained vertical throughout the experiment. Conversely, preparations assembled in horizontal cells turned upright showed stripes for samples turned in the first few minutes and then the horizontal pattern. These preparations have hence retained a primitive "memory" of their conditions during the first few minutes after initiation of microtubule formation. The final morphology of the sample depends on whether the cells were horizontal or vertical at a critical period before the formation of the structure, about 6 min after initiation of assembly. The process can be described as a bifurcation between two different morphological states, with time as the bifurcation parameter.

The microtubular solutions are gels of viscosity of about 5000 poise (*22*), and this prevents any possible convection currents from arising in the sample. Furthermore, samples prepared in vertical cells with a constant temperature difference of 5°C between the bottom and top of the cell or by mixing of the reactants preequilibrated at 35°C also formed stripes. There is thus no evidence to suggest that convective currents play an important role in the self-organizing process.

Microtubules scatter light and their absorbance at about 350 nm is often used to assay their formation (*23*). The assembly kinetics measured by this method frequently show a monotonic increase to a steady-state value. This is not the case here; the absorbance increases to a maximum after a few minutes and then slowly declines to a lower value (Fig. 1B). This behavior, which is a consequence of the reaction kinetics, is not due to denaturation or a shortage of reactants. Maxima of this type are typical of nonlinear phenomena containing feedback mechanisms (*24*). At the maximum, the microtubular composition of the sample is unstable with respect to time. The position of the instability occurs at about the bifurcation time, and the slow decline that follows (Fig. 1) is approximately concurrent with the period of self-organization. To ensure that the absorbance variation corresponds to a bulk change in the relative concentrations of free tubulin and microtubules, I followed the assembly kinetics of a rectangular area 6 mm by 9 mm using small angle neutron scattering (*17, 25, 26*). The variation in the intensity of the microtubule scattering showed the same qualitative dependence as the absorbance. This confirms that the instability is of a chemical nature involving the relative concentrations of microtubules and tubulin. The direction of the gravitational field has a decisive effect on the sample at the moment of the chemical instability and results in a bifurcation between two morphological pathways.

Stationary striped microtubular structures such as these have been reported under different sample and buffer conditions (*14–16*), and in all cases the assembly kinetics show an instability. Slowing down the assembly kinetics so that the assembly overshoot did not arise inhibited the formation of stripes. Displacing the time at which the absorbance maximum occurred also displaced the bifurcation time. These observations suggest that the self-organizing process and the chemical instability are related phenomena associated with the kinetics of assembly; this in turn suggests a reactive contribution to the self-organizing process.

Reaction diffusion theories (*3–9*) of chemically dissipative processes predict that differences in concentration will occur with a periodicity (L) approximately equal to the distance over which groups of molecules diffuse before reacting, according to terms involving $L^2 = D/R$, where D is the diffusion constant and R is the reaction rate. Increasing the reaction rate by increasing the concentration will hence decrease the repeat distance. In agreement with this, the stripe periodicity decreased with increasing tubulin concentration. In the presence of a GTP regenerating system, the reaction rate can be measured with ^{31}P nuclear magnetic resonance (NMR) by the rate of depletion of the organic phosphate signal (Fig. 2A). For samples prepared at 29°C and 35°C, respectively, the reactivity increased by a factor of 2.1. If the rate of diffusion remains unchanged, the spacing should decrease by approximately 1.45, the square root of this value. In agreement with this, the spacing decreased by a factor of 1.47 (Fig. 2A).

Molecular diffusion also affects the structures by a term involving the total diffusion length (*3–9*), and periodicities will only arise if this length is greater than 5.784 times the wavelength of the periodicity (*9*). Figure 2B shows preparations at a lower tubulin concentration (3 mg/ml), assembled in vertical cells differing only in thickness (0.5 to 5 mm). Stripes of 0.7-mm separation are formed in 5-mm cells. However, their formation is progressively inhibited as the sample thickness diminishes, and in 0.5-mm cells no patterning occurs. The pattern also depends on the vertical height of the sample, and stripes do not appear until the sample height is greater than approximately 5.8 times the stripe separation. These observations strongly suggest that the self-organizing mechanism

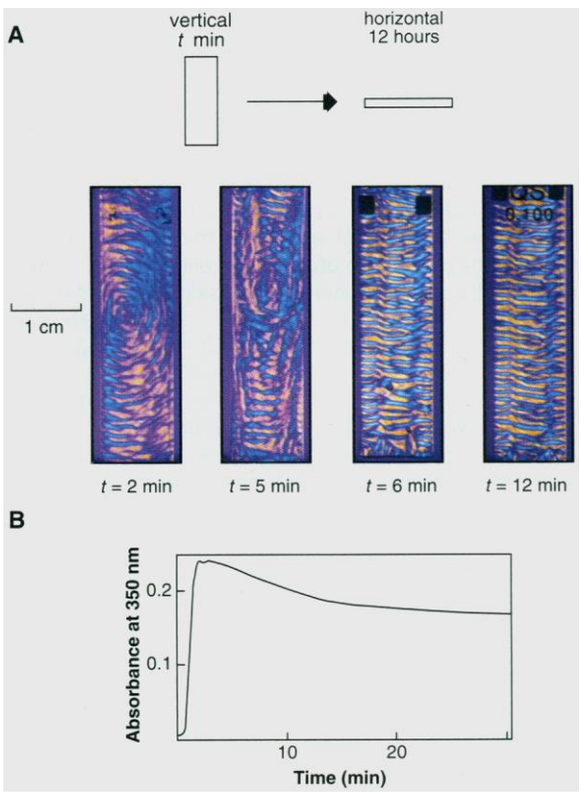

Fig. 1. Bifurcation properties (**A**) and chemical instability (**B**) in microtubular solutions formed from phosphocellulose-purified tubulin (10 mg/ml) (*20*) in the presence of 1 mM GTP and a GTP regenerating system [20 mM acetyl phosphate and acetate kinase (1.65 μg/ml)] in a D_2O-based buffer (*32*). (A) The photographs show the final stationary morphologies observed in samples rotated from upright to horizontal at different times, t, during the first 20 min (*33*). The final morphology depends on the sample configuration at a critical moment (t = 6 min) before the formation of the structure. The morphological transition does not occur abruptly at 6 min but is progressive over about 2 min. This can be seen in the intermediate behavior shown at t = 5 min. (B) The kinetics of microtubule assembly, as measured by the absorbance at 350 nm through a central vertical slit (1 mm by 10 mm), show a maximum demonstrating the existence of an instability at approximately the bifurcation time. The direction of the gravitational field has a decisive effect on the sample at the moment of the chemical instability and results in a bifurcation between two morphological pathways.

contains both reactive and diffusive contributions, in agreement with the mechanism first proposed by Turing (5).

Patterns of similar appearance are observed at different distance scales. Individual stripes (≈0.5 mm) are comprised of smaller bands of about 100-μm separation (Fig. 3). This shows that structures of dimensions comparable to living cells can be formed. Similar looking arrangements also appear over distances of ~3 mm. For the preparation having the composition given in Fig. 2A, the different repeat distances were determined as 90 μm, 0.53 mm, and 3.0 mm. Each scaling factor is approximately equal, and the average factor is 5.77. This empirical value is close to the theoretical value (5.784) for the minimum diffusion length, which suggests that repeat patterns of different periodicity might be governed by this ratio. Self-similar structures frequently appear as a result of nonlinear chaotic processes. Mechanisms of the type observed here may be of importance in biology because they provide a means to generate and replicate a form over different distance scales.

For systems containing more than two coupled reactants, reaction diffusion theories (3–9) predict that the formation of stationary periodic variations in concentration is preceded by chemical waves in the sample. In the present case, spontaneous ordering is observed not as differences in concentration as such, but as periodic differences in microtubule orientation. It is hence necessary to show that the microtubular self-organizing process is associated with differences in concentration. Microtubules grow by the addition of tubulin at one end, their directional growth being fastest where the concentrations are such that the reaction rates are highest. Calculations (27) show that orientational ordering and concentration gradients are interrelated phenomena. As a result of this coupling, which could itself be nonlinear, spontaneous periodic changes in orientation may arise concurrent with the reaction diffusion process. That this is the case is shown by the following observations.

Immediately after the instability, the left- and right-hand sides of samples assembled upright show different colors (yellow or blue) of birefringence (Fig. 4A) and hence differ in having either obtuse or acute microtubule orientations. The stripes then form when small alternating regions of the sample undergo a reversal of orientational ordering. This is seen as a birefringent color change; blue stripes appear in the yellow zone and yellow stripes appear in the blue zone. In neutron small angle scattering (Fig. 4B) of a horizontal band having the approximate dimension of a stripe, the process is manifested as a change in the direction of the microtubular scattering on the detector from an acute to an obtuse arc. Simultaneous with this reordering, the intensity of the microtubular scattering declines, rises, and declines again (Fig. 4B). The microtubular reordering, which is itself the stripe-forming process, is hence concurrent with a chemical wave, involving different concentrations of microtubules and free tubulin, crossing the sample area under investigation. The neutron scattering results also show higher counts for the sample area undergoing orientational reordering than for the area that does not reorder. The sample areas that reorder repeat themselves periodically as the stripes, implying that periodic differences in microtubule concentration arise throughout the sample during the self-organizing process.

The application of theoretical concepts of nonlinear dissipative mechanisms to biology has to some extent been retarded by the absence of simple biochemical examples.

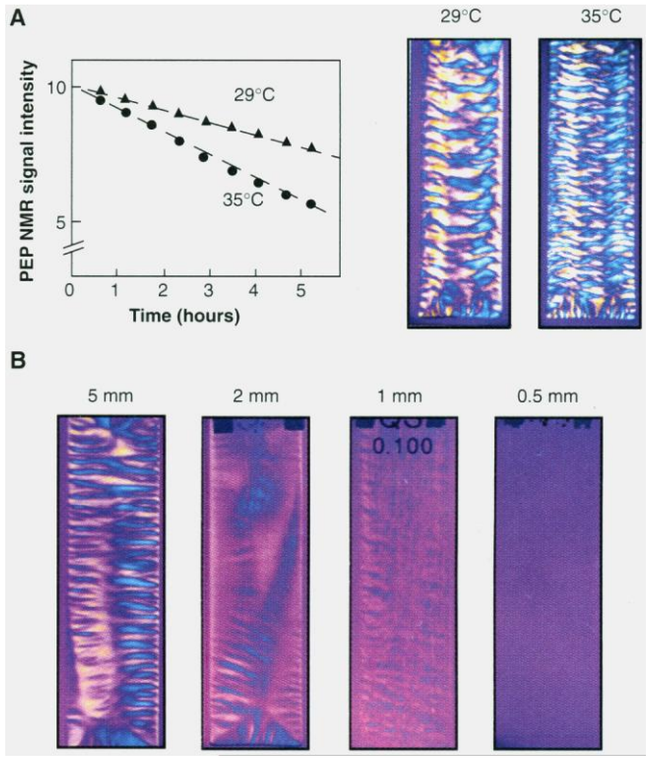

Fig. 2. Dependence of the sample morphology on (**A**) the reaction rate and (**B**) the sample dimensions or diffusion length. (A) Dependence of the spacing on the square root of the reaction rate. The photographs show the stationary birefringent structures formed in 2-mm optical pathlength vertical cells at 29°C and 35°C. Acute and obtuse microtubular orientations give rise to blue or yellow interference colors, respectively. The average spacing decreases by a factor of 1.47, from 0.78 to 0.53 mm. The graph at left shows the dependence of the ^{31}P NMR signal (40 MHz) on the rate of organic phosphate depletion during the self-organizing process. The slope, which is proportional to the reaction rate, increases by 2.1 when the assembly temperature is increased from 29° to 35°C. The initial sample composition was tubulin (10 mg/ml), GTP (1 mM), phosphoenol pyruvate (PEP; 10 mM), and pyruvate kinase (45 μg/ml). Owing to the high chemical stability of PEP, a pyruvate-based regenerating system was preferred for these measurements. (B) Microtubules formed at 35°C in vertical cells of the same height and width but of different optical pathlength. The preparation initially contained tubulin (3 mg/ml), GTP (1 mM), acetyl phosphate (20 mM), and acetate kinase (1.65 μg/ml). Shown are photographs of the sample birefringence at about 12 hours after initiation of microtubule formation.

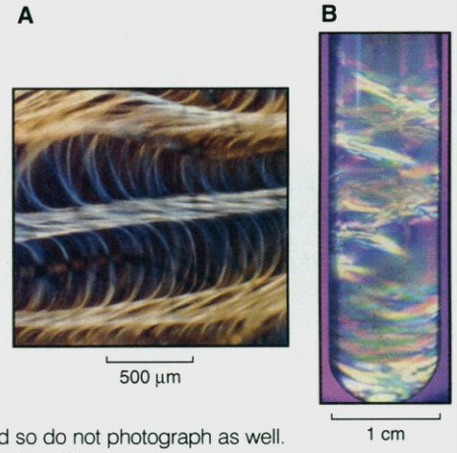

Fig. 3. Self-similar organizations appear at different distance scales. Each individual stripe (≈0.5 mm) is comprised of pseudohelical bands of about 100-μm separation, but also form part of a larger structure having a repeat distance of about 3 mm. (**A**) A sample prepared from a solution containing tubulin (16 mg/ml), GTP (1 mM), acetyl phosphate (20 mM), and acetate kinase (1.65 μg/ml) assembled in a vertical cell and observed with elliptically polarized light. (**B**) A solution assembled in a 10-mm tube and observed through cross polars with a wavelength retardation plate. Acute and obtuse microtubule orientations give rise to blue and yellow colors, respectively. The photograph is of a sample used for NMR measurements and was assembled in a vertical magnetic field of 9.2 T. Similar structures arise without a magnetic field, but they are not as well formed and so do not photograph as well. The sample composition is the same as that given in Fig. 2A.

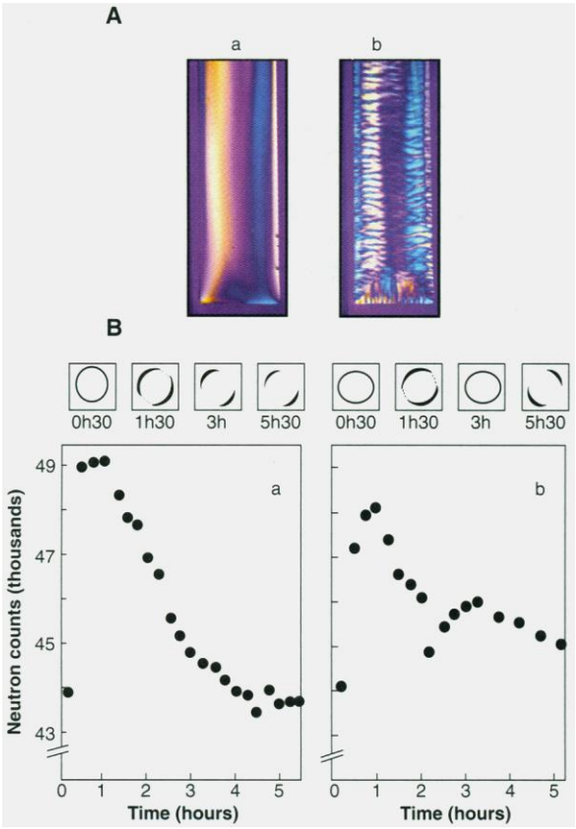

Fig. 4. Chemical waves and differences in microtubule concentration during stripe formation. (**A**) Sample birefringence at (a) 20 min and (b) 5 hours after initiation of assembly. Acute and obtuse microtubule orientations give rise to yellow and blue colors, respectively. The extensive yellow and blue birefringent regions seen in (a) subsequently split into the smaller yellow and blue stripes seen in (b). Hence, in some regions the microtubules take up and retain one orientation, whereas in regions where the color change occurs, the microtubules reverse their orientation. (**B**) Preparations having the composition described in Fig. 1 were warmed from 4° to 35°C in upright 1-mm optical pathlength cells and were examined through a small arbitrarily positioned horizontal slit 0.5 mm by 4 mm. Neutron scattering spectra (*34, 35*) showing the known microtubular scattering curve (*17, 26*) were recorded every 12 min. The graphs (a) and (b) show the time dependence of the microtubular scattering intensity for two different samples. The icons just above these plots show the distribution of scattered neutrons onto the two-dimensional detector and indicate the microtubular orientational ordering. In (a), which corresponds to a region where there is no reversal of the microtubular orientation, there is a slow decrease of about 15% in the scattered intensity concurrent with the progressive orientation of the microtubules. The same process starts to occur in (b). However, after about 2 hours, the scattered intensity suddenly decreases; the microtubules disorient until they are once again isotropic and then progressively reorient in the opposing configuration. At the same time, the microtubular scattering intensity rises and then begins to drop again. These spectra show that a wave of microtubule partial disassembly-reassembly-disassembly crosses the sample area under investigation. This occurs concurrently with the reversing of microtubule orientation, which is itself the stripe-forming process. Moreover, in (a) and (b) the scattering intensities, which are initially similar, differ while microtubule reorientation, that is to say stripe formation, is proceeding. This implies the existence of periodic differences in microtubule concentration throughout the sample, concurrent with stripe formation.

The microtubular system presented behaves in the manner expected for a dissipative space structure formed by a Turing type mechanism. It may be of value as an experimental model for advancing the understanding of biological self-organization and for comparison with theory.

Theoreticians have proposed that bifurcations and biochemical dissipative space structures, as well as alternative theories (*28*), might account for various aspects of morphogenesis. The microtubular solution just after bifurcation could be described as being "determined" but not yet "differentiated." In developing eggs of the fly *Drosophila*, body segmentation is related to the formation of regular periodicities in the concentration of proteins produced by specific genes (*29, 30*). Such stripe formation may involve dissipative mechanisms.

The reorganization of the cellular microtubular system involves the disassembly and reassembly of microtubules (*11*). As a result, cell biologists have invested considerable effort into understanding the process of microtubule assembly, mainly in terms of linear phenomena (*31*). The present results show that complex biological phenomena occur as a result of nonlinear mechanisms. Identifying and understanding such processes are a prerequisite to investigating their possible role in living organisms.

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32. Buffer consisted of 100 mM MES, 1 mM EGTA, and 1 mM $MgCl_2$ in D_2O at pH 6.75.
33. Preparations were placed in 20 optical cells and microtubules formed after the solution was warmed from 4° to 35°C at time zero. Consecutive cells, initially vertical, were turned horizontal at 1-min intervals and then left for 12 hours for the structures to form. After this period, the samples were observed through cross polars (0° and 90°) with a wavelength compensater (550 nm) at 45° (*21*). The compensater produces a uniform mauve birefringent background seen at the edges of the sample. Microtubule orientations such that their birefringence adds on to the birefringence of the compensater produce a blue wavelength shift, whereas orientations that subtract cause a yellow shift. Samples prepared with the cells that were vertical during the first 12 hours show stripes similar to those shown at t = 6 min and 12 min, corresponding to periodic variations in the microtubule orientation alternating from acute to obtuse. Samples prepared in cells that were horizontal all the time show a different morphology, similar to that seen at t = 2 min and consistent with circular or spiral variations in the orientation.
34. Measurements were carried out on the D11 camera at the Institut Laue-Langevin, Grenoble, with a sample to detector distance of 2.5 m and 10 Å wavelength neutrons.
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