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Ground-based methods reproduce space-flight experiments and show that weak vibrations trigger microtubule self-organisation

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Abstract

The effect of weightlessness on physical and biological systems is frequently studied by experiments in space. However, on the ground, gravity effects may also be strongly attenuated using methods such as magnetic levitation and clinorotation. Under suitable conditions, in vitro preparations of microtubules, a major element of the cytoskeleton, self-organise by a process of reaction–diffusion: self-organisation is triggered by gravity and samples prepared in space do not self-organise. Here, we report experiments carried out with ground-based methods of clinorotation and magnetic levitation. The behaviour observed closely resembles that of the space-flight experiment and suggests that many space experiments could be carried out equally well on the ground. Using clinorotation, we find that weak vibrations also trigger microtubule self-organisation and have an effect similar to gravity. Thus, in some in vitro biological systems, vibrations are a countermeasure to weightlessness.

Keywords: Weightlessness; Magnetic levitation; Clinorotation; Weak vibrations; Self-organisation; Reaction–diffusion; Complex systems; Microtubules

1. Introduction

Conditions of reduced weight, equivalent to between 10^{-2} to 10^{-4} times gravity (g), can be obtained in spacecraft under free-fall conditions [1] and the effect of weightlessness on physical, chemical and biological systems is frequently studied this way. Nevertheless, on earth, ground-based methods such as clinorotation [2,3] or magnetic levitation [4] can substantially attenuate its effects. In astronauts, weightlessness results in reduced bone mass and modified immune responses [5,6]. Recently, it has been shown that brief periods of exposure to weak vibrations can result in an increase in bone mass [7]. A substantial body of evidence demonstrates that modifications in cellular processes occur when cells – in particular immune [1,8] and bone cells [9,10] – are placed under conditions of weightlessness and a number of experiments indicate a participation of the cytoskeleton in this process [10–12].

Weak external fields, such as gravity and vibrations, are not normally considered as intervening in chemical and biochemical reactions. One possible manner by which they can is through certain types of reaction–diffusion processes. Since the late 1930's, theoreticians have predicted that under appropriate conditions, a non-linear coupling of reactive processes with molecular diffusion can lead to the progressive development of a stationary chemical pattern [13–17]. Some chemical systems have been established as behaving this way [18–23]. In some reaction–diffusion systems, it has been predicted that self-organisation can depend on weak external fields, such as gravity, which break the symmetry of the process [24,25]. Their presence, at a critical moment or bifurcation time early in the process, can determine the morphology that subsequently develops. The formation of microtubules, a major element of the cytoskeleton, shows this type of behaviour [26,27]. Under appropriate conditions, preparations self-organise by reaction

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and diffusion and this self-organisation is triggered by external factors, such as gravity [28,29], magnetic fields [30], or sheering [29].

Microtubules [31,32] are long (several μm) tubular shaped super-molecular assemblies, having inner and outer diameters of 16 and 24 nm, comprised of the protein, tubulin. They have two major cellular roles; they organise the cell interior, and they permit and control the directional movement of intracellular particles and organelles from one part of the cell to another. Microtubules are readily formed in vitro by warming a preparation of purified tubulin in the presence of excess guanosine triphosphate (GTP) from about 7 to 36 °C. Chemical reactions occur, GTP is hydrolysed to guanosine diphosphate (GDP), and the tubulin assembles into microtubules within 2–3 min. After microtubule form, this reaction continues by processes in which the complex, tubulin–GTP, is added to the growing end of a microtubule and tubulin–GDP is lost from the opposite shrinking end.

Under suitable conditions, in vitro microtubule preparations progressively self-organise over approximately 5 h by a process of reaction and diffusion to form a series of stripes of about 0.5 mm separation [26]. Microtubules, in each striped band, are highly oriented, at either 45° or 135° to the stripe direction, with adjacent stripes having opposing orientations. This orientational pattern coincides with an identical concentration pattern; the microtubule concentration drops by about 25% and then rises again every time the microtubule orientation flips from acute to obtuse [33]. Within each band, another series of stripes of about 100 μm separation occurs, and these in their turn contain further sets of stripes of 20, 5, and 1 μm [28]. Self-organisation also arises when samples are prepared in miniature containers of dimensions (2–200 μm) comparable to cells and embryos [34].

Striped morphologies occur when microtubules are assembled in upright rectangular sample containers, but a different pattern arises when they are assembled in the same container lying flat down [35]. The morphology that forms depends upon the orientation of the sample with respect to gravity at a critical moment early in the process (6 min) before any self-organised pattern is visible [36]. Samples subject to weightlessness, produced in a free-falling spacecraft for the first 13 min of the process, did not self-organise [28].

Here, we report ground-based experiments, in which both clinorotation and magnetic levitation were used to reduce gravity effects. They show the same behaviour as observed in space. Gravity and vibrations give rise to related phenomena. Gravity produces a constant acceleration, whereas vibrations are oscillations in the magnitude and direction of acceleration. Under conditions of near weightlessness produced in a clinostat, we show that the presence of weak vibrations, for a short period following microtubule assembly, also triggers self-organisation. Hence, in some in vitro biological systems, weak vibrations will counteract weightlessness.

2. Experimental

Tubulin, isolated and purified as previously described [37], was transferred into buffer comprised of 100 mM MES (2-*N* morpholino ethanesulphonic acid), 1 mM EGTA (ethylene glycol-bis-(*B*-aminoethyl) *N*, *N*, N1, N1 tetra-acetic acid), and 1 mM $MgCl_2$, in D_2O at pH 6.75 and stored in liquid nitrogen. Before an experiment, tubulin aliquots from the same batch were thawed out and diluted in cold buffer to 10 mg ml^{-1} in the presence of 2 mM GTP. Microtubules were formed by warming the solution to 36 °C. Self-organisation is conveniently observed by placing the sample between crossed polars with a wavelength retardation plate at 45° between them. Macroscopic regions comprised of microtubules oriented at 45° give rise to a blue interference colour whereas regions containing microtubules oriented at 135° are yellow.

Magnetic levitation experiments were carried out in a superconducting magnet operating at 15.62 T. Clinorotation experiments were carried out using a commercial clinostat manufactured by CCM, the Netherlands and kindly loaned to us by J. van Loon (Dutch Experiment Support Centre, Free University, Amsterdam, the Netherlands).

3. Results

3.1. Magnetic levitation

Magnetic levitation experiments have been reported on both physical and biological systems [4,38–42]. The method is based upon the following. A magnetic field gradient interacts with matter to produce a force proportional to the product of the field (B_z) at position z, the field gradient (dB/dz) and the magnetic susceptibility of the material (χ). This force is repulsive for diamagnetic substances, which are of negative magnetic susceptibility. The value and direction of a magnetic field gradient can be adjusted so that the magnetic force counterbalances that of gravity [4,38–42]. For water and organic materials this requires a magnet operating at a field of about 15 T. According to Albrecht–Bueler, an object at the centre of the earth has no weight due to the cancelling out of gravitational forces in all directions [43]. Likewise, because magnetic fields act on diamagnetic matter at a level of the electrons in individual molecules, magnetic levitation can counteract gravity and cause a substantial reduction in weight.

Gravity may act upon matter in several manners. One possible effect is to cause mechanical deformations due to 'weight' [44]. Gravity will also interact with any density differences present in the sample to cause a buoyancy force that can lead to transport in the vertical direction. In principle, magnetic levitation will counterbalance both of these effects.

Magnetic field conditions required for the levitation of microtubule preparations were determined in the following way. We placed a drop of microtubule preparation in a vertical superconducting magnet at the position of maximum repulsive force with the magnetic field (B_0) set to the maximum value of 17 T. We then progressively decreased the field until the microtubule drop fell. For a value of the field intensity slightly higher than this value (15.62 T) the drop could be maintained in levitation. At this height, z, 6.2 cm above the centre of the magnetic field, the magnetic field intensity (B_z) was 11.9 T and the product $B_z \cdot dB/dz = 1430\ T^2\ m^{-1}$.

The sample volume for which the magnetic force counteracting gravity is homogenous to within a few percent is limited to a region about 2 cm high. The samples were hence contained in glass cells measuring 2 cm by 1 cm by 1 mm rather than in 4 cm long cells as used in the space experiment. Samples were contained in a preheated holder maintained at 36 °C. The holder contained two samples, one above the other. With the holder positioned in the magnet, the lower sample was under conditions of magnetic levitation, whereas the upper sample was outside of the magnetic field under normal 1 g conditions.

Magnetic fields also interact with the anisotropy in the diamagnetic susceptibility of microtubules to produce a torque. Under appropriate conditions, this torque can orient the microtubules and hence trigger or modify their self-organisation. We carried out experiments which showed, in agreement with previous reports [45], that this orienting effect occurs only during the first 2–3 min after instigating microtubule formation, when both the sample viscosity and the microtubule length are low. Hence, to avoid the situation where the magnetic field gradient used to levitate the sample itself triggers self-organisation, we initially assembled (36 °C) the microtubules, for the first three minutes, outside of the magnetic field. The sample was then lowered into the magnet, thus placing it under conditions of magnetic levitation three minutes before the critical time (6 min) when gravity acts on it. The sample was removed from the magnet 12 min later (15 min after instigating microtubule formation), left for 5 h at 36 °C, and then photographed. As shown in Fig. 1, self-organisation is strongly inhibited. This behaviour should be contrasted with that of the reference 1 g sample positioned outside the magnet and which

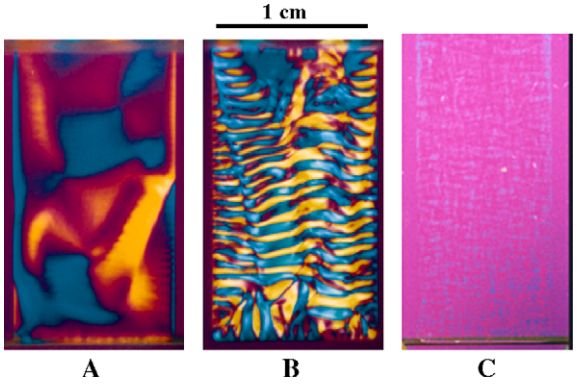

Fig. 1. Effect of magnetic levitation on microtubule self-organisation. A solution of tubulin (10 mg ml^{-1}) in the presence of a large excess of GTP (2 mM) was placed in glass cells, 20 by 10 by 1 mm. Microtubules were assembled by warming the sample to 36 °C for 3 min and placing it in the super-conducting magnet (also at 36 °C) under conditions of magnetic levitation (see text for details). The sample was removed from the magnet 15 min after instigating microtubule formation. The sample was maintained at 36 °C and photographed 5 h later. As shown in A), self-organisation is strongly inhibited. This observation contrasts with the sample shown in B). This sample was assembled simultaneously, at another position on the sample holder under normal 1 g gravity conditions, outside the magnetic field and photographed at the same time as A). The product of the magnetic field and field gradient was 1430 $T^2\ m^{-1}$ and the magnetic field at the centre of the sample was 11.9 T. For comparison, the photograph C) shows the result of an equivalent space-flight experiment [28]. Samples were photographed through linear cross polars with a wavelength retardation plate at 45°.

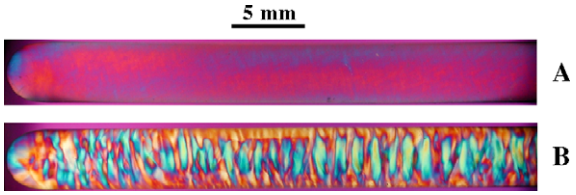

Fig. 2. Effect of near weightlessness produced by clinorotation on microtubule self-organisation. In A) microtubules were assembled in an 8 cm long tube of 4 mm internal diameter, rotating around the horizontal axis at 60 rpm, for the first 15 min following microtubule assembly, then photographed 5 h later. Self-organisation does not occur. This contrasts with the 1 g reference sample shown in B) assembled simultaneously at another position on the clinostat not undergoing rotation, then photographed at the same time as A). For the clinorotated sample, no structures were detected when the sample was placed at an oblique angle so as to obtain a partial view down the long axis of the sample.

self-organised in the normal manner. The optical density at 350 nm is proportional to microtubule concentration. From this, we found that under conditions of magnetic levitation, microtubules assemble to the same extent as at 1 g. The results of this experiment compare favourably with those obtained in space-flight [28] (Fig. 1C).

3.2. Clinorotation

In biological systems, clinorotation has been employed to attenuate the effects of gravity for a number of years [2,3,43,44,46–49]. The method consists of rotating the sample about the horizontal axis at constant velocity [2,3,44,47]. In this case, there are no forces present to counteract gravity. However, for a particle or region of density difference, 'falling' under gravity, rotation continually changes the 'direction of fall' so that the net directional transport due to gravity cancels out. Hence, this simple devise can strongly attenuate one of the major effects of gravity. Klaus [44] call this, 'functional weightlessness'. In practice, speeds of rotation about 60 rpm are often effective. However, the centrifugal force of acceleration that sample rotation causes limits the reduction in the force of acceleration to which the sample is subjected. For example, at 60 rpm, 1 mm off the axis of rotation, the force of acceleration is $4 \cdot 10^{-3}$ g [3]. For this reason, we choose to work on samples contained in tubes of 4 mm internal diameter. Sample geometry and dimensions affect the microtubule reaction–diffusion process and in short cylindrical samples under conditions of weightlessness this factor can itself trigger self-organisation [29,50]. To avoid this occurring, we assembled microtubules in tubes 8 cm long. In this case, on the ground, striped morphologies form for both vertical and horizontal configurations.

The commercial apparatus that we used allowed both rotating and stationary samples. The clinostat was first warmed to 36 °C in a hot room. Two samples, containing tubulin and GTP at 4 °C, were placed in it and warmed to 36 °C. One sample was rotated about the horizontal axis at 60 rpm, whereas the other (1 g reference) sample remained stationary. Microtubules form within the first few minutes after heating. The microtubule preparations have a high viscosity of about 10^4 P and sample rotation does not cause mixing. After 15 min,

sample rotation was stopped, the samples left for 5 h at 36 °C, and then photographed. Microtubules were found to assemble to the same extent under clinorotation as at 1 g. However, samples subject to clinorotation for the first 15 min did not self-organise (Fig. 2A). This contrasts with the behaviour of stationary (1 g) samples, and which self-organised in the normal manner (Fig. 2B). These experimental results bear a close resemblance to those obtained in space-flight [28].

3.3. Effect of weak vibrations

The acceleration caused by terrestrial gravity on a body is constant at 9.8 m s^{-2}. In the case of vibrations, the magnitude of acceleration periodically varies between zero and a maximum value. In physical systems that contain density differences, such as thin fluid layers, granular materials, or some 2-phase fluids, vibrations can result in self-organisation of the system, under both 0 and 1 g conditions [51–53]. To see whether under conditions of weightlessness, vibrations might also trigger microtubule self-organisation, we attached a small vibrator and battery to the outside of the sample tube. The vibrator had a frequency of 125 Hz and caused a displacement perpendicular to the long axis of the tube of approximately 10 μm. We estimate the maximum acceleration as about 1.25 m s^{-2} (0.13 g). Although clinorotation averages out gravity effects, in the present experiment it will not average out those from vibrations because the vibrator is attached to the sample tube and rotates with it.

The experiment was carried out in the clinostat, under conditions close to weightlessness, with the vibrator functioning for the first 15 min only. We found, as shown in Fig. 3A, that self-organisation comparable to that under normal gravity conditions occurs. On the contrary, the solution did not self-organise when the experiment was repeated with the vibrator in position but not working (Fig. 3B). These experiments give no information as to the threshold acceleration for vibrations to induce stripe formation.

Clinorotation acts by inhibiting gravity driven transport arising from the interaction of gravity with any density differences that may be present in the preparation. The fact that clinorotation inhibits microtubule self-organisation means that gravity and vibrations trigger self-organisation by way of a directional transport term that they cause. For this to occur, density fluctuations must be present in the sample at the critical period when gravity and vibrations act.

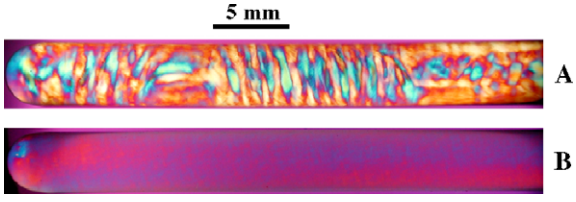

Fig. 3. Effect of weak vibrations on microtubule self-organisation. Conditions close to weightlessness were produced in a clinostat, as shown in Fig. 2A, by rotating the sample at 60 rpm for the first 15 min following microtubule formation. A vibrator was attached to the sample, which when operated caused a displacement of 10 μm, with a frequency of 125 Hz, perpendicular to the long axis of the sample. In A) the vibrator was functioning for the first 15 min after instigating microtubule formation and the sample photographed 5 h later. Self-organisation has occurred similar to that which arises under normal 1 g gravity conditions. This contrasts with the sample shown in B), taken under the same conditions of clinorotation, except that the vibrator was switched off.

4. Discussion

Microtubules possess a reactive polarity, often growing from one end by the addition of tubulin-GTP, whilst liberating a trail of tubulin–GDP from the opposite shrinking end. Excess GTP present in solution converts the liberated tubulin–GDP back to tubulin-GTP, after which it is again available to be incorporated into the growing ends of neighbouring microtubules. Self-organisation is believed to occur in the following way [27,29,54]. Because the reactive addition of tubulin increases with tubulin-GTP concentration, neighbouring microtubules will preferentially grow into trails of high tubulin-GTP concentration. For the microtubule reaction dynamics (and rates of tubulin diffusion) thought to be present under the conditions used here, individual microtubules modify the rate and direction of growth of their neighbours by way of the chemical trails they produce. Microtubules, when they first assemble from the tubulin preparation, are in a growing phase. There is almost no shrinking and their distribution is uniform and isotropic. However, the rapid depletion of free tubulin, caused by their rapid growth, gives rise to unfavourable reactive conditions that brings about their partial disassembly. The strong microtubules shrinking that then occur result in the formation of the chemical trails outlined above. The isotropic arrangement of microtubules is now unstable, for if a few microtubules take up a preferred orientation then their neighbours will progressively grow into the same direction, and so on. At this critical bifurcation time, any small external factor that breaks the symmetry of the reaction–diffusion process by favouring a privileged direction of microtubule growth or orientation triggers self-organisation. The chemical trails of tubulin outlined above that arise at the bifurcation time also correspond to strong density fluctuations. They interact with gravity (or vibrations) to cause a buoyancy force capable of inducing a directional transport that breaks the symmetry of the reaction–diffusion process. By promoting microtubule growth along a specific direction, gravity and vibrations trigger self-organisation. The fact that self-organisation does not occur upon clinorotation, which averages out directional transport caused by gravity, is consistent with this explanation. Vibrations, although they have a time-averaged acceleration that is zero, correspond at any one time to an acceleration of finite value, but whose direction and magnitude continually vary. Energy is dissipated into the system. In the present case, vibrations trigger self-organisation because they act along one axis only, and so break the symmetry of the reaction–diffusion process. If vibrations were present equally in all directions, then there would be no symmetry breaking, and we would not expect self-organisation to occur.

There are many reports that cellular functions, such as growth rates, apoptosis, signalling pathways and gene

expression, may be modified when cells, in particular lymphocytes and osteoblasts, are placed under conditions of weightlessness [1,10,11,55]. Many experiments point to an involvement of the cytoskeleton and microtubules [10–12]. Recent experiments on various cell lines cultured under low gravity conditions show modifications in cytoskeletal organisation. In particular, under conditions of reduced gravity, human lymphocyte (Jurkat), epithelial breast cancer (MCF-7), utricular hair, and glial cells show a disorganised microtubule network compared to 1 g control experiments [12,49,56,57]. In cells, microtubule organisation results from, and is dependent upon, the reaction dynamics of microtubules. One of the characteristic properties of microtubule self-organisation by reaction–diffusion is its dependence and triggering by gravity and other external factors. The in vivo observation outlined above, consistent with the in vitro observations reported here, hence raises the possibility that in some cases microtubules in living cells can self-organise by reaction–diffusion processes. Further experiments are required to determine whether this is the case. Microtubule organisation is a fundamental cellular process and the viability of a cell is compromised when it does not occur correctly. If the behaviour reported here also occurs in vivo, then it poses the question as to the long-term survival of certain organisms in space without a corrective action to replace terrestrial gravity.

The effect of vibrations on cellular microtubule organisation has not been studied under conditions of weightlessness. However, in space-flight experiments, samples are subject to vibrations during launch, and because of this, the effect of launch vibrations has been studied under normal 1 g conditions. Simulated launch vibrations have been found to modify microtubule organisation in human immune (Jurkat) cells [11] and they have also been reported as affecting osteoblast cells [58]. Bone cells are directly mechanosensitive and vibrations are known to modify both mRNA and gene expression [58].

5. Conclusions

The experiments reported here demonstrate firstly that the effect of weightlessness on microtubule self-organisation can be studied using ground-based equipment. These methods, when applicable, have substantial advantages over space flight experiments. Experiments can be set up and carried out in a matter of days, and the methods permit extensive laboratory preparation and analysis immediately before and after experiment. In addition, the effects of launch vibration or sample recovery do not complicate the interpretation of results. The methods are inexpensive, rapid, readily available, do not endanger life, and experiments are readily repeated. We conclude that many space experiments, either manned or unmanned, could be better carried out using ground-based apparatus.

Recently it has been shown that exposure to brief periods (10 min) of vibration, of magnitude and frequency similar to those used here, can result in an increase in bone mass [7]. Weak vibrations are hence under investigation as a possible therapy for osteoporosis [59,60]. The observation that weak vibrations trigger microtubule self-organisation in vitro raises the possibility that they could also counteract the effect of weightlessness on microtubule organisation in vivo. They could hence find application for correcting for weightlessness in various types of organisms exposed to long periods of weightlessness. For astronauts, they could be a simple way to help diminish disorders such as reduced bone mass and depressed immune systems that weightlessness causes. How vibrations act at a sub-cellular level is not established. The manner by which vibrations and other weak external fields trigger microtubule self-organisation by breaking the symmetry of the reaction–diffusion process could form an underlying molecular basis for this behaviour and raises the question as to whether effects on living organisms, both therapeutic and harmful, can result from exposure to brief periods of external physical stimuli.

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