GRAVITY DEPENDENCE OF MICROTUBULE SELF-ORGANISATION

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ABSTRACT

This article presents a molecular mechanism by which living cells can be affected by weightlessness. Weak external forces such as gravity are not normally considered as intervening in chemical and biochemical reactions. One manner by which they might, is by participating in certain types of reaction-diffusion mechanisms that lead to macroscopic self-organisation. Under appropriate conditions, at a critical moment early in the process, self-organisation can depend on the presence of a weak external force such as gravity. The in vitro formation of microtubules shows this type of behaviour. Samples progressively self-organise by way of a combination of microtubule reaction dynamics and molecular diffusion and the morphology that forms depends upon the presence of gravity early in the process. In this system, ground experiments, with either a rotating clinostat or under conditions of magnetic levitation, demonstrate that the effect of weightlessness can be studied without the expense and trouble of space flight. At a molecular level, the dependence of microtubule self-organisation on gravity is due to its interaction with macroscopic concentration and density fluctuations created by microtubule contraction and elongation in a manner that shows analogies with the way ant population's self-organise. Numerical simulations of the reaction-diffusion process predict macroscopic self-organisation in qualitative agreement with experiment and account for how it is triggered by weak external factors such as gravity. There is a growing body of evidence that microtubule organisation by these processes occurs in vivo during embryogenesis and the cell cycle.

INTRODUCTION

The fact that weightlessness affects biological processes at a molecular level often elicits either disbelief or wonder. Nevertheless, the accumulated conclusions of numerous experiments conducted over more than 30 years clearly demonstrate that this is indeed the case (Cogoli and Gmünder 1991, Hammond et al 1999, various authors 1999, Lewis 2002, Hughes-Fulford 2002). The reasons for this disbelief or wonder is that many biologists have become accustomed to regarding biological processes in terms that ultimately depend only on the properties of individual molecules. Within such a framework it is not easy to account for how a biochemical reaction in a biological object, such as a cell or an embryo, might be dependent on an external physical force as weak as terrestrial gravity.

However, another approach is slowly developing. Until the advent of the computer, it was difficult to predict the behaviour of a population of individual units that mutually effect one another. Since then, scientists from various disciplines have progressively discovered that in such populations, new properties emerge. These phenomena do not come about from any intrinsic property of the individual unit as such, but on the contrary arise by the way that the individuals ‘talk to one another’ and behave as a collective ensemble. The properties arise from the non-linear dynamics that describe the behaviour of such coupled systems. In recent years, systems of this type (Gleick 1987, Nicolis and Prigogine 1987, Coveney and Highfield 1995, Camazine et al. 2001) have been termed ‘complex’ and the phenomena that arise as ‘emergent’. Some scientists ask whether some global properties of biological systems might be accounted for in terms of the ‘emergent’ properties of ‘complex’ systems.

In many ‘complex’ systems, self-organisation occurs as an ‘emergent' property. A particular feature of some of these systems is that self-organisation can be strongly affected by the presence of weak external factors that break the symmetry of the system and so modify its collective behaviour. In this article, I shall summarise some of the ‘emergent' properties that arise in a ‘complex' system comprised of a population of microtubules. Under appropriate conditions these preparations spontaneously self-organise by a process of reaction and diffusion and this self-organisation is strongly gravity dependent.

Living systems provide many examples of self-organisation by collective processes (Parish and Edelstein-Keshet 1999, Camazine et al. 2001). Fish schools, bird clouds, wasp swarms, ant colonies, and colonies of certain types of unicellular organisms and bacteria, all self-organise in this way. Structures and organisations develop, not by action at the level of the individual, but rather by way of dynamic processes in which the individuals are strongly coupled to one another and behave as a collective ensemble. A striking feature of this behaviour is that the same types of morphology often arise in spite of large differences in the size and nature of the individual unit. For example, star galaxies, hurricanes, New Mexico bat colonies, slime mould amoebae, and microtubules, all form spiral shapes. In fish schools, or bird clouds, tubular or tore-like morphologies occur. Striped arrangements often arise; when they do, they are nearly always the result of an outside external perturbation that induces a directional bias on the actions of the individual. For example, an insect nest may develop a striped morphology if it is exposed to an air current at some stage during its construction.

A well-studied example of this type of behaviour is the formation of ant colonies. There are many similarities between the collective behaviour of ants (Holldobler and Wilson 1991) and microtubules. A moving ant leaves behind itself, trails of chemicals, known as pheromones which can attract or repulse other ants. An ant encountering a trail of an attractive pheromone will
change its direction to follow the trail. This ant, will, in its turn deposit more pheromone on the trail thus reinforcing it (Hollolobler and Wilson 1991). The self-amplification of trails of attractive and repulsive pheromones leads to the self-organisation of the ant population.

One of the advantages of this type of process is that ants can rapidly establish the shortest route between a food supply and their nest (Goss et al 1989, Nicolis and Deneubourg 1999, Camazine et al 2002). Consider a situation where there are two identical food supplies close to an ant colony. One source is slightly closer to the nest than the other. As ants return to the nest with food, they leave chemical trails that are followed by other ants. These ants reinforce the trails, and so more and more ants follow the paths to the two food supplies. However, from the closer of the two sources it takes less time for an ant to return to the nest. This leads to a larger number of ants taking this path, thus reinforcing the strength of its chemical trail, compared with the longer path. Hence, progressively more and more ants take the shorter path to the closer food supply until they all follow this route. If the food supplies are at exactly equal distance, then a weak external factor will suffice to favour one pathway over the other and hence determine which of the two food sources the ants consume. It is easy to see that the choice as to which route develops is determined at an early stage of the process before the reinforcement of the pathway has gone very far. As pathway reinforcement progresses, then it will take the application of an increasingly stronger external factor to induce a change in which the alternate pathway develops. In the absence of such an effect, the deciding external factor need only be present for a critical period early in the process. Once pathway reinforcement has started it will continue until the food source is consumed. This is a simple example of a bifurcation in a non-linear dynamic system. At the bifurcation time, the distribution of ants is unstable to weak external factors.

The construction of ant nests is based on the same principles. However, so as to synchronise the actions of all the individuals over space and time, in addition to local chemical communication between the ants, a global bias over the entire colony is also required. The queen fulfils this function by producing a specific pheromone that diffuses from around her. Ants will only build when this pheromone concentration is within a certain range. When there are no perturbations around the queen, the pheromone diffusion is uniform in all directions and the base of the nest will be circular. However, this is rarely the case and weak external factors, such as air currents, temperature gradients, or the initial ground topology, all provide external biases that effect the morphology of the structure that develops. Likewise, the construction by the ants, of a wall or gallery, modifies the pheromone diffusion profile and this, in its turn, also effects the future shape of the nest.

The behaviour described above is a typical example of 'emergent' properties in a 'complex' system. A question that arises is how processes of this type might come about at a molecular level by way of biochemical reactions within biological objects such as an egg or a cell.

Since the 1930's, certain theoreticians (Kolmogorov et al. 1937, Rashevsky 1940, Turing 1952, Glansdorf and Prigogine 1971, Nicolis and Prigogine 1977, Prigogine and Stengers 1984, Nicolis and Prigogine 1989) have proposed that some particular types of chemical or biochemical reactions might show non-linear dynamic properties by way of a coupling between reactive processes and molecular diffusion. Contrary to what is normally observed, they predicted that a chemical pattern comprised of periodic variations in the concentration of some of the reactants could spontaneously arise from an initially homogeneous solution. Structures of this type are called reaction-diffusion or Turing-like structures. They also go under the name of dissipative structures. The latter term was widely used by Prigogine and co-workers because a dissipation of chemical energy is required to drive and maintain the system sufficiently far-from-chemical equilibrium that self-organisation occurs. Even though such terms were not used at the time, what these theoreticians predicted was that biological self-organisation could arise as an 'emergent' phenomenon in a 'complex' system by molecular processes of reaction and diffusion.

In addition to self-organisation, such reaction-diffusion systems can also show bifurcation properties (figure 1) (Nicolis and Prigogine 1977, Nicolis and Prigogine 1989). At a critical moment early in the process, the system may bifurcate between dynamic pathways leading to self-organised states of different morphology. The presence of a small effect such as an external field, at a bifurcation point of the bistable type, can determine the morphology of the state that subsequently forms. Once the bifurcation has occurred, the system evolves progressively along the selected pathway to the pre-determined morphology. It behaves as though it retained a memory of the conditions prevailing at the bifurcation. Kondepudi and Prigogine (Kondepudi and Prigogine 1981, Kondepudi 1982) explicitly calculated that in some reaction-diffusion systems, terrestrial gravity could cause a bifurcation of this type. The property of bifurcation is intimately related to the fact that these dynamic systems are far-from-equilibrium and may show instabilities. The bifurcation point in any out of equilibrium system, and at which point the system is sensitive to weak external fields coincides with a condition of instability in the homogeneous state.

These concepts, although a subject of interest and debate for many years, are only now beginning to be progressively accepted by biologists. One of the reasons for this long delay is that until recently there were no examples of simple in vitro biological systems known to self-organise this way. Under appropriate conditions, in vitro preparations of microtubules, a major component of the cytoskeleton, show this type of behaviour. The microtubule preparations spontaneously self-organise by a combination of reaction and diffusion, and the morphology of the state that forms depends upon gravity at a critical bifurcation time early in the process (Tabony and Job 1990, Tabony and Job 1992a, Tabony and Job 1992b, Tabony 1994, Tabony 1996, Tabony and Papaseit 1998, Tabony et al 2000, Tabony et al 2001b, Tabony et

Figure 1. Schematic representation of bifurcation phenomena in a chemical system. The horizontal axis represents the distance of the system from equilibrium, and the vertical axis a state variable. Near equilibrium, the system has a linear behaviour, and the thermodynamic state is the only permitted stable solution. When far-from-equilibrium, the system has a non-linear behaviour. At a critical point shown by the dotted line, and which corresponds to the bifurcation point, the thermodynamic state becomes unstable, and new stable non-linear states arise. At this point, a weak field, such as gravity, can result in a small separation between the different non-linear pathways, and thus favour the formation of one of the possible non-linear states.

MICROTUBULE ASSEMBLY AND REACTION DYNAMICS

Microtubules (Alberts et al. 1983, Dustin 1984) have two major roles: they organise the interior of the cell, and they permit and control the directional movement of intracellular particles and organelles from one part of the cell to another. They participate in many fundamental cellular functions including, the maintenance of shape, motility, signal transmission, and they play a determining role in the organisational changes that occur during the early stages of embryogenesis.

Microtubules are tubular shaped super-molecular assemblies several microns long, having inner and outer diameters of 16 nm and 24 nm. They arise from the self-assembly of a proteins, tubulin α and β, by way of reactions involving the hydrolysis of guanosine triphosphate (GTP) to guanosine diphosphate (GDP). Microtubules may be formed in vitro by warming a solution containing purified tubulin and GTP from about 4°C to 36°C. Once microtubules are formed after a few minutes, chemical activity continues through processes whereby tubulin is added and depleted from the opposite ends of the microtubules by reactions involving GTP hydrolysis. There is hence a continual consumption or dissipation of chemical energy through the system. The in vitro formation of microtubules from tubulin generally involves a rapid initial increase in the number of microtubules. In many cases, this is followed by a regime in which the amount of microtubules in co-existence with free tubulin attains a stationary state. In other cases, a stationary state is not attained and the microtubule mass either decreases to a lower level or shows damped oscillations.

One of the particularities of microtubules is that due to differences in reactivity at opposite ends, they frequently grow from one end whilst shrinking from the other. Since the rates of growth and shrinking are often comparable, individual microtubules change position and appear to move at speeds of several microns per minute. The shrinking end of a microtubule leaves behind it a chemical trail of high local concentration in tubulin (Papaseit et al 2000, Tabony et al 2001a, Glade et al 2002a, Glade et al 2002b). Likewise, the growing end of a microtubule creates a region depleted in tubulin. Neighbouring microtubules will preferentially grow into regions of high tubulin concentration whilst avoiding the regions of low concentration. The chemical trails produced by individual microtubules activate and inhibit the growth of their neighbours. Thus, neighbouring microtubules "talk to each other" by depleting and accentuating the local concentration of active chemicals and this coupling of reaction with diffusion progressively leads to macroscopic variations in the concentration and orientation of the microtubules. Self-organisation arises as an 'emergent' phenomenon in a 'complex' system (Tabony et al. 2002c).

MICROTUBULE SELF-ORGANISATION

Tubulin solutions at concentrations of the order of 10 mg/ml, made up in a suitable buffer (Tabony and Job 1990), are assembled into microtubules by warming the solution from 4°C to 35°C in the presence of an excess of GTP. Microtubules form rapidly within 2-3 minutes. Progressively, over a period of about 5 hours, the initially homogenous solution spontaneously self-organises to form a macroscopic structure. Figure 2 shows the striped arrangement of about 0.5 mm separation that arises when microtubules are assembled in spectrophotometer cells 40 mm by 10 mm by 1 mm. Once formed, the structure is stationary, and the solution remains stable for about 3 days. After this time, the reaction runs out of chemicals, the microtubules progressively disassemble and the tubulin denatures.

The preparations are of high optical birefringence (Figure 2) and this indicates that the microtubules are highly aligned with respect to one another. In each striped band the microtubules are highly oriented at either 45° or 135°, but adjacent stripes differ in having alternating orientations. This pattern of variations in orientation can be observed by placing the sample between crossed linear polaris with a wavelength retardation plate placed at 45° between them (Figure 2C). The retardation plate produces a uniform mauve background. Microtubule orientations, such that their birefringence adds to the birefringence of the wavelength plate, produce a blue wavelength shift, whereas orientations that subtract cause a yellow shift. Sample regions made up of microtubule orientations that are either acute or obtuse, differ by producing yellow or blue interference colours respectively. In addition to these changes in orientation, periodic variations in microtubule
concentration, of about 30% of the mean, also occur from stripe to stripe (Papaseit et al 1999, Tabony et al 2002a) (Figure 3). The concentration pattern coincides with the pattern of variations in microtubule orientation.

The patterns are more complicated than it appears at first sight for the 0.5 mm stripes contain within them another series of stripes of about 100 µm separation. These stripes, in their turn, contain another striped arrangement of about 20µm separation. At distances below this, there are additional levels of organisation of about 5 µm and 1 µm separation. Some of these structures are shown in Figure 4. An additional level of ordering of several mm in periodicity is observed when samples are made up in larger sample containers (Tabony 1990, Tabony and Papaseit 1998, Tabony et al 2000). These large stripes in turn contain the lower levels of organisation already mentioned. Hence similar types of pattern spontaneously arise over distances ranging from a few microns up to several centimetres. The range of dimension over which these morphologies occur is typical of those found in many types of higher organisms. Cells are about 10 µm in size, eggs are often about a millimetre, and a developing mammalian embryo is several centimetres long. When microtubules are assembled in small sample containers about 100 µm in size, self-organisation still occurs but with periodicity's that are consistent with the size of the sample. This observation demonstrates the microtubule self-organisation by the processes described here also occurs in containers of the dimensions of a cell or an embryo.

Striped morphologies occur when the microtubules are prepared in upright sample containers, but a different pattern arises when they are prepared in the same containers lying flat (Figure 5) (Tabony and Job 1992, Tabony 1994). This behaviour is attributed to the determining role of the direction of gravity during structure formation. So as to test this hypothesis, microtubules were assembled in sample cells lying flat down on the turntable of a record player turning at 33 rpm and with the long axis of the sample positioned along the direction of the centrifugal field (0.14 g). A striped morphology once again forms (Figure 6), and the direction of the stripes is perpendicular to that of the applied centrifugal field.

Once formed after 5-6 hours, the structures are independent of their orientation with respect to gravity. To establish at what moment the sample morphology depends upon the gravity direction, microtubule formation was simultaneously instigated in twenty different rectangular shaped samples placed upright. Consecutive cells were turned from vertical to horizontal at intervals of one minute, and the samples examined 12 hours later, after the structures had formed (Tabony 1994) (Figure 7). Twenty minutes after instigating microtubule formation, when the last sample was turned from vertical to horizontal, there are no obvious signs of a striped structure. Since the structure forms while the cells are flat, one might expect that they would all form the horizontal
pattern. This is the case for samples inverted during the first 3-4 minutes. However, samples that were upright for six minutes or more, formed striped morphologies identical to preparations that remained vertical all the time. The final sample morphology depends upon whether the sample was horizontal or vertical at a critical time, six minutes after instigating assembly, and at an early stage in the formation of the self-organised structure. The process can be described as a bifurcation between pathways leading to two different morphological states, and in which the direction of the sample with respect to gravity determines the morphology that subsequently forms.

Figure 5. Different stationary morphologies form depending upon whether the sample cells are horizontal or vertical. Microtubules were assembled in a 1 mm optical pathlength cell positioned respectively vertical (A) or horizontal (B) during the entire period of structure formation. The structures, once formed are independent of the orientation of the cell with respect to gravity. Samples were photographed through linear cross polars with a wavelength retardation plate at 45°.

Figure 6. Effect of weak centrifugal fields on sample morphology. Microtubules were assembled with the sample placed face down on the turntable of a record player with its long axis along the direction of the centrifugal field (0.14 g). The striped morphology that forms resembles that which arises when the sample is vertical. Samples were photographed through linear cross polars with a wavelength retardation plate at 45°.

To determine whether the self-organising process is directly dependent on gravity, microtubules were assembled under conditions of weightlessness (Papaseit et al 2000, Tabony et al 2002b). The experiment was carried out during the flight of a sounding rocket of the European Space Agency. This provided approximately 13 minutes of weightlessness before the payload fell back to earth and was recovered. Since on the ground, the sample morphology is determined by the sample orientation with respect to gravity 6 minutes after instigating microtubule assembly, 13 minutes of low gravity should suffice to investigate the effect of weightlessness on the self-organising process.

Flight samples were contained in an experimental module divided into two compartments; a weightlessness compartment and a 1 g on-board centrifuge compartment. The samples formed in the centrifuge part of the module formed stripes when the centrifugal field was parallel to the long axis of the cell, and a circular morphology when it was perpendicular. These morphologies resemble those that form on the ground and show that self-organisation was unaffected by payload re-entry and recovery. In contrast to this behaviour, the samples formed under conditions of weightlessness showed practically no self-organisation (Figure 8). Hence, under these conditions, the presence of gravity for the first 13 minutes after instigating microtubule assembly actually triggers self-organisation and determines the subsequent behaviour.

A similar behaviour can also be obtained in ground-based experiments using methods that substantially reduce the effects of the gravity vector, such as clinorotation (Briegleb 1992) or magnetic levitation (Beaugnon 1991, Geim 1998). Glade (Glade and Tabony, 2001) carried out experiments using several different
types of clinostat and found that the simplest devise, and which rotated the sample about only the horizontal axis, gave the best results. With this apparatus, when the sample dimensions perpendicular to the axis of rotation were kept to below 5 mm and the sample positioned to within 1 mm of the axis of rotation, the behaviour was close to that observed in space flight (figure 9).


Figure 8. Microtubule structures as formed during space flight. Microtubules were assembled when conditions of weightlessness were obtained in the payload. Photographs A) and B) show the self-organised morphologies that arise for samples assembled on a 1g on-board centrifuge with the centrifugal field, parallel (A) and perpendicular (B), to the long axis of the sample cell. The centrifuge was stopped after 13 minutes, immediately prior to re-entry, and the samples left under 1 g conditions for a further 5 hours while the structures developed. The photographs C), also taken after 5 hours, shows that almost no self-organisation occurs when samples are subject to weightlessness during the first 13 minutes of the experiment. D) is a photograph, taken after 5 hours, of a sample in which an air bubble in the neck of the sample cell traversed the sample during re-entry. Microtubules were oriented along the bubble trajectory, thus triggering partial self-organisation perpendicular to the path of the bubble.

It needs to be stressed in these experiments that weightlessness affects microtubule self-organisation but not microtubule self-assembly. Microtubules assemble to the same extent and show the same assembly kinetics under conditions of weightlessness as at 1 g and regardless as to whether weightlessness arises by ground based methods or by space flight.

One of the numerous disadvantages of space experiments is that problems can occur due to air bubbles. In the sounding rocket experiment mentioned above, although care was taken to prevent it, in some of the samples small air bubbles formed in the neck of the sample container (Tabony et al 2001 Tabony et al 2002a, Tabony et al 2002b). For these samples, during re-entry, when the sample was subject to high centrifugal forces, the air bubble was pushed through the sample. In one sample, this process was filmed. A strongly birefringent lined formed along the trajectory of the air bubble showing that the bubble oriented the microtubules along its path. Subsequently, striped regions limited in extent, developed perpendicular to this trajectory (figure 8D).

Hence, orienting the microtubules at an early stage in the process can also trigger self-organisation.

A different manner of orienting the microtubules is to apply a strong uniform magnetic field. When microtubules were assembled in flat horizontal cells, exposed to a horizontal magnetic field of 10 Tesla for only the first 15 minutes of the self-organising process, then instead of the circular 'flat' morphology, a striped 'vertical' morphology formed. This demonstrates that orienting the microtubules at the bifurcation time by way of a magnetic field modifies the self-organised morphology in a way similar to a change in the gravity direction.

The sum of these different experiments shows that any effect, which at the bifurcation time leads to a partial orientation of microtubules, triggers self-organisation.

Another factor that strongly affects self-organisation is sample shape. This is implicit in the fact that for rectangular shaped samples, 40 mm by 10 by 1 mm, different morphologies arise when the microtubules are assembled with the sample placed flat down or upright. Sample shapes effect self-organisation because microtubule reaction dynamics are modified in the region close to the boundary. When the shape of the boundary is strongly asymmetric it produces a privileged direction for microtubule growth that can suffice to trigger self-organisation. This effect is more fully described by Glade et al (Glade et al 2002a, Glade et al 2002b). Long asymmetric sample shapes favour self-organisation. For certain sample shapes, this effect can suffice to trigger self-organisation without any assistance from gravity. This is illustrated in Figures 9 and 10, and which shows that partial self-organisation occurs in cylindrical 'egg' shaped containers exposed to weightlessness for the first fifteen minutes following microtubule assembly. Although in this case, even though weightlessness does not completely prevent self-organisation, it nevertheless slows it down substantially and the self-organised morphology is significantly perturbed.

Figure 9. Clinorotation gives results close to space flight. Self-organisation of microtubules in 5 mm diameter 'egg' shaped containers at different times, a) 30 min; b) 1 hour; c) 3 hours; d) 5 hours, following microtubule assembly. The series of photographs A) were taken under 1 g conditions whereas the series B) were photographed after assembling the microtubules on a rotating clinorotator turning at 60 rpm for the first 15 minutes following assembly. The photograph, Bb, taken after 5 hours compares favourably with that obtained in space flight after the same time (see Figure 10).
Fig. 10. Effect of sample geometry on self-organisation. Microtubule structures as formed in 5 mm diameter 'egg' shaped containers assembled during space-flight and photographed 5 hours after instigating microtubule assembly; A) on the 1g 'on-board' centrifuge; B) under conditions of weightlessness. In sample containers of this shape, some self-organisation occurs even under conditions of weightlessness.

MOLECULAR MECHANISM AND NUMERICAL SIMULATIONS

The kinetics of formation of the self-organised microtubule preparations show an 'over-shoot' about 6 minutes after instigating microtubule formation (Tabony 1994, Papaseit et al 2000, Tabony et al 2002b) (Figure 11). The bifurcation point in any out-of-equilibrium system, and at which point the system is sensitive to weak external factors, must coincide with a condition of instability in the homogenous state. In the microtubule system, the 'overshoot' describes a chemical instability in the relative proportions of free tubulin and microtubules. This occurs at the bifurcation time, at the moment that the system is gravity dependent. The microtubules assemble, and then partially disassemble by about 20% before approaching a constant level after about 60 minutes.

Numerous experiments demonstrate that self-organisation results from the chemical dynamics of microtubule assembly and disassembly (Tabony 1994, Tabony 1996, Tabony and Papaseit 1998, Tabony et al 2000, Tabony et al 2002b) Microtubules are continually growing from one end and shrinking from the other. The shrinking end of a microtubule leaves behind it a chemical trail of high tubulin concentration. Likewise the growing end produces regions depleted in tubulin. A neighbouring microtubule will preferentially grow into a region of high tubulin concentration whilst avoiding regions of low concentration. The chemical trails produced by a given microtubule, will modify and determine the direction of growth of its neighbours. Thus neighbouring microtubules "talk to each other" by depleting and accentuating the local concentration of active chemicals. The coupling of reaction with diffusion progressively leads to macroscopic variations in the orientation and concentration of the microtubules.

When the microtubules first form from the tubulin solution they are still in a growing phase. There is almost no disassembly from their shrinking ends and the microtubules are distributed uniformly through the solution in an isotropic manner. However, the rapid initial growth of the microtubules results in a reduction of the free tubulin concentration in the solution. The depletion of tubulin by the growing microtubules triggers their own partial disassembly and this manifests itself as the 'overshoot' shown in the assembly kinetics. When disassembly does start to occur, just prior to the bifurcation time, it leads to the formation of the chemical trails outlined above. The isotropic arrangement of microtubules is now unstable. At this point in time (the bifurcation time), orienting just a few microtubules will induce their neighbours to grow along the same orientation. Once started, the process mutually reinforces itself with time and leads to self-organisation. Hence, in agreement with experiments, any small effect that partially orients microtubules over the entire sample, or leads to a privileged direction of microtubule growth, will trigger self-organisation.

To test this hypothesis, Glade carried out numerical simulations of a population of growing and shrinking microtubules (Glade et al 2002a, Glade et al 2002b). These simulations incorporated as parameters, experimentally realistic microtubule reaction dynamics and the experimentally determined tubulin diffusion constant. Simulations involving a few microtubules, demonstrated both the formation of the tubulin trails outlined above and promotion of the growth of neighbouring microtubules along the same orientation. When the simulations were extended to a population of about 10⁴ microtubules on a two-dimensional reaction space, 100 µm by 100 µm, then after 2-3 hours of reaction time, a self-organised structure comprised of regular bands of about 5 µm separation developed. This structure is comparable with the experimental self-organised structure that arises over a similar distance scale (Figure 12).

During initial simulations, the authors noticed that the direction of the stripes was always along the diagonal of the reaction space and this suggested that a directional bias had been unwittingly built into the algorithm. This turned out to be the case and arose from a small asymmetry in the way that the tubulin diffusion was
digitised. When the asymmetry in diffusion was removed, macroscopic self-organisation did not come about. A bias was then reintroduced into the algorithm that broke the symmetry of the system by one of two ways. Either by orienting some of the microtubules at the bifurcation time (this would resemble the manner that magnetic fields act on the system) or by making tubulin diffusion anisotropic at the bifurcation time (this would resemble the manner that gravity acts on the system). Gravity triggers self-organisation in the following way. At the bifurcation time, gravity interacts with the strong density fluctuations produced by the partial disassembly of the microtubules. This interaction causes a 'drift' term that breaks the symmetry of the transport processes. By promoting microtubule growth along a specific direction over the entire sample, self-organisation is triggered.

Figure 12. Reaction-diffusion simulations for a population of approximately $10^4$ microtubules. The diagonal stripes shown in A) are triggered by a small asymmetry in tubulin diffusion. B) shows a simulation in which this asymmetry is no longer present. Although concentration inhomogeneities are present there is no macroscopic self-organisation. In C) the simulation is identical to B) except that diffusion is now twice as fast along the y-axis as along the x-axis. Self-organised stripes develop in which the microtubules are oriented perpendicular to the direction of the stripes. D) experimentally observed self-organised structure over the same distance scale.

**MICROTUBULAR REACTION - DIFFUSION PROCESSES IN VIVO.**

In humans, weightlessness depresses the immune system and reduces bone formation. These, and other effects, are thought to arise at a cellular level. Terrestrial gravity is not normally considered as intervening in chemical or biochemical processes and so the question arises as to how it might be that gravity does affect cellular function? A possible mechanism by which gravity may intervene in biochemical mechanisms is by way of the bifurcation properties of microtubule reaction-diffusion processes outlined in this article. One of the questions that now needs answering is whether such processes also occur in vivo; in particular do they arise either during embryogenesis or the cell.

For several decades, experiments in space have been furnishing an increasing body of evidence that various cellular processes, such as growth rates, signalling pathways and gene expression are substantially modified when various cell types are placed under conditions of weightlessness. A substantial number of experiments point to an involvement of the cytoskeleton.

One of the characteristic properties of the reaction-diffusion process is that microtubule self-organisation can be either triggered or modified by changes in the gravity vector. The major effect that these *in vitro* studies suggest is that microtubule organisation might be strongly modified when cells are placed under conditions of weightlessness.

Recently, researchers have observed substantial modifications in the organisation of the microtubules under conditions of weightlessness. Human lymphocyte (Jurkat) cells cultured in space show a disorganised microtubule network compared to ground control experiments (Lewis et al. 1998). Likewise, for human breast cancer cells (MCF-7) cultured in space (Vassy et al. 2001), the author’s report that many cells exhibit a strongly disorganised microtubule network (Figure 13). Glial cells cultured under conditions of reduced gravity produced on a random positioning machine (Uva et al. 2002) show a disorganised microtubule network and weightlessness has been reported to modify microtubule organisation in rat utricular hair cells (Gaboyard et al. 2002). Experiments of this type will be discussed in more detail in other articles in this edition.

Rashevsky, Turing, Prigogine and co-workers, and others, first developed their theories as a possible underlying physical-chemical explanation for biological self-organisation. They were the first to predict a way by which a macroscopic chemical pattern could spontaneously develop in an initially unstructured egg.

It has long been known that gravity is involved in the early developmental stages of certain types of egg (Morgan 1904, Ancel and Vintemberger 1948). For example, serious malformations result when *xenopus* eggs are rotated by 90°, at a critical time when the so-called ‘grey crescent’ forms (Cooke 1986). In *xenopus* eggs, grey crescent formation and its gravity dependence are an essential factor in determining the body plan of the organism. The grey crescent is comprised of a macroscopic array of aligned microtubules (Zischkind and Elinson 1990) and it is quite plausible that both its formation and gravity dependence result from the type of reaction-diffusion processes outlined above.
Another example where microtubule reaction-diffusion processes may arise during embryogenesis, is the formation of striped patterns in *drosophila* fruit fly eggs. In these eggs, the early stages of development occur by consecutive nuclear divisions in a non-compartmentalised space (Foe and Alberts 1983). Organisation of the cytoplasm by microtubules is known to play a major role in the morphogenetic processes that occur. Between nuclear divisions 10 to 14, cells progressively form at the surface of the embryo. These cells remain open towards the inside of the egg, until the end of the 14th nuclear division. Just prior to this, for around 5 minutes when the ventral and cephalic furrows appear at gastrulation, the distribution of microtubules in the egg displays a striped arrangement (Figure 14) (Calliani 1989, Papaseit et al 1999, Tabony et al. 2002a). Although the contrast is low, twelve stripes can be counted, and there may be 2 additional stripes of lower intensity. The stripes occur in the central part of the egg only; the end regions are not striped. This pattern coincides with and arises at the same time, as the pattern formed by the segmentation gene product, *engrailed*, which plays a major role in determining the body segmentation pattern (St. Johnson and Nusslein-Volhard 1992) of the larva that develops from the egg.

As outlined above, microtubule self-organisation *in vitro* by reaction-diffusion processes is affected by sample shape. When microtubule self-organised structures are prepared in cylindrical containers, whose shape mimic that of a *drosophila* egg, the morphology shown in figure 15 arises (Papaseit et al 1999, Tabony et al. 2002a). As for the microtubule pattern in the *drosophila* egg, there are two stripe-free regions at each end of the sample, separated by a striped central zone. The exact morphology of the *in vitro* pattern depends on the length of the sample. Below a certain critical length, the striped central region does not form. For longer samples, the end stripe-free zones remain of the same length and the number of stripes in the central region increase with sample length. For samples of appropriate length, morphologies containing 7 blue and yellow birefringent stripes arise. When observed by fluorescence, this number doubles to give fourteen stripes in the microtubule concentration (Figure 15). This pattern closely resembles the microtubule pattern in *drosophila* eggs.

**Figure 13.** Microtubule organisation in human breast cancer (MCF 7) cells. *A*) at 1g; *B*) under weightlessness. The microtubules, as in the case of in vitro preparations described above, do not self-organise under low gravity conditions Reproduced from Vassy et al (Vassy et al 2001) by permission of FASEB J.

**Figure 14.** Microtubules patterns observed by immuno-fluorescence, in *A*) whole, and *B, C*) ligated *drosophila* eggs. The positions of the ventral and cephalic furrows, indicating gastrulation, are shown in A). Ligation divides the egg into two unconnected fragments, and development continues in one, or both of the fragments.

*Drosophila* eggs can be shortened, shortly after they are laid, by ligation. Within ten minutes a membrane forms that separates the egg into two unconnected fragments. Development can occur in either one, or sometimes both, parts of the egg. The dependence of the microtubule pattern on sample length is a feature of the *in vitro* pattern. Papaseit (Papaseit et al. 1999, Tabony et al. 2002c) examined microtubule patterns in ligated *drosophila* eggs as a function of egg fragment length (figure 15). Although it is not easy to count the exact number of stripes, nevertheless, as for the *in vitro* microtubule preparations, the microtubule pattern is clearly comprised of two stripe-free regions separated by a striped central region. Also, as for the *in vitro* microtubule patterns, the length of the end zones is independent of fragment length and approximately the same as in unligated eggs. For the case of the ligated egg shown in Figure 14c, both fragments have continued to develop. However, the shorter fragment does not show any stripes. This is consistent with the fact that in this...
The behaviour of the microtubule pattern in the *drosophila* eggs resembles that observed for *in vitro* microtubule self-organisation in samples of different length, and this suggests that similar reaction-diffusion processes might be occurring in both cases.

In addition to the cases outlined above, there is also circumstantial evidence that microtubule reaction-diffusion processes might arise in peripheral nerve cells and during plant cell development (Tabony 1996).

The results outlined above show how a very simple biological system comprised of only tubulin and GTP, is capable of showing a strong gravity dependence. It must be stressed, contrary to what is often erroneously stated, that gravity does not affect the assembly of tubulin into microtubules. The level of assembly of tubulin into microtubules is the same under conditions of weightlessness as under terrestrial gravity. Gravity participates in the microtubule self-organising process and under appropriate conditions triggers self-organisation. The gravity direction breaks the symmetry of the initially homogenous state and leads to the emergence of form and pattern in a way that has many analogies with the manner that weak external factors affect ant colonies.

At this stage it is not clear whether these processes are widespread in biology, or if they are limited to microtubules. It may be that the specific type of reaction-diffusion mechanism encountered here, based on reactive growth and shortening of tubes or rods, is a mechanism that is particularly suited to self-organisation. Since other elements of the cytoskeleton, such as actin and intermediate filaments show reaction dynamics of the same type, it is likely that they will behave in a way similar to microtubules.

Although gravity triggers self-organisation, it also needs to be borne in mind that other factors, in particular geometrical factors, can also trigger it. Depending on circumstances, these other factors may either reinforce or oppose the action of gravity. Hence, in some cases the effect of weightlessness may be merely to perturb and slow down self-organisation rather than prevent it completely. In other cases, the different triggering factors, including gravity may oppose and counterbalance one another in such a way that under 1g conditions, self-organisation does not occur. In such circumstances, when the effect of gravity is removed, then self-organisation can occur due to the remaining triggering factors whose effects are no longer cancelled out by gravity. In the case of intermediate filaments of the cytoskeleton, Cogoli-Greuter has reported (Cogoli-Greuter et al. 1998) an example of this type of behaviour. Considerations of this type can account for why different cell types show differing sensitivities towards weightlessness.

The fact that gravity affects microtubule organisation demonstrates that it intervenes in a fundamental cellular process. It will hence indirectly affect other cellular processes, which in their turn depend upon microtubule self-organisation. There is a mounting body of evidence that processes of this type occur *in vivo* and play a role in embryogenesis and the cell cycle. Microtubule organisation is an essential biological process and if it does not occur correctly, then the consequences on other cellular processes will be many, varied and can eventually lead to cell death. This raises the question of the long-term survival of certain organisms in space without a corrective action to replace terrestrial gravity.

At a practical level, one of the striking features of this work is that very simple and inexpensive apparatus and methods, such as turning the sample from vertical to horizontal, spinning on a record player, or rotating about the horizontal axis at 60 rpm, can lead to substantial effects. In the present case, the understanding derived from these simple experiments is comparable with those

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**Figure 15. Effect of sample length on microtubule patterns.**

A) Birefringent patterns formed by microtubules assembled in cylindrical ‘egg’ shaped sample containers of different length. B) Microtubule concentration variations as detected by fluorescence. The overall morphology resembles the microtubule pattern in *drosophila* eggs.

**CONCLUSIONS**

The results outlined above show how a very simple biological system comprised of only tubulin and GTP, is capable of showing a strong gravity dependence. It must...
obtained from experiments carried out in space, and without the associated difficulties and expense. Conditions of weightlessness produced on the ground by methods such as clinorotation and magnetic levitation result in the same behaviour as observed in space experiments. These methods have the advantage over space experiments of being inexpensive, rapid, readily available, simple, easily reproducible, do not endanger life, and their conclusions are not complicated from effects that might arise from launch vibrations or payload re-entry and recovery.

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